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TOLL-LIKE RECEPTOR EXPRESSION AND FUNCTION AT THE OCULAR SURFACE

by

RACHEL REDFERN RUMP, O.D.,

A DISSERTATION

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Approved by:

_____
Alison McDermott, Ph.D. (chair)

_____
William Miller, O.D., Ph.D.

_____
Deborah Otteson, Ph.D.

_____
Stephen Pflugfelder, M.D.

Committee in Charge

Dedication

This work is dedicated to my loving husband Kevin and our wonderful son Gavin.

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Abstract

Purpose: Ocular surface inflammation presents from various etiologies (e.g. dry eye and *Pseudomonas aeruginosa* (PA) infection), yet little is known about the role of toll-like receptors (TLR) which, upon activation, stimulate inflammation. The objective of this research was to determine: (1) baseline expression of TLRs in ocular surface cells, if TLR activation or products thereof (antimicrobial peptides, AMPs) can modulate TLR expression, and if TLR agonists can stimulate the expression of AMPs human β–defensin (hBD-2) or LL-37 which can kill PA; (2) if ocular surface cell TLR expression is modulated in response to dry eye associated conditions, desiccation and in dry eye patients; (3) TLR expression in mice with experimental dry eye (EDE), AMP expression in EDE mice and dry eye subjects and if topical TLR agonist application modulates the inflammatory response in mice with EDE *in vivo*.

Methods: (1) TLR expression was examined by RT-PCR, flow cytometry or immunostaining. Cells were exposed to AMPs or TLR agonists to determine TLR mRNA expression or the antimicrobial activity of the culture media against PA. (2) TLR mRNA or protein expression was examined by real-time RT- PCR or western blotting in cells cultured with hyperosmolar stress (HOS), or dry eye associated cytokines (IL-1α/β, TNFα, TGFβ), in a human corneal organ culture model following desiccation and in conjunctival impression cytology (CIC) samples from human dry eye patients. (3) TLR and/or AMP expression was determined at the ocular surface and/or lacrimal gland in mice with EDE using quantitative RT-PCR and immunostaining. AMP mRNA expression was examined in human dry eye CIC samples. A TLR agonist cocktail was

applied to the ocular surface of mice with EDE. Central corneal thickness (CCT), recruitment of inflammatory cells into to the corneal stroma and corneal fluorescein staining were determined by *in vivo* imaging and corneal epithelial AMP expression was determined by quantitative RT-PCR and immunostaining 24 hours later.

Results: (1) Ocular surface cells express most TLRs with the exception of TLR8. Some AMPs but not TLR agonists modulated TLR mRNA expression. TLR agonists increased the secretion of hBD-2 and LL-37 into the growth media, which was able to significantly kill PA. (2) HOS significantly increased TLR4 mRNA but decreased TLR4 protein. Dry eye associated cytokines had no effect on TLR expression. TLR4 and TLR5 were upregulated in response to desiccation. TLR9 expression was downregulated in response to HOS, desiccation and in dry eye subjects. (3) EDE modulated TLR mRNA and protein expression by the ocular surface and lacrimal gland, compromised corneal epithelial integrity and decreased the expression of mRNA and protein of CRAMP, the LL-37 mouse homolog. hBD-2 was significantly upregulated in dry eye subjects. TLR agonist treatment downregulated mBD-4 protein in the cornea, decreased CCT, but did not increase inflammatory cell recruitment into the stroma.

Conclusions: (1) TLRs are expressed on the ocular surface and their activation triggers the production of LL-37 and hBD-2, with LL-37 being particularly important in killing PA. (2) Dry eye and associated conditions modulated TLR expression which may alter the ocular surface immune/inflammatory response. (3) EDE significantly disrupts the mouse corneal epithelium, increases TLR expression and decreases the expression of CRAMP. Mice with EDE were resistant to TLR induced corneal inflammation

Table of Contents

List of Figures

List of Tables

List of Publications

Published

Redfern RL, Reins RY, McDermott AM Toll-Like Receptor Activation Modulates Antimicrobial Peptide Expression by Ocular Surface Cells. *Exp Eye Res*. In Press. (Chapter 2).

Redfern RL, McDermott AM, 2010.Toll-like receptors in ocular surface disease. *Exp Eye Res*. 90, 679-87. (Portion of Chapter 1)

Other Publications

Huang LC, **Redfern RL**, Narayanan S, Reins RY, McDermott AM. *In vitro* activity of human beta-defensin 2 against Pseudomonas aeruginosa in the presence of tear fluid. *Antimicrob Agents Chemother*. 2007 Nov;51(11):3853-60.

McDermott AM, **Redfern RL**, Zhang B, Pei Y, Huang L, Proske RJ. Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2003 44(5):1859-65.

McDermott AM, **Redfern RL**, Zhang B. Human beta-defensin 2 is up-regulated during re-epithelialization of the cornea. *Curr Eye Res*. 2001 22(1):64-7.

List of Abbreviations

Abbreviation	Expansion
AC	allergic conjunctivitis
AKC	atopic keratoconjunctivitis
AMPs	antimicrobial peptides
AP-1	activating protein-1
CCT	central corneal thickness
CIC	conjunctival impression cytology
CpG	cytosine-phosphate-guanosine
CRAMP	cathelicidin-related antimicrobial peptide
EDE	experimental dry eye
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hBD	human β-defensin
hCAP-18	human cationic antimicrobial protein-18
HCEC	human corneal epithelial cells
HNP	human neutrophil peptide
HOS	hyperosmolar stress
HRT	Heidelberg Retinal Tomography
HSP	heat shock protein
HSV	herpes simplex virus
IFN	Interferon
IL	Interleukin
IOBA-NHC	normal human conjunctival epithelial cell line
IP-10	interferon-inducible protein
IRAK	IL-1R-associated kinase
IRF	interferon regulatory factor
KCS	keratoconjunctivitis sicca
LL-37	human cathelicidin
LPS	lipopolysaccharide

MAPK	mitogen activated protein kinase
mBD	mouse β-defensin
MMP	matrix metalloproteinase
MRSA	methicillin-resistant Staphylococcus aureus
NF-κB	nuclear factor kappa B
OSDI	ocular surface disease index
PA	Pseudomonas aeruginosa
PAMP	pathogen associated molecular patterns
SA	Staphylococcus aureus
SD-OCT	Spectralis Spectral Domain Optical Coherence Tomography
SIGIRR	single Ig IL-1R-related molecule
SS	Sjögren's syndrome
TGF	transforming growth factor
TIR	Toll/IL-1 receptor
TLR	toll-like receptors
TNF	tumor necrosis factor
UT	untreated
VKC	vernal keratoconjunctivitis

Chapter 1 - Introduction

1.1 Toll-Like Receptors

Toll-like receptors (TLRs) are a family of highly conserved glycoprotein pattern recognition receptors that recognize conserved motifs on pathogen associated molecular patterns (PAMPs) on bacteria, viruses, fungi and protozoa. TLRs are expressed on a wide variety of cell types including epithelia, endothelia, antigen presenting cells and lymphocytes. They are type I transmembrane glycoproteins that have an extracellular leucine-rich domain and a cytoplasmic domain that is homologous to the signaling domain of the interleukin (IL)-1 receptor, referred to as the Toll/IL-1 receptor (TIR) domain. The latter mediates activation of intracellular signaling pathways, leading to functional changes including cytokine, chemokine and adhesion molecule expression.

To date, 10 functional human TLRs have been identified; their microbial ligands and signaling pathways are depicted in Figure 1.1. TLR1, 2, 4, 5, 6, and 10 are typically located at the cell surface. TLR2 forms heterodimers with TLR1 or TLR6 which recognize a variety of microbial lipoproteins. TLR2/6 and TLR2/1 heterodimers recognize bacterial diacyl and triacyl lipopeptides respectively (von Aulock *et al.*, 2003; Takeda *et al.*, 2002). TLR4 forms a complex with MD-2 and CD14 and recognizes lipopolysaccharide (LPS) from Gram-negative bacteria (Beutler 2000). TLR5 recognizes flagellin, a component of bacterial flagella (Hayashi *et al.*, 2001). TLR10 is able to dimerize with TLR1 and TLR2, but the microbial ligand for this receptor has yet to be identified (Hasan *et al.*, 2005). TLR 3, 7, 8, and 9 are typically located intracellularly, on endosomal membranes and recognize nucleic acids. TLR3 recognizes double stranded RNA, a by-product of viral replication (Alexopoulou *et al.*, 2001), whereas TLR7 and 8 recognize viral single stranded RNA

(Diebold *et al.*, 2004; Heil *et al.*, 2004). TLR9 responds to unmethylated cytosine-phosphate-guanosine dinucleotide (CpG) motifs found in both bacterial and viral DNA (Hemmi *et al.*, 2000; Tabeta *et al.*, 2004).

Although TLRs were first recognized for their capacity to bind PAMPs recently a number of endogenous ligands have come to light. Many of these are molecules indicative of tissue trauma, such as intracellular components of ruptured cells, nucleic acids, heat shock proteins and extracellular matrix breakdown products such as hyaluronan fragments, fibrinogen and high-mobility group box 1 proteins (Kluwe *et al.*, 2009). Thus, TLRs may be part of a surveillance system to monitor tissue injury and progress of re-modeling as well as infection. On the downside, TLR activation by endogenous ligands is also associated with disease; activation of TLR9 by endogenous DNA is implicated in the development of autoimmune disorders such as systemic lupus erythematosus in both humans and murine models of the disease (Lamphier *et al.*, 2006).

With the exception of aforementioned self-nucleic acid signaling via TLR9, endogenous TLR ligands trigger TLR2 or TLR4 activation. Owing to similarities among the cytokine effects of these endogenous ligands and TLR2/4 microbial agonists, it has been suggested that contamination with bacterial LPS or lipoprotein is actually responsible for at least some of the effects attributed to endogenous ligands (Tsan and Gao 2007). Thus, studies claiming identification of an endogenous TLR ligand need to be scrutinized to ensure adequate controls were in place to account for possible bacterial product contamination.

All TLRs, except TLR3, signal via the adaptor molecule myeloid differentiation protein 88 (MyD88) which associates with the TLR cytoplasmic domain via a homophilic

interaction between the TIR domains (Figure 1.1). IL-1R-associated kinase (IRAK)-4 and IRAK-1 are recruited, activated IRAK-4 phosphorylates IRAK-1 which ultimately leads to the activation of transcription factors activating protein (AP)-1, nuclear factor κB (NFκB) and interferon regulatory factor (IRF)-5. This stimulates the expression of multiple genes such as cytokines, chemokines and adhesion molecules. TLR3 signals via a MyD88 independent pathway that leads to the activation of adaptor protein, TIR domain-containing adaptor protein-inducing interferon (IFN)-β (TRIF), thus leading to the activation of transcription factors IRF-3 and IRF-7 that subsequently induce expression of IFN–α/ β and IFN inducible genes such as RANTES and interferon-inducible protein (IP)-10. Figure 1.1 shows a general overview of TLR signaling, for comprehensive details of the pathways the reader is referred to a review article by Albiger *et al.* (2007).

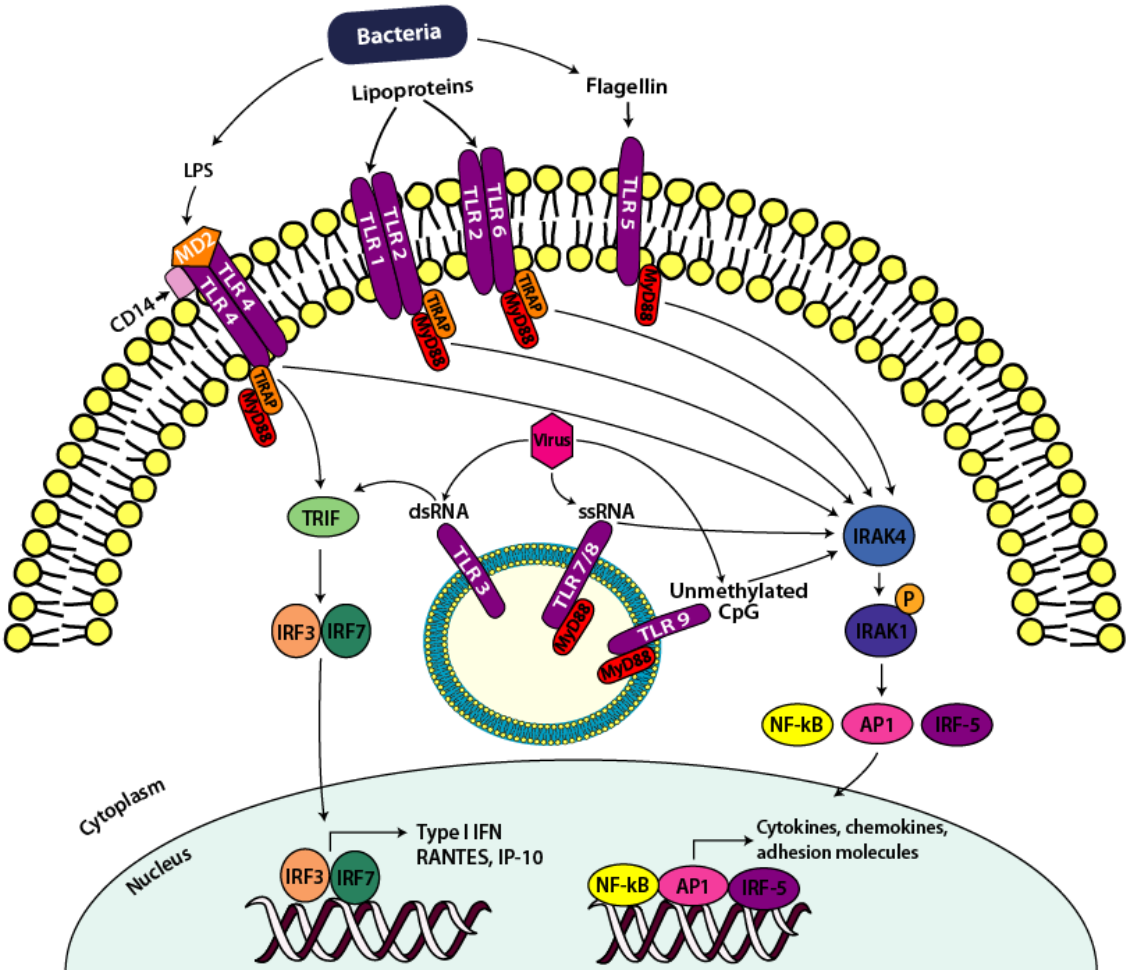


Figure 1.1 Simplified overview of TLR signaling

Cell surface TLR2, 4 and 5 recognize bacterial PAMPs lipoproteins, LPS and flagellin respectively, whereas intracellular TLR3, 7/8, and 9 recognize microbial dsRNA, ssRNA and unmethlylated CpG motifs respectively from either replicating or infecting viruses or bacteria in the endosome of the cell. The activation of TLRs initiates a MyD88-dependent (all TLRs except TLR3) or TRIF-dependent (TLR3 and TLR4) pathway. The MyD88-dependent pathway utilizes adapter molecule TIRAP (except TLR7, 8 and 9) leading to IRAK-4 and IRAK-1 recruitment, activated IRAK-4 phosphorylates IRAK-1 which ultimately leads to the activation of transcription factors AP-1, NFκB and IRF-5. TLR3

and TLR4 signal via a MyD88-independent pathway that is mediated via the adaptor protein, TRIF, which leads to the activation of transcription factors IRF-3 and IRF-7 that induce the expression of type I IFN genes.

1.2 Expression of TLRs at the Ocular Surface

A summary of findings regarding cornea and conjunctival TLR expression from several different published sources cited below is presented in Figure 1.2. The first report of the localization of TLRs to the ocular surface came in 2001 when Song *et al.* showed that freshly isolated and telomerase immortalized human corneal epithelial cells (HCEC) express TLR4. Subsequently, the expression of mRNA for TLRs 1-10 has been detected in the corneal epithelium from subjects undergoing various ocular surgeries and from cadaver corneas; however, not all subjects expressed all TLRs and the relative expression between subjects was variable, with TLR7 and 8 tending to be lower (Jin *et al.*, 2007; Redfern *et al.*, 2006 (Chapter 2); Ueta *et al.*, 2005; Wu *et al.*, 2007). Similar results have been observed with primary cultured HCEC and cell lines (Kumar *et al.*, 2004; Redfern *et al.*, IOVS 2006; 47: ARVO E-Abstract 4372; Wu *et al.*, 2007). Expression at the protein level has been confirmed for TLR2, 3, 4, 5 and 7 (Hozono *et al.*, 2006; Kumar *et al.*, 2006a; Li *et al.*, 2005; Song *et al.*, 2001; Ueta *et al.*, 2004, 2005; Wu *et al.*, 2007; Zhang *et al.*, 2003).

Studies of the distribution and functionality of some TLRs at the ocular surface has produced contrasting results in a number of instances. Ueta *et al.* (2004, 2005) observed intracellular TLR2 expression in HCEC that was unresponsive to peptidoglycan. However, Kumar *et al.* (2004, 2006b) found cell surface TLR2 expression, stimulation of which activated NFκB and upregulated cytokine and antimicrobial peptide (human β-defensin-2, hBD-2) expression. Similarly, Kumar *et al.* (2006a) observed functional (as determined by NFκB activation and IL-6 and IL-8 secretion) intracellular expression of TLR3 by HCEC. However Ueta *et al.* (2005) reported that TLR3, while functional, was

expressed at the cell surface. TLR3 is commonly found intracellularly on endosomal membranes, although surface expression has been documented for other cell types including fibroblasts (Matsumoto *et al.,* 2003) and cytokine exposed keratinocytes (Begon *et al.,* 2007). Several studies support expression of functional TLR4 by HCEC (McNamara *et al.,* 1999; Song *et al.,* 2001; Wu *et al.,* 2007). However, others report intracellular expression of TLR4 that was unresponsive to LPS, leading to the suggestion that this would contribute to an "immunosilent environment" to prevent unnecessary responses to commensal flora (Kumar *et al.,* 2006b; Ueta *et al*., 2004).

As these aforementioned studies were carried out primarily with cultured cells, the variability in the resulting data may relate to donor variation (for primary cultures), culture conditions and differences in how cell lines were derived. Notably Blais *et al.* (2005) observed that whereas LPS alone had little effect on IL-6 and IL-8 secretion, addition of CD14 or LPS binding protein increased their secretion, suggesting that culture conditions can have a significant influence on the responsiveness of cells to LPS. Furthermore, lack of MD-2 expression is responsible for the inability of some HCEC to respond to LPS (Zhang *et al.,* 2009).

Blais *et al.* (2005) found TLR4 and MD-2 expression in the basal and wing but not the superficial epithelial cells of human corneal tissue sections. Zhang *et al.* (2003) reported a similar distribution of TLR5, a finding confirmed by Hozono *et al.* (2006) and by our lab (Figure. 1.2). This suggests that TLR4 and 5 will only be activated when there is a breach in the epithelium, thereby preventing inappropriate inflammatory responses when the epithelium is intact (Zhang *et al*., 2003). Interestingly, Hozono *et al.* (2006) showed that flagellin from ocular pathogenic bacteria, but not that from ocular non-

pathogens or intestinal pathogens, activated gene transcription and cytokine production in HCEC; however the mechanism underlying this is not understood.

Few studies have specifically addressed TLR expression in corneal layers other than the epithelium. Ebihara *et al*. (2007) detected TLR2 and 4, but not 3 or 9 in keratocytes from human cadaver corneas. In contrast, all TLR mRNAs were expressed except 8 (Redfern *et al*., 2006, Chapter 2) in stromal cells, however no distinction was made between keratocytes and resident immune cells. Cultured corneal fibroblasts express TLR1, 3, 4, 6, 7, and 9 and functional studies have shown that TLR3 (Liu *et al.,* 2008) and TLR4 (Kumagai *et al.,* 2005) activation in corneal fibroblast results in cytokine secretion. Such stromal TLR expression is expected to provide immediate surveillance against microbial infection following a breach in the ocular surface that penetrates the entire epithelial barrier.

In regards to the conjunctiva, immunohistochemistry have revealed expression of TLR2, 4, and 9 in both the epithelium and stroma, with staining being more intense in the stroma (Bonini *et al.,* 2005). These findings were confirmed by Li *et al.* (2007) who additionally observed immunostaining for TLR1, 3 and 5. Studies with samples collected by impression cytology and in cultured cells show that conjunctival epithelial cells typically express TLR1-6 and 9, have variable expression of TLR7 and do not express TLR 8 or 10 (Cook *et al.,* 2005; Redfern *et al.,* 2006; Chapter 2). Similar results were also observed for limbal epithelial cells (Li *et al.,* 2007). Activation of TLR1/2, 3, 4 and 5 has been shown to trigger primary conjunctival epithelial cell cytokine secretion (Li *et al.,* 2007; Chung *et al.,* 2009). However, Talreja *et al.* (2005) found that in a conjunctival

epithelial cell line TLR4 agonist, LPS was unable to stimulate cytokine secretion due to lack of expression of MD-2.

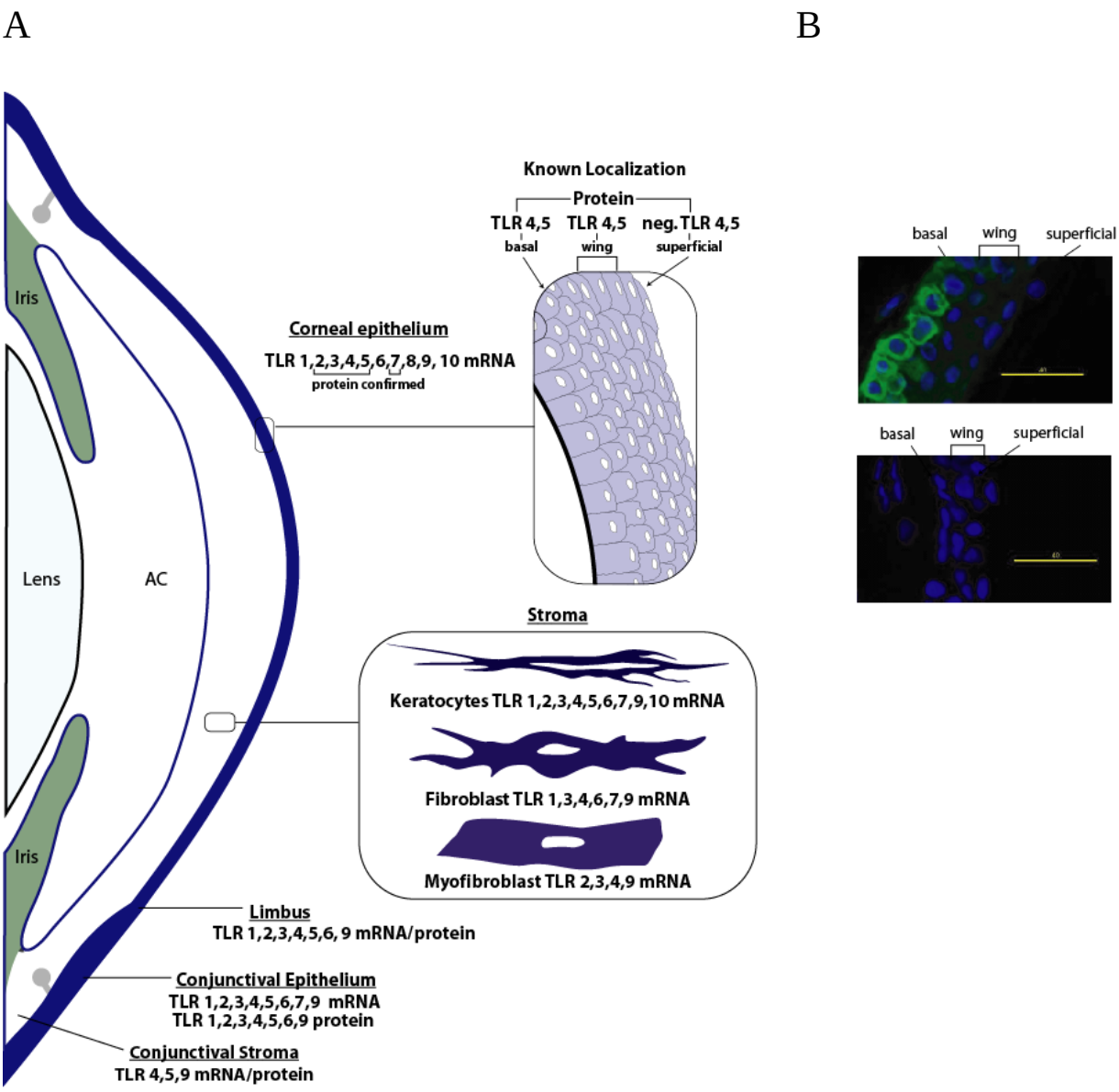


Figure 1.2 Expression of TLRs at the ocular surface

This summary is based on published data from multiple sources discussed in the text for human tissue sections and primary cultured cells. AC: anterior chamber, neg: negligible expression. (B) TLR 5 expression. TLR5 (green) is localized to basal and wing epithelial cells in normal human corneas (top image). The isotype control antibody revealed no

background staining (bottom image). 4,6'-diamidino-2-phenylindole (DAPI) was used to stain nuclei (blue). Scale bars represent 40 microns.

In summary, despite conflicting data from some laboratories, the current literature indicates the expression of multiple TLRs by corneal and conjunctival cells. They are capable of responding to invading pathogens providing a valuable defense mechanism to reduce microbial infection. However, TLR activation has the potential to do more harm than good, as it can lead to a robust inflammatory response which may contribute to disease processes as discussed below and summarized in Table 1.

Table 1.1 Association of TLRs with Ocular Surface Diseases

Disease	TLR
Herpes Simplex keratitis	TLR2,3,4,7,9
Pseudomonas keratitis	TLR4,5,9
Fungal keratitis	TLR2,4
Vernal keratoconjunctivitis	TLR4,9
Atopic Keratoconjunctivitis	TLR2
Sjögren's syndrome	TLR1,2,3,4
Non- Sjögren's syndrome	TLR2,4,5,9

1.3 TLRs in Ocular Surface Disease

1.3.1 Infection

Given that the major function of TLRs is pathogen recognition, it follows that these receptors play an important role in the ocular surface immune response to infectious agents. TLR activation by pathogens on the ocular surface would be expected to result in production of cytokines and chemokines important for stimulating immune and inflammatory cell infiltration into the area to alleviate the microbial load and resolve the infection. Further, TLR activation may facilitate initiation of the acquired immune response by enhancing MHCII and co-stimulatory molecule expression on antigen presenting cells resident in the cornea and conjunctiva (Hamrah and Dana 2007; Yamagami *et al.*, 2007). These two arms of the immune response may protect the ocular surface from microbial infection. However, the sequelae from the inflammatory response may result in damage to the ocular surface over and above that from the initial infection. Involvement of TLRs in various ocular infections is discussed here.

1.3.1.1 Herpes Simplex Keratitis

Herpes simplex virus (HSV)-1 is a frequent cause of corneal blindness that commonly necessitates a corneal transplant and is one of the leading causes of unilateral infectious corneal blindness worldwide (Liesegang, 2001). In the US and France, the incidence of new cases of ocular HSV-1 infection is estimated to be 8.4–13.2 per 100,000 persons annually with an overall incidence, including recurrences, of 20.7–31.5 episodes per 100,000 persons (Labetoulle *et al.*, 2005; Liesegang *et al.*,1989). Infection of the corneal

epithelium and stroma leads to a robust inflammatory response which may produce sight-threatening keratitis. Although this response helps reduce viral load, it also contributes to ocular surface damage, resulting in corneal scarring and vision loss. Notably, the absence of TLR2 or TLR4 and to a lesser extent TLR9 in transgenic knockout mice resulted in significantly diminished vision impairing HSV corneal lesions compared to wild-type (WT) mice (Sarangi *et al.,* 2007). Furthermore, mice lacking the adapter molecule MyD88, which is required for signaling of all TLRs (except TLR3), were resistant to lesion development. Despite this, they were unable to control the HSV infection and most succumbed to lethal encephalitis (Sarangi *et al.*, 2007). These findings suggest that TLR participation in particular, contributes to reducing the viral load but also promotes sight-threatening inflammation in HSV infection.

Jin *et al.* (2007) showed that all TLRs were expressed in human corneas with active HSV infection, but in particular TLR4, 7, 8 and 9 mRNA expression and TLR2 and TLR9 protein were upregulated relative to healthy corneas. Also, corneas with prior HSV infection showed an upregulation of TLR7 and downregulation of all other TLRs. Unfortunately, the authors did not correlate TLR expression with particular cell types, making it difficult to fully interpret these observations.

Infection of corneal epithelial cells with HSV-1 (KOS strain) caused two peaks of activation of NFκB and mitogen activated protein kinase (MAPK) (Li *et al.,* 2006). The first (1-4 hours post-infection) was associated with increased expression of IL-6, IL-8, TNFα and IFNγ. The second phase (8hrs post-infection) was associated with enhanced expression of TLR7 but downregulation of TLR3, results in keeping with Jin *et al.* (2007). These observations suggest that TLRs may function sequentially; with TLR3

being activated first and TLR7 subsequently upregulated as a consequence of the infection (Li *et al.,* 2006). Cook *et al.* (2004) have suggested that persistent TLR activation by HSV antigens and DNA may lead to prolonged expression of cytokines/chemokines and contribute to pathology after the active infection has subsided.

Together, these data suggest that TLRs 3, 4, 7 and 9 participate in the epithelial response to HSV infection and activation of specific TLRs leads to the production of antiviral molecules that can directly participate in protecting the ocular surface. TLR activation is important for resolution of viral infection as demonstrated by the success of Imiquimod, a TLR7 agonist approved by the FDA for treating human papilloma viral infections which notably also has had some success in treating HSV infections in clinical studies (Miller *et al.*, 2008). However, whereas activation of some TLRs is clearly beneficial, excessive inflammation at the ocular surface can also result, leading to sight-threatening inflammation during the attempts to control the viral infection.

1.3.1.2 Bacterial Keratitis

Pseudomonas aeruginosa (PA) and *Staphylococcus aureus* (SA) are two of the most common isolates from patients with microbial keratitis (Pachigolla *et al.*, 2007), with PA being the most common cause of bacterial keratitis in extended-wear contact lens users (Green *et al.*, 2007). Several studies have found that contact lens wear is the highest risk factor for the development of serious bacterial keratitis (Kerautret *et al.*, 2006). In the US, the overall annual rate of microbial keratitis with visual acuity loss in silicone hydrogel contact lens wearers is 3.6 per 10,000 (Schein *et al.*, 2005). Notably, the number of ocular isolates of methicillin-resistant Staphylococcus aureus (MRSA) has increased from 4.1% in 1998 to 1999 to 16.7% in 2005 to 2006 (Freidlin *et al.*, 2007). If not treated promptly,

microbial keratitis can lead to epithelial defects, stromal ulceration, scarring and vision impairment. Cultured corneal and conjunctival epithelial cells respond to ocular surface pathogens, their extracts and known PAMPs by the production of cytokines and chemokines characteristic of TLR activation which *in vivo*, is expected to recruit immune and inflammatory cells to destroy the invading pathogen. Furthermore the activation of ocular surface epithelial cells by pathogens and TLR agonists also leads to the production of antimicrobial peptides such as hBD-2 and the cathelicidin LL-37 (Kumar *et al.,* 2006b, 2007; McNamara *et al.,* 1999; Redfern *et al.,* 2006; Chapter 2), which are discussed in more detail in section 1.4. These peptides can kill pathogens by causing membrane disruption through an electrostatic interaction of the positively charged peptide and the negatively charged microbial membrane (Radek & Gallo 2007). Thus, in addition to facilitating effector cell recruitment, TLR activation at the ocular surface is expected to lead to local production of antimicrobial peptides that can assist in eliminating pathogens.

Several studies have found that SA can cause severe keratitis in infected individuals and in animal models as characterized by bacterial invasion of the underlying stroma and intense neutrophil infiltration which results in corneal opacification and potentially loss of vision (Hume *et al.,* 2001; Girgis *et al.,* 2003; Hume *et al.,* 2005; Bourcier *et al.* 2003, Sloop *et al.,* 1999). In an experimental mouse model of SA keratitis, exposure of corneal epithelium to SA increased neutrophil recruitment to the corneal stroma, corneal thickness and corneal haze in normal C57Bl/6 mice, mice deficient TLR4 or TLR9, but not in mice deficient in TLR2 or MyD88, suggesting that SA-induced corneal inflammation is mediated by TLR2 and MyD88 (Sun *et al.,* 2006). The same group also reported that UV killed SA and Pam3Cys (TLR2 synthetic ligand) stimulated

the phosphorylation of MAP kinases, JNK, p38 MAPK and ERK. Blockade of JNK, but not that of p38 or ERK phosphorylation, had an inhibitory effect on IkB alpha degradation and CXC chemokine production (Adhikary *et al.,* 2008). Furthermore, they found that corneal inflammation was significantly impaired in mice deficient in JNK1 compared with control mice, suggesting that JNK has an essential role in TLR2-induced corneal inflammation.

Extensive study of the underlying mechanism of the pathogenesis of PA keratitis in experimental models has revealed that mice can be divided in two groups based upon their immune response to the pathogen (Hazlett, 2004). BALB/c mice are resistant to PA infection because they mount a Th2 based response that facilitates recovery and corneal healing. In contrast, C57BL/6 mice are susceptible to PA infection as they mount a Th1 based immune response leading to corneal perforation. Comparison among these mouse strains provides a unique opportunity to understand the immune response to PA and involvement of TLRs. In 2005 Huang *et al*., reported that silencing TLR9 by siRNA in C57BL/6 mice resulted in less severe inflammation, reduced PMN infiltration, but consequently increased bacterial load. These data suggested that TLR9 activation is required to adequately eliminate bacteria but that it also contributes to corneal destruction. Subsequently, the same group also reported that corneal TLR4 expression is increased in PA infection and deficiency of this receptor in BALB/c mice resulted in a susceptible rather than resistant phenotype (Huang *et al.,* 2006a). These observations suggest that TLR4 is critical for resistance to PA keratitis.

Additional animal studies have shown that single Ig IL-1R-related molecule (SIGIRR) and ST2 are also required for resistance to PA infection (Huang *et al.,* 2006b,

2007). SIGIRR and ST2 are negative regulators of TLR signaling that act by sequestering MyD88 and IRAK. Blocking SIGIRR or ST2 activity was associated with more serious clinical disease, indicating that although TLR signaling is required for resistance to PA keratitis, if its activity cannot be adequately regulated by SIGIRR/ST2, ocular surface damage ensues. Thus, TLR participation in PA keratitis is essential for eliminating the organism, but is problematic as it contributes to corneal destruction. Such duality makes it difficult to envision modulating TLR activity as a means to control corneal damage.

In an interesting *in vitro* study, Maltseva *et al.*, (2007) reported that MyD88 dependent increase in corneal epithelial hBD-2 expression caused by exposure to PA supernatant was abrogated by the presence of a contact lens, thus giving new insight into the mechanism by which contact lens wear predisposes to PA keratitis. Additional *in vivo* studies have shown that defensins and LL-37 play an important role in protecting the ocular surface from PA infections. In particular, mice deficient in cathelicidin-related antimicrobial peptide (CRAMP), the murine homolog of LL-37 are more susceptible to PA keratitis, had significantly delayed bacterial clearance and an increased number of infiltrating neutrophils in the cornea (Huang *et al.*, 2007). A similar finding was reported in BALB/c mice following knock down of mBD-2 or mBD-3, but not of mBD-1 or mBD-4, by siRNA (Wu *et al.*, 2009a, 2009b). Furthermore Wu *et al.* also found that silencing mBD2, mBD3 or both defensins resulted in a significant upregulation of TLR2, TLR4 and MyD88 but not TLR5 or TLR9 (Wu *et al.*, 2009b). A recent study by Kumar *et al.* (2008) also has revealed an interesting therapeutic possibility. They observed that pre-treatment with the TLR5 agonist flagellin markedly reduced the severity of subsequent PA infection in C57BL/6 mice. This was in part due to induction of corneal expression of the

antimicrobial molecules, nitric oxide and cathelin-related antimicrobial peptide (the murine homolog of LL-37). They also observed similar results *in vitro*, as flagellin pre-treatment enhanced PA induced expression of hBD-2 and LL-37 in HCEC (Kumar *et al.*, 2007). These observations raise the possibility of utilizing TLR activation as a prophylactic means of preventing an overwhelming inflammatory response and corneal destruction in PA keratitis.

1.3.1.3 Fungal Keratitis

Fungal keratitis is characterized by a severe inflammatory response initiated by virulence factors which is then exacerbated by the host response that may lead to destruction of the cornea and poor visual outcome. Fungal keratitis typically occurs in tropical or subtropical climates such as in southern Florida, and *Fusarium* is the most common isolate (41%) followed by Candida (14%) and Apergillus (12%) (Iyer *et al.*, 2006). In fact, fungal keratitis can account for up to 52% of microbial keratitis cases in CL wearers in Florida (Tuli *et al.*, 2007). A study from the Massachusettes Eye and Ear Infirmary in the Boston area, found that the number cases of fungal infections has doubled from 1999-2002 to 2004-2007 with approximately 1.0 case per month (Jurkunas *et al.*, 2009). In early 2006, multiple reports of *Fusarium* keratitis among CL wearers were submitted to the Centers for Disease Control and Prevention and later found to be associated with Bausch & Lomb ReNu Multiplus (Rochester, NY) brand contact lens solution (Chang *et al.*, 2006). Since the recall of this solution, the number of cases has dropped (Chang et. al., 2006) but in some cities fungal keratitis is the leading cause of microbial infections. In a three year retrospective study of patients with microbial keratitis in Brazil, *Fusarium* was the most

common isolate from patients with microbial keratitis but ocular trauma was found to be the major risk factor in these cases (Furlantto *et al.,* 2010).

Little is known of the precise role of TLRs in fungal keratitis but they have been implicated in fungal recognition and subsequent cytokine production. Known fungal TLR ligands include zymosan which activates TLR2/TLR6 heterodimers, whereas mannan activates TLR4 (Roeder *et al.,* 2004). Interestingly whereas TLR4 activation induces chemokine release and leukocyte recruitment, TLR2 activation results in the production of anti-inflammatory IL-10 and T-regulatory cell proliferation which may represent an attempt to circumvent host defense mechanisms (Netea *et al*., 2006).

As noted above, members of the genus *Fusarium* are the most frequently isolated organisms in patients with fungal keratitis (Iyer *et al.,* 2006). Inactivated hyphae of *Fusarium solani* upregulated the expression of TLR2, 3, 4, and 6 mRNA, TLR2 and 4 protein and increased secretion of IL-6 and IL-8 in HCEC (Jin *et al.,* 2008). Further, a recent *in vivo* study of contact lens associated *Fusarium* keratitis has shown that mice deficient in TLR4 but not TLR2 have impaired responses to *Fusarium* indicating that TLR4 plays a role in controlling growth and replication of the pathogen (Sun *et al*., 2009).

Aspergillus fumigatus is another fairly common culprit in fungal keratitis. Guo and Wu (2009) found that exposure of HCEC to *Aspergillus fumigatus* antigens upregulated TLR2 and TLR4 and stimulated the release of IL-1α and IL-6. Furthermore, they recently reported that exposure of HCEC to *Aspergillus fumigatus* antigens resulted in the release of IL-10 that was inhibited by treatment with TLR2, and TLR4 antibodies (Zhao *et al.,* 2009). Together, these results suggest that TLR2 and TLR4 are involved in the ocular

surface response to fungal pathogens but much remains to be understood of their specific roles.

In summary, TLRs play a critical role in recognizing and responding to various microbes on the ocular surface. The absence of specific TLRs can result in uncontrolled microbial infection that can be fatal in some animal models. In contrast, excessive TLR activation can stimulate sight-threatening inflammation. Further understanding of the involvement of specific TLRs would shed light on how a balance between microbial clearance and an appropriate inflammatory response can be achieved, allowing for the development of potential therapeutic paradigms to optimize anti-microbial effects while minimizing damaging inflammatory responses.

1.3.2 Ocular Surface inflammation

Because activation of TLRs leads to the production of inflammatory cytokines, it is reasonable to hypothesize that these receptors may play a role in mediating some of the events in inflammatory ocular surface disorders. In such a scenario it is envisaged that TLR activation would, most likely, be via various endogenous ligands and/or normal flora bacteria rather than pathogens. For example, a sudden excess of endogenous ligand may lead to TLR over-activation, or a breach of the superficial epithelial layers may provide access to TLRs normally hidden from ocular surface commensals. The involvement of TLRs in two ocular surface disorders characterized by inflammation, allergic conjunctivitis and dry eye, are discussed here.

1.3.2.1 Allergic conjunctivitis

Allergic conjunctivitis (AC) refers to hypersensitivity disease affecting the eye lids, conjunctiva and sometimes the cornea. The term covers various clinical forms including

seasonal allergic conjunctivitis, vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). A number of studies have linked TLRs with systemic allergic disease (Bauer *et al.*, 2007), thus it is reasonable to suggest that these versatile receptors may have a role in ocular allergy too. Bonini *et al.* (2005) evaluated TLR2, 4, 9 expression in conjunctival biopsy specimens from 10 patients with a normal ocular surface undergoing cataract surgery and 9 patients with VKC. All three TLRs were detected by immunostaining in the conjunctival epithelium and stroma of both normal and VKC patients, with staining being more intense in the stroma. Comparing the two groups, TLR4 expression was increased, TLR9 was decreased and there was no significant change in TLR2 expression in VKC versus normal conjunctiva. In VKC stroma, some TLR4 staining was localized to CD4+ve T-cells, eosinophils and mast cells; also some TLR9 staining co-localized to CD4+ve T-cells and eosinophils but not mast cells. Positive staining was also found on cells with fibroblast-like morphology. It remains to be determined if these changes in TLR expression are the "cause or effect". Signaling through TLR4 has been shown to induce a Th2 mediated allergic response in a mouse model (Eisenbarth *et al.*, 2002). Increased expression of TLR4 may be accounted for by cells infiltrating the conjunctiva in response to VKC and it is possible that enhanced TLR4 expression and activation may lead to overproduction of pro-inflammatory cytokines and chemokines that exacerbate the ongoing inflammatory response.

Cook *et al.* (2005) noted increased immunostaining for TLR2 in conjunctival impression cytology samples from patients with AKC. Their accompanying *in vitro* investigation revealed that SA and IFNγ upregulated TLR2 expression in cultured conjunctival epithelial cells and that SA and TLR2 agonists stimulated IL-8 and TNFα

production. As SA colonization is common in AKC, they suggested that SA maybe responsible for TLR2 upregulation and activation, and that TLR2 mediated cytokine production may contribute to the ongoing inflammatory response. The upregulation of TLR2 in patients with AKC raises an interesting possibility that TLR2 can also be activated by endogenous ligands. For example, HSP70, an endogenous ligand for TLR2 is expressed in the conjunctival epithelium in patients with AKC (Berra *et al.,* 1994) and conceivably could contribute to inflammation by activating over-expressed TLR2.

In seeking to understand the mechanisms underlying ocular allergy, Fukushima *et al.* (2006) treated mice with TLR2 agonist, Pam3CSK4, to determine if it could affect the development of experimental immune-mediated blepharoconjunctivitis induced by short ragweed pollen. Interestingly, treatment during the efferent phase significantly suppressed eosinophil infiltration and this was attributed to apoptosis of CD4+ T-cells. Previously, in a similar mouse model, it had been observed that oligonucleotides with unmethylated CpG motifs (TLR9 agonist) administered after ragweed sensitization inhibited the immediate hypersensitivity response and later infiltration of inflammatory cells and also induced a ragweed specific Th1 response (Magone *et al.,* 2000; Miyazaki *et al.,* 2000). These observations suggest that the development of AC may be modulated by TLR agonists. Indeed, a large body of evidence has accumulated indicating that TLR9 ligands are effective in the prevention and treatment of animal models of a variety of allergic disorders and a number of TLR9 agonists are in clinical trials for allergic rhinitis and asthma (Hayashi & Raz, 2006; Kanzler *et al.,* 2007).

1.3.2.2 Dry eye and TLR expression

Dry eye is a common multi-factorial disorder in which inflammation plays a major role. The core mechanisms are driven by tear hyperosmolarity (Gilbard *et al.,* 1978; Farris 1994; Bron *et al.,* 2002) and tear film instability which stimulates an increase in proinflammatory cytokines at the ocular surface (Afonso *et al.,* 1999; Pflugfelder *et al.,* 1999; Solomon *et al.,* 2001). The resulting inflammation exacerbates tear film instability and hyperosmolarity creating a vicious cycle (2007 Report of the Dry Eye Workshop). At its most severe (dry eye associated with the autoimmune disorder Sjögren's syndrome (SS)), dry eye carries an increased risk of corneal ulceration (Vivino *et al.,* 2001) and infection (Derk and Vivino 2004) which may result in vision loss. In SS, the activity of self-reactive lymphocytes leads to damage and destruction of the salivary and lacrimal glands leading to severe dry mouth and dry eye. Kawakami *et al*. (2007) studied TLR expression by immunohistochemistry in labial salivary glands and observed increased expression of TLR2, 3 and 4 and also the adaptor molecule MyD88 in samples from patients with SS. Staining was localized to acinar cells, ductal epithelial cells and infiltrating mononuclear cells. They also observed that TLR agonists stimulated production of IL-6 and expression of CD54 (ICAM-1) in a cultured salivary gland cell line and thus concluded that TLR activation may contribute to the inflammatory microenvironment in SS. Spachidou *et al.,* (2007) also reported TLR1-4 expression and upregulation of CD54, CD40 and MHC I in response to TLR activation in epithelial cells from labial salivary gland biopsies. Furthermore, the same group found that expression of TLR1, 2 and 4 was significantly greater in cells from SS patients, suggesting the active participation of TLRs in the pathophysiology of SS. A gene array study also revealed

upregulation of TLR8 and 9 in labial salivary glands from SS patients (Gottenberg *et al.*, 2006). In a mouse model of SS, Killedar *et al.*, (2006) observed that TLR3 and 7 and several of their downstream effectors were upregulated in the submandibular glands early in the disease course. Together these observations point to a role for TLRs in salivary gland inflammation in SS and considering similarities between the two secretory systems similar findings are expected in the lacrimal glands. In keeping with this, TLR4 mRNA expression was increased in the lacrimal glands and cornea in the SS (Murphy Roth Large/ lymphoproliferation (MRL/lpr)) mouse model (Christopherson PL, *et al.* IOVS 2005; 46: ARVO E- Abstract 4462).

Although SS causes severe dry eye, non-SS dry eye is more prevalent. In a pilot study, TLR2 but not TLR4 mRNA was upregulated in conjunctival cells in patients with dry eye (Barabino S, *et al. IOVS* 2006; 47: ARVO E- Abstract 5594). However the authors did not observe a concomitant increase in TLR2 protein expression. In studies here it was observed that dry-eye related cytokines (IL-1α and β, TNFα and TGFβ) had no effect on ocular surface epithelial cell TLR expression, although hyperosmotic culture media increased expression of TLR4, decreased expression of TLR9 but had no effect on TLR5. Furthermore, we found that a desiccating environment upregulated HCEC expression of TLR4 and 5, but downregulated TLR9 (Chapter 3). These data indicate that dry eye conditions differentially modulate TLR expression and are suggestive of TLR participation in the pathophysiology of non-SS as well as SS dry eye.

As noted above, it is unknown if altered ocular surface TLR expression in inflammatory conditions is cause or effect. However, regardless of the etiology a change in expression pattern may have both beneficial and detrimental effects. Upregulated

expression may confer an enhanced ability for pathogen recognition, whereas reduced expression may lead to an inadequate response and therefore increased risk of infection. However, the latter may be compensated for by the fact that many pathogens are recognized in more than one way, by interactions with multiple TLRs and interaction with other PRRs. For example, mice deficient in TLR4 still respond to PA (Huang *et al.*, 2006a), presumably in part through the activation of TLR5 by PA flagellin. Enhanced TLR expression may lead to inappropriate and exacerbated inflammatory responses thus contributing to disease processes such as allergy and dry eye, whereas reduced expression would be expected to be anti-inflammatory. In general, activation of the various TLRs leads to a similar response by ocular surface epithelial cells i.e. cytokine/chemokine and antimicrobial peptide production. Therefore it is possible that downregulation of the expression of some TLRs is a compensatory response for the upregulation of other TLRs, to try to minimize hyper-responsiveness. Much is yet to be learned about the contribution of TLRs to ocular allergy and dry eye. In particular, studies investigating endogenous TLR ligands (e.g. heat and stress shock proteins, high mobility group box 1 protein etc) are currently lacking but will be very important in furthering our understanding of the role of TLRs in the pathophysiology of ocular surface inflammation.

As discussed here current evidence indicates that TLRs are important molecules expressed at the ocular surface. They have a primary role in detecting the presence of various pathogens leading to activation of innate immune responses such as production of antimicrobial peptides and recruitment of immune and inflammatory cells by chemokine and cytokine production. Unfortunately, TLR action may be a double edge-sword as the consequence of their activation may contribute to ocular surface destruction during

infection and the exacerbation of various ocular surface inflammatory conditions such as allergy and dry eye.

1.4 Antimicrobial Peptides

The ocular surface is constantly exposed to a myriad of pathogens yet despite this unrelenting challenge, the cornea and conjunctiva rarely succumb to infection. The ocular surface is well equipped with multiple defense mechanisms to ward off potential pathogens, including an intact ocular surface epithelium that forms a physical barrier from the external environment and enzymes and other proteins in the tear film that have potent antimicrobial activity (Sack *et al.,* 2001). In addition to these, a number of cationic antimicrobial peptides (AMPs) have also been identified at the ocular surface.

Antimicrobial peptides are small peptides, typically consisting of less than 50 amino acids with the majority of them considered to be cationic, as they carry an overall positive charge due an excess of positively charged amino acids (McDermott, 2009). They exert their antimicrobial activity through electrostatic interactions with negatively charged microbial membranes and this interaction is salt sensitive for some AMPs (Zasloff, 2002). They are an essential component of the innate immune system and have been identified among a wide array of organisms including bacteria, fungi, plants, invertebrates and vertebrates.

Of the AMPs, defensins and cathelicidins represent the two distinct classes that are most studied. Defensins contain six cysteine residues connected by three intramolecular disulphide bonds that lead to the formation of a β-sheet structure. Based on the location of disulphide bonds, defensins are further classified into α-defensins and β-defensins. There are six human α-defensins, four human neutrophil peptides (HNP)-1-4 which, as their

name implies, were first discovered in neutrophils (Ganz *et al.,* 1985; Selsted *et al.,* 1985, Wilde *et al.,* 1989), and human defensin (HD)-5 and 6 which were first discovered in Paneth cells in the intestine (Jones and Bevins, 1992, 1993). Four human β-defensins, (hBD)-1-4 have been well characterized and are expressed in a variety of cell types, but are most often found in epithelial cells (Lehrer and Ganz, 2002). LL-37 is the only cathelcidin expressed in humans and as its name implies, it begins with two leucine (L) residues and has 37 amino acids. LL-37 is derived from the inactive precursor, human cationic antimicrobial protein-18 (hCAP-18) which was first found in neutrophils where it is stored in secretory granules as hCAP-18 and upon secretion is cleaved by proteinase-3 into the active form, LL-37 (Sorensen *et al.,* 2001).

In addition to their antimicrobial effects, defensins have been shown to modulate a variety of cellular activities including chemotaxis of T cells, dendritic cells (Chertov *et al.*,1996; D. Yang *et al*.,1999), and monocytes (Territo *et al.*,1989), stimulation of epithelial cell and fibroblast proliferation (Murphy *et al*.,1993; Aarbiou *et al*.,2002; Li *et al.* 2006), stimulation of cytokine production (Chaly *et al.,* 2002; Van Wetering *et al.*,1997), and stimulation of histamine release from mast cells (Scott *et al.,* 2002; Niyonsaba *et al.,* 2002). LL-37 has been shown to be chemotactic for neutrophils, mast cells, monocytes, T lymphocytes and is thought to stimulate inflammation through modulating chemokine and cytokine production by macrophages and histamine release from mast cells (Befus *et al.,* 1999; Niyonsaba *et al.,* 2001). Huang *et al.* (2006) reported that LL-37 can induce HCEC migration and secretion of IL-8, IL-6, and IL-1β and TNF-α. Similarly, Li *et al.* (2008) found that defensin HNP-1, hBD-2 and hBD-3 stimulated the production of cytokines and chemokines such as IL-6, IL-8 and RANTES in a normal

human conjunctival epithelial cell line (IOBA-NHC) which in turn are likely involved in the recruitment of inflammatory cells following injury or insult to the ocular surface.

Defensins and LL-37 are expressed on the ocular surface and may arise from different cell types. Alpha defensins arrive at the ocular surface via infiltrating neutrophils, β-defensins are secreted from the epithelia, while LL-37 can be secreted by both cell types. hBD-1 and hBD-3 and LL-37 are constitutively expressed by both corneal and conjunctival epithelia whereas hBD-2 expression is inducible by conditions mimicking injury, inflammation and in response to bacterial products (Gordon *et al.*,2005; McDermott *et al.*, 2001, 2003; McNamara *et al.*,1999; Narayanan, *et al.*,2003).

Recent *in vivo* studies in animal models have shown that cathelicidin and defensins play an important role in protecting the ocular surface from PA infection. Which as noted earlier (section 1.3.1.2) is the leading cause of infectious keratitis associated with contact lens users (Fleiszig and Evans, 2002). Futher, there has been emergence of multi-drug resistant PA strains (Rossolini & Mantengoli, 2005) which has prompted studies to investigate the potential role of AMP in PA infections. As discussed earlier (section 1.3.1.2), mice deficient in CRAMP (Huang *et al.*, 2007a), mBD-2 or mBD-3 (Wu et. al, 2009a, 2009b) were susceptible to PA keratitis, moreover the induction of corneal antimicrobial peptide expression as a result of pre-treatment with flagellin reduced the severity of subsequent PA infection in C57BL/6 mice (Kumar *et al.*, 2008). Taken together, these suggest that AMPs are an essential component of immune response to protect against PA infections.

1.5 Dry Eye

Dry eye syndrome is an ocular surface condition that affects millions of individuals every year and is one of the leading causes for visits to the eye doctor (Brewitt and Sistani, 2001). Clinically, patients often report symptoms of ocular surface irritation such as sharp pain, grittiness, itching, redness, and burning. Patients with severe dry eye, have an increased risk for corneal ulceration and melting (Vivino *et al.,* 2001) and ocular infection (Derk and Vivino, 2004) which may result in vision loss. Although dry eye typically does not result in blindness, patients often report a decreased quality of life and reduced ability to work and perform daily tasks such as reading and driving. Despite its common occurrence, the pathogenesis of dry eye is not fully understood. Dry eye is considered to be a multifactorial condition and inflammation is thought to be a significant component of dry eye. This is apparent by the improvement in ocular signs and symptoms with anti-inflammatory therapies, such as cyclosporine A, corticosteroids, and doxycycline (Pflugfelder, 2004).

Dry eye results from either a deficiency of the lacrimal gland to produce tears or excessive tear evaporation (typically from meibomian gland disease), which leads to damage to the ocular surface and symptoms of ocular discomfort (Lemp, 1995). Dry eye can occur in men or women of any racial group at any age; however several studies have found a higher risk of dry eye in women (Smith *et al.,* 2004; Schaumberg *et al.,* 2003). Compared with Whites, Hispanic and Asian women were more likely to report severe symptoms, but not be clinically diagnosed with dry eye. There were no significant differences by income, however more educated women were less likely to have dry eye (Schaumberg *et al*., 2003).

Additionally, Sjögren's syndrome (SS), a chronic severe form of dry eye affects women nine times more frequently than men. Sjögren's syndrome is a systemic, autoimmune inflammatory disorder characterized by lymphocytic infiltration of most notably the lacrimal gland resulting in a very severe form of dry eye (Derk and Vivino, 2004). Additionally these patients also typically have dry mouth, joint pain and fatigue. Several cytokines were identified on the ocular surface of SS patients such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β (Pflugfelder *et al.,* 1999) suggesting that these cytokines may have a role in the pathogenesis of the ocular surface alteration.

Mouse models are essential to investigate the mechanisms of dry eye disease *in vivo*. In efforts to recapitulate dry eye that occurs in humans, several animal models have been created by various means, including surgical removal of the lacrimal glands, ocular surface desiccation by mechanically inhibiting blinking, and pharmacologic inhibition of tear secretion (Fujihara et. al., 1998 and 2001; Burgalassi *et al*., 1999, Song *et al.,* 2003). A botulinum toxin B-induced mouse model has also been developed in which the toxin is used to block cholinergic muscarinic receptors in the lacrimal gland producing dry eye (Suwan-apichon *et al.,* 2006) due to aqueous deficiency. Inhibiting tear secretion in mice by systemic administration of the muscarinic cholinergic antagonist scopolamine and a low humidity is one of the most widely accepted and established models of dry eye (Dunsun *et al.,* 2002). This model produces dry eye that mimics human dry eye disease and was used in this study to examine dry eye in the mouse model.

Previous studies have found that ocular surface irritation can be attributed to an increase in osmolarity of the tear film (Balik, 1952). Elevated tear film osmolarity has

been linked to the signs and symptoms of dry eye, whether the increase results from decreased tear production or increased evaporation. In 1970, Mishima confirmed tear osmolarity of dry eye patients was elevated by about 25mOsm/L compared to normals. In 2005, Lou *et al.,* demonstrated that hyperosmolarity stimulated the expression of pro-inflammatory cytokines IL-1α, TNF-α and matrix metalloproteinase (MMP)-9 on the ocular surface *in vivo* in a murine model. Taken together, these results suggest that hyperosmolar tears as seen in dry eye stimulate an inflammatory reaction by the production of proinflammatory cytokines and matrix MMP-9. The latter, has destructive effects on the ocular surface, it is known to lyse components of the corneal epithelial basement membrane and tight junction proteins, such as ZO-1 and occludin which maintain corneal epithelial barrier function (Pflugfelder *et al.,* 2005; De Paiva *et al.,* 2005). The increased MMP-9 activity in dry eye is in part responsible for increased corneal epithelial desquamation (punctate epithelial erosions) and corneal surface irregularity (Pflugfelder *et al.,* 2005). This can have devastating effects on vision and the health of the eye, as the cornea must provide an intact smooth refracting surface for clear vision and to prevent harmful agents such as bacteria, viruses and noxious agents from invading the eye.

Another important pathologic finding in dry eye is increased apoptosis or programmed cell death of the ocular surface epithelia (Yeh *et al.,* 2003). Apoptosis occurs through two pathways, an extrinsic pathway involving death ligands (e.g., TNF-α, Fas ligand) and an intrinsic pathway that is initiated by DNA damage. Both pathways result in mitochondrial damage and activation of caspases, such as caspase 3. Apoptosis is thought to be involved in the pathogenesis of keratoconjunctivitis sicca (KCS), a severe form of

dry eye commonly seen in Sjögren's syndrome patients. In these patients and other patients with dry eye, the lacrimal gland and the ocular surface become irritated and inflamed through the production of cytokines that interfere with the normal neural connections that drive the tearing reflex (Stern *et al.,* 1998). The ocular surface becomes poorly lubricated and has altered barrier function manifesting clinically as increased permeability to fluorescein dye (Pflugfelder *et al.,* 1998). Ultimately dry eye patients have hyperosmolar tears with increased levels of proinflammatory cytokines and MMPs. Additionally the ocular surface displays various pathological findings such as epithelial cell apoptosis and desquamation and goblet cell loss. The cause of these pathologic changes is not well characterized; however inflammation appears to play a significant role.

1.6 Significance and Research Goals

Ocular surface inflammation is a major health concern and presents from various etiologies including dry eye, ocular allergies and microbial infections. A PA infection is particularly devastating to the ocular surface and is the most common cause of contact lens related microbial keratitis. In these infections, the immune response may reduce the microbial load however the sequelae from the inflammation may result in the rapid destruction of the cornea beyond that from the initial infection. Given that the major function of TLRs is pathogen recognition, it follows that these receptors play an important role in the ocular surface immune response to PA. TLR activation on the ocular surface has the potential to stimulate a robust sight threatening inflammatory response. Further understanding of the involvement of specific TLRs would shed light on how a balance between microbial clearance and an appropriate inflammatory response can be achieved,

allowing for the development of potential therapeutic paradigms to optimize antimicrobial effects while minimizing damaging inflammatory responses.

Dry eye syndrome is also a significant health concern that reduces the quality of life of millions of individuals every year. Ironically, despite its common occurrence, there are few therapeutic interventions available and treatment is often palliative and moreover the pathogenesis is still not fully understood. TLRs may play a role in mediating some of the events in dry eye inflammation. In such a scenario it is envisaged that TLR activation would, most likely, be via various endogenous ligands and/or normal flora bacteria rather than pathogens. For example, a sudden increase in the number of TLRs and/or endogenous ligands may lead to TLR over-activation, or a breach of the superficial epithelial layers may provide access to additional TLRs normally hidden from ocular surface commensals. Therefore determining the potential role for TLR in the pathogenesis of dry eye may lead to the development of much needed novel therapeutic options.

The overall goal of this study was to investigate TLR expression on the ocular surface and to determine the role of TLRs in pathogenesis of PA infection and dry eye to advance the development of novel therapeutic options for these sight threatening conditions. Upon commencing this research project, little was known regarding TLR expression on the ocular surface and their potential involvement in PA infections and dry eye syndrome. Therefore TLR expression was determined in various ocular surface cells and if TLR activation can modulate the expression of functionally active antimicrobial peptides and their own expression (chapter 2). Secondly, TLR expression was determined in (1) patients with dry eye, (2) animals with experimental dry eye and (3) in ocular

surface cells in response to dry eye associated conditions *in vitro* and using an organ culture desiccation model (chapters 3-4).

The first specific aim of the current research was to determine TLR mRNA and/or protein expression in various ocular surface cells, if TLR activation stimulates the production of functionally active AMP and if AMPs and TLR agonists modulate TLR mRNA expression in ocular surface cells (chapter 2). The next objective was to determine which dry eye associated factors modulate the expression of TLRs. To do this, several studies were designed using dry eye subjects, an established mouse model of dry eye, and to isolate various components of dry eye several *in vitro* studies were designed in ocular surface cells. Specifically, in chapter 3, the second specific aim was to establish TLR4, 5 and 9 expression patterns in human conjunctival epithelial cells from subjects with dry eye and age- and gender-matched normal subjects and to determine if dry eye associated conditions (hyperosmolar stress, cytokines or desiccation) modulate TLR4, 5 and 9 expression in ocular surface cells *in vitro* and in an organ culture desiccation model. Using an established mouse model, my third specific aim was to determine if experimental dry eye (EDE) modulates TLR and/or AMP expression in C57BL/6 mice and dry eye subjects and to determine if topical application of a TLR agonist cocktail to the ocular surface in mice with EDE can stimulate an increase in inflammation in the mouse cornea and modulate the expression of mouse AMPs (chapter 4).

Determining the involvement of TLR on the ocular surface is important to further our understanding of the immune response that occurs in dry eye and PA infections. Given that TLRs have been implicated in several ocular surface diseases as described in chapter 1, the data gathered here may also be applied to the potential roles of TLR in other ocular

surface inflammatory conditions. The goal of this study is understand how TLR expression is modulated in ocular surface inflammatory conditions and also the functional consequence of a change in TLR expression on the ocular surface.

Chapter 2 - Toll-Like Receptor Activation Modulates Antimicrobial Peptide Expression by Ocular Surface Cells

2.1 Introduction

The ocular surface is constantly exposed to a myriad of pathogens yet despite this unrelenting challenge, the cornea and conjunctiva rarely succumb to infection. This is due to the fact that the ocular surface is well equipped with multiple defense mechanisms to ward off potential pathogens, including an intact ocular surface epithelium that forms a physical barrier to the external environment and enzymes and other proteins in the tear film that have potent antimicrobial activity (Sack *et al.*, 2001). A number of cationic AMPs have also been identified in human corneal and conjunctival epithelial cells such as human β-defensin (hBD) -1-3 and cathelicidin (LL-37), with the latter derived from the cleavage of human cationic antimicrobial protein (hCAP)-18. hBD-1, hBD-3 and LL-37 are constitutively expressed by both corneal and conjunctival epithelia whereas hBD-2 expression is inducible by conditions mimicking injury, inflammation and in response to bacterial products (Gordon *et al.*, 2005; McDermott *et al.*, 2001, 2003; McNamara *et al.*, 1999; Narayanan, *et al.*,2003).

In addition to their antimicrobial effects, AMPS have been shown to modulate a variety of cellular activities as described in Section 1.4. Some of these include stimulation

of chemotaxis of various immune cells (Chertov *et al.*, 1996; D. Yang *et al.*, 1999, Territo *et al.*, 1989), cell proliferation (Murphy *et al.*, 1993; Aarbiou *et al.*, 2002; Li *et al.* 2006), and cytokine production and histamine release (Chaly *et al.*, 2002; Van Wetering *et al.*, 1997; Scott *et al.*, 2002; Niyonsaba *et al.*, 2002; Befus *et al.*, 1999).

The ability of the ocular surface to respond to pathogens is in part attributed to a family of receptors called TLR which recognize conserved motifs on PAMPs on microbes leading to the production of inflammatory cytokines and co-stimulatory molecules, thus initiating innate and adaptive immunity (Janeway and Medzhitov, 2002). Toll-like receptors are expressed on a wide variety of cell types. In humans there are 10 functional TLRs, each having distinct ligands as described in Section 1.1. A link between TLR activation and upregulation of AMP expression has been established in a number of tissues. For example, in respiratory epithelial cells, stimulation of TLR2 with peptidoglycan (PGN) or lipopeptide upregulated hBD-2 expression (Hertz *et al.*, 2003; Homma *et al.*, 2004). Peptidoglycan also induced hBD-2 expression in intestinal epithelial cells (Vora *et al.*, 2004) and PGN and yeast wall particles induced hBD-2 expression in human keratinocytes (Kawai *et al.*, 2002). Also LL-37 expression can be induced by stimulation through TLR2, TLR4, and TLR9 in monocyte-derived macrophages (Rivas-Santiago *et al.*, 2008)

Upregulation of β-defensins and LL-37 through the activation of TLRs may play a role in the innate and adaptive immune system by providing the ocular surface with direct defense against various pathogens and stimulating the recruitment and activation of immune and inflammatory cells. To further investigate the link between TLR activation and AMPs, TLR expression was determined in various ocular surface epithelial cells, in

the process confirming some previous observations by others, and extended to stromal cells. As they are a first-line of defense against pathogens invading the ocular surface, both corneal and conjunctival epithelial cells were studied. Three sources of corneal epithelial cell were investigated: a cell line, primary cultured cells and cells from cadaver corneas. Comparisons among these are helpful to elucidate effects due to culture conditions/cell transformation and hence identify the actual TLR profile that most likely is present *in vivo*. Similarly, a conjunctival epithelial cell line and conjunctival epithelial cells isolated by impression cytology from human subjects were used to study TLR expression. Furthermore, as penetrating corneal injury will allow pathogens direct access to the corneal stroma, TLR expression by keratocytes in cadaver corneas and by their repair phenotype, the corneal fibroblast, in cell culture was investigated. Specific TLR agonists stimulated the expression of AMP (defensins and LL-37) mRNA and the production of functionally active AMPs that are effective in killing PA was examined. To further investigate the consequence of TLR activation, TLR expression was examined in ocular surface cells treated with AMPs and TLR agonists.

2.2 Materials and Methods

Corneal and conjunctival cells

Human corneas unsuitable for transplantation were obtained from eye banks within 2-7 days of death. The tissue was obtained in accordance with the guidelines of the Declaration of Helsinki regarding research involving human tissue. The average donor age was 67 ± 11 years. Human corneal epithelial cells and stromal keratocytes were isolated as previously described by McDermott *et al.*, (2003) and Pei *et al.*, (2006) respectively. The epithelial cells were maintained in EpiLife medium (Invitrogen; Portland, OR). For some

experiments, the isolated stromal cells were cultured in the presence of 10% FBS to induce transformation into the corneal fibroblast phenotype (Fini, 1999). Scraped epithelium, freshly isolated stromal cells and primary cultured cells (passage 1 for epithelial cells, passage 1-2 for fibroblasts) were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction and analysis of TLR1-10, defensin and LL-37 mRNA expression by RT-PCR. Normal human conjunctival (IOBA-NHC) epithelial cells (Diebold *et al.,* 2004) were cultured in DMEM-F12 (1:1 vol/vol), containing 10% FBS, 2 ng/ml mouse epidermal growth factor (EGF), 1 µg/ml bovine insulin, 0.1 µg/ml cholera toxin, 5 µg/ml hydrocortisone, 2.5 µg/ml amphotericin B, and a penicillin streptomycin mixture (5000 U/mL and 5000 µg/ml, respectively). SV40 HCEC were maintained in SHEM (DMEM-Ham's F12, 1:1 by volume) supplemented with 10% FBS, mouse EGF (0.01 µg/ml), bovine insulin (5ng/ml), cholera toxin (0.1 µg/ml) and penicillin and streptomycin antibiotics (5000 U/ml and 5000 µg/ml, respectively). Cultured cells were maintained at 37^{o}C in 5% CO_2. IOBA-NHC (passages ranged between 72-89) and SV40 HCEC (passages ranged from 17-38) were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction and analysis of TLR1-10, hBD-1-3 and LL-37 mRNA expression by RT-PCR.

Conjunctival impression cytology

All procedures involving human subjects were in accordance with the Tenets of the Declaration of Helsinki and were approved by the University of Houston Institutional Review Board. Informed consent was obtained from all subjects and none had a history of ocular surface disease. The average donor age was 32 ± 5 years of age. A drop of 0.5%

proparacaine (Bausch and Lomb; Rochester, NY) was instilled on the eye. A 13 X 6.5mm sterilized polyether sulfone membrane (Pall Gelman; East Hills, NY) was placed on the bulbar conjunctiva (superior/inferior temporal) for 2-5 seconds, removed, and placed in 350µls of RNeasy RLT lysis buffer (Qiagen; Valencia, CA) and vortexed for 60 seconds. The membrane was removed and the samples stored at -80°C until RNA extraction and analysis of TLR1-10 mRNA expression by RT-PCR.

Exposure to TLR agonists and antimicrobial peptides

To determine if TLR activation upregulates AMP or TLR expression, SV40 HCEC, IOBA-NHC or primary HCEC were grown to subconfluency in a 12 well plate. The media was then replaced with antibiotic-free and serum-free (SV40 and IOBA-NHC) or supplement-free (primary HCEC) media and the cells were incubated overnight at 37°C. The cells were then treated with various TLR agonists using the concentrations recommended by the manufacturer (Invivogen, San Diego, CA), TLR1/2 Agonist: Pam3CSK4 (1 µg/ml); TLR2 Agonist: HKLM (10^8 cells/ml); TLR3 Agonist: Poly(I:C) (1 µg/ml); TLR4 Agonist: LPS *E. coli* K12 (1 µg/ml); TLR5 Agonist: Flagellin *S. typhimurium* (1 µg/ml); TLR6/2 Agonist: FSL1 (1 µg/ml); TLR7 Agonist: Imiquimod (1 µg/ml); TLR9 Agonist: ODN2006 (5 µM); 10ng/ml of IL-1β (positive control for hBD-2) or serum and antibiotic-free media alone for 24 hours at 37°C. Following TLR agonist treatment, the media was collected and centrifuged (1400 X g for 2 minutes) to remove cell debris, snap frozen and stored at -80°C until further analysis by antimicrobial assay or immunoblotting. The cells were harvested in RNeasy RLT lysis buffer, snap frozen and stored at -80°C until RNA extraction for analysis of hBD1, 2, 3 or LL-37 mRNA

expression. To determine if AMPs modulate TLR mRNA expression, SV40 HCEC, IOBA-NHC or primary HCEC were treated with 3 μg/ml of hBD-2 (Peprotech; Rocky Hill, NJ), 5 μg/ml of LL-37 (American Peptide Company; Sunnyvale, CA) or serum-free (SV40 and IOBA-NHC) or supplement-free (primary HCEC) media and incubated for 24 hours at 37°C. The cells were harvested in RNeasy RLT lysis buffer, snap frozen and stored at -80°C until RNA extraction and RT-PCR for TLR1-10 mRNA expression.

Reverse transcription-polymerase chain reaction for detection of TLR1-10, hBD1-3 and LL-37

Total RNA from HCEC (scraped, cultured and SV40 cell line), keratocytes, stromal fibroblasts, and IOBA-NHC cells was extracted using an RNeasy Mini Kit (Qiagen). For conjunctival impression cytology (CIC) samples, RNA was extracted using an RNeasy Micro Kit. RNA elution columns were DNase treated prior to RNA elution to avoid genomic DNA contamination. To detect TLR (1-10), hBD-1-3 and LL-37 expression, one step RT-PCR was carried out with a Superscript I kit using 0.25 μg of RNA and 25 pmol of primers per reaction. Amplification of the cDNA was performed for 40 cycles of: denaturation at 94°C for 50 seconds; annealing at 58°C for 30 seconds; extension at 72°C for 1 minute. TLR1-10 (Ueta *et al.*, 2004), glyceraldehyde 3-phosphate dehydrogenase **(**GAPDH), hBD1-3 (Narayanan *et al.*, 2003) and LL-37 (Huang *et al.*, 2006) primers were used as previously described. PCR products were analyzed by agarose (1.3%) gel electrophoresis with ethidium bromide staining. A digital image was captured using an Alpha Imager gel documentation system (Alpha Innotec; San Leandro, CA). For samples exposed to AMPs, the pixel intensity of TLR PCR products was determined and

normalized to GAPDH, the internal control, and calibrated to non-treated samples. TLR PCR products that demonstrated a change in expression following antimicrobial peptide treatment were analyzed on an agarose gel after 30, 35, 40 and 45 cycles to confirm linear amplification. The data were analyzed by Student's *t*-test with values of $P \leq 0.05$ being considered significant. The RT-PCR products were sequenced (Seqwright; Houston, TX) to confirm their identities. Human spleen and U937 cell RNA were used as positive controls for TLR mRNA transcript expression. No PCR product was obtained in controls in which either the RNA or reverse transcriptase was omitted.

Quantitative RT-PCR for detection of GAPDH, hBD-2, LL-37, TLR4, 5 and 9

Total RNA from SV40 HCEC and IOBA NHC cells was extracted using a RNeasy Mini Kit (Qiagen) as describe above. cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen). Samples containing no reverse transcriptase or water in place of RNA (no template control) served as negative controls. Quantitative RT-PCR was used to quantitate mRNA expression of GAPDH, hBD-2, LL-37, TLR4, 5 and 9. Primers were as follows: GAPDH Forward: 5'GACCACAGTCCATGCCATCA3
, GAPDH Reverse: 5'CATCACGCCACAGTTTCC3', hBD-2 Forward:
5'GACTCAGCTCCTGGTGAAGC3'
hBD-2 Reverse: 5'TTTTGTTCCAGGGAGACCAC3', LL-37 Forward:
5'GGACAGTGACCCTCAACCAG3', LL-37 Reverse:
5'AGAAGCCTGAGCCAGGGTAG3'
TLR4 Forward: 5'AATCCCCTGAGGCATTTAGG3', TLR4 Reverse:
5'AAACTCTGGATGGGGTTTCC3', TLR5 Forward:

5’ACTGACAACGTGGCTTCTCC3’ TLR5 Reverse: 5’GTCAATTGCCAGGAAAGCTG3’, TLR9 Forward: 5’CCTTCCCTGTAGCTGCTGTC3’, TLR9 Reverse: 5’GACTTCAGGAACAGCCAGTTG3’. Amplification of cDNA was performed with Brilliant SYBR Green QPCR Master Mix (Stratagene; La Jolla, CA) using the specific primers listed at optimized concentrations. Amplified gene products were normalized to GAPDH, the internal control and calibrated to non-treated samples. The relative change of treated versus control samples was then determined with the value of control samples being normalized to one. The data were analyzed by Student's *t*-test with values of $P \leq 0.05$ being considered significant. Dissociation melt curves were analyzed to ensure reaction specificity. For each experiment, the samples were analyzed in triplicate and the mean relative quantity of TLR expression was calculated. Data are representative of a minimum of three experiments and were analyzed using an unpaired Student’s *t*-test where $P \leq 0.05$ was considered a significant difference.

Immunostaining for TLR3, 5 and 9 in human corneas and primary cultured fibroblasts

Human corneas unsuitable for transplantation were obtained from eye banks and embedded in OCT upon receipt, frozen, and then 10 µm cryosections were cut and fixed with acetone. Primary cultured fibroblasts (passage 3-6) were cultured into an 8 well chamber slide until subconfluency. Fibroblasts and human cornea cryosections were incubated with blocking solution (10% goat serum, 0.05% gelatin, 5% bovine serum albumin and 0.05% Tween-20 diluted in PBS) for two hours at room temperature. After

blocking, the sections were incubated with either 1 µg/ml rabbit anti-TLR9 (Abcam; Cambridge, MA), 10 µg/ml mouse anti-TLR3 (Imgenex; San Diego, CA) or 10 µg/ml mouse anti-TLR5 (Imgenex) antibody at 4°C overnight and then with 5µg/ml Alexa 546 or 6.6 µg/ml of 488-conjugated second antibody (Invitrogen) in blocking solution for one hour at room temperature. As a negative control, some sections were incubated with the relevant isotype control instead of the primary TLR antibody. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and the sections viewed with a DeltaVison imaging system (Applied Precision; Issaquah, WA).

Flow cytometry for TLR3 and 9

Freshly isolated stromal keratocytes were harvested as described above for immunostaining and placed into culture to differentiate into corneal fibroblasts and used at passage 3. Cultured SV40 HCEC and fibroblasts cells were pelleted, resuspended and permeabilized in 3% BSA/0.1% Triton-X to determine TLR intracellular expression. Equal amounts of cells were aliquoted into 15 ml conical plastic tubes and blocked with 3% BSA for 30 minutes. The cells were then incubated for an additional 30 minutes with either 1 µg/ml rabbit anti-TLR9 (Abcam) or 10 µg/ml mouse anti-TLR3, then with Alexa 488-conjugated second antibody in blocking solution. Flow cytometry was performed on a FACS Canto II (BD Biosciences; San Jose, CA) and the data were analyzed using FlowJo flow cytometry analysis software.

Antimicrobial assay

Pseudomonas aeruginosa (PA) strain ATCC19660, a cytotoxic strain which can induce severe ocular infection in experimentally infected mice, was tested in this study and prepared as previously described (Huang *et al.,* 2006). The antimicrobial activity of media collected from primary HCEC treated with TLR3, 5 and 6/2 agonists was tested against PA based on a previously described protocol (Kumar *et al.,* 2006). Primary HCEC cells grown in antibiotic-free media were treated for 20 hours with a combination of TLR3 agonist[PolyI:C (1 µg/ml)], TLR5 agonist [Flagellin *S. typhimurium* (1 µg/ml)] and TLR6/2 agonist [FSL1 (1 µg/ml)] or media alone in a six well plate; the culture media was collected and centrifuged (1400 X g for 2 minutes) to remove cell debris and used immediately or snap frozen and stored at -80°C until further analysis. One milliliter of this media was inoculated with 200 colony forming units of PA19660 and the cultures were incubated at 37°C for 4 hours while shaking; the culture media of untreated HCEC served as the control. At the end of the incubation, 10 µl of serial dilutions of each reaction mixture were spread evenly over the surface of nutrient broth plates using sterile plastic spreaders. After incubation at 37°C for approximately 16 hours, a digital image was captured of each plate with an Alpha Imager documentation system (Alpha Innotec). The number of colonies was counted by using the colony count software of the Alpha Imager. The data were analyzed by Student's *t*-test with $P \leq 0.05$ being considered significant.

The culture media from TLR agonist treated cells did indeed have significant antimicrobial activity against PA. Therefore, to determine if hBD-2 and LL-37 peptides were responsible for this activity a series of additional experiments was performed. Previous studies have shown that in the presence of salt, the antimicrobial activity of hBD-2 and LL-37 against PA is reduced. In 150 mM NaCl, the EC_{50} for hBD-2 against PA

(ATCC strain 27853) is decreased by 13 fold (Huang *et al.*, 2007b) whereas LL-37 appears to be less susceptible to the effects of salt with only a 3.5 fold reduction of the EC_{50} (Huang *et al.*, 2006). Because of this effect, the antimicrobial activity of the peptides in primary HCEC culture media which contains 130 mM NaCl was determined. Here, fresh EpiLife media was incubated with 200 cfu/ml of PA and either 3 µg/ml or 3 ng/ml of purified synthetic LL-37, or 10 µg/ml of recombinant hBD-2 or media alone and the antimicrobial activity was determined. In keeping with the previous observations, the data showed that LL-37 retained more antimicrobial activity when added to culture media than hBD-2. To determine if LL-37 was responsible for the antimicrobial activity against PA, the growth media following TLR agonist treatment was incubated with an antibody against LL-37 which binds to the functional C-terminal end of the peptide and abolishes its antimicrobial activity. In these experiments, primary HCEC were treated with either a combination of TLR3, 5, 6/2 agonists or media alone for 20 hours. The culture media was collected and centrifuged (1400 X g for 2 minutes) to remove cell debris and then incubated with either preimmune rabbit serum or rabbit anti-human LL-37 C-terminal antibody (donated by Dr. R.I. Lehrer) diluted 1 to 200 at 4°C overnight and then the antimicrobial activity against PA was determined as described above. The data were analyzed by Student's *t*-test with $P \leq 0.05$ being considered significant.

Immunoblot Analysis for hBD-2 and LL-37 and hBD-2 ELISA

Primary HCEC were treated with media or a combination of TLR3, 5 and 6/2 agonists and a portion of the culture media was then blotted onto a polyvinylidene difluoride membrane using a microfiltration apparatus (Biodot; Irvine, CA) to detect the presence of hBD-2

(McDermott *et al.*, 2001) or LL-37 (Huang *et al.*, 2006) as previously described. The remaining cells were lysed in 100 µl of ice cold RIPA buffer containing protease inhibitors (Roche; Nutley, NJ) and scraped free from the dish to ensure complete removal of all cells. Briefly for hBD-2 immunoblotting, after loading the sample, the membrane was then fixed in 10% formalin for one hour at room temperature. For both hBD-2 and LL-37 immunoblots, nonspecific binding sites were blocked by incubation in 5% blotto in TBS containing 0.05% Tween (TTBS). Membranes were then incubated with primary antibody against either hBD-2 (donated by Dr. T. Ganz) diluted 1 to 1000 or LL-37 (donated by Dr. R.I. Lehrer) diluted 1 to 5000 in 5% blotto with TTBS. After an overnight incubation at 4^{o}C, the membranes were then incubated with a horseradish peroxidase linked second antibody and immunoreactivity was detected by enhanced chemiluminescence (ECL Plus Western Blot Detection kit; GE Healthcare, Piscataway, NJ). The results were documented with an Alpha Imager documentation system. A standard curve was generated by plotting the density of the peptide standard versus concentration to determine the amount of LL-37 peptide in the samples. A portion of the culture media and the lysate were also collected as described above and analyzed for the presence of hBD-2 with an ELISA kit (Peprotech; Rocky Hill, NJ) as per the manufacturer's instructions.

2.3 Results

TLR expression by corneal and conjunctival cells

Owing to discrepancies between data from previous studies, possibly because different cell sources (cell lines vs. primary culture) were used, TLR mRNA expression was determined in epithelial cell lines and cultures and in other corneal cells. The results are

summarized in Table 2.1. TLR1-3, 5-6 and 9 were expressed in all three of the sources of HCEC. TLR4 mRNA was only detected in SV40 HCEC by RT-PCR but in subsequent experiments low levels of expression were detected in primary HCEC by quantitative RT-PCR (data not shown). Expression of TLR7 mRNA was detected in SV40 HCEC and primary cultured HCEC but not in freshly scraped cells. TLR10 was not expressed by any of the HCEC with the exception of a weak expression in two of the five SV40 HCEC cultures that were tested. Stromal keratocytes expressed TLR1-7 and TLR9 and 10 whereas cultured corneal fibroblasts, the keratocyte repair phenotype, similarly expressed TLR1, 3, 4, 6, 7, 9 and 10 but did not express TLR2 and TLR5. IOBA-NHC cells expressed TLR1-4, 6-7 and TLR9 and conjunctival cells collected by impression cytology expressed TLR1-7, and TLR9-10. Notably, TLR1, 3, 6 and 9 were expressed on all cell types tested suggesting that they may represent a first line response to microbial infections. TLR8 was not detected in any of the corneal or a conjunctival cell tested but was detected and its sequenced confirmed in human spleen samples, the positive control.

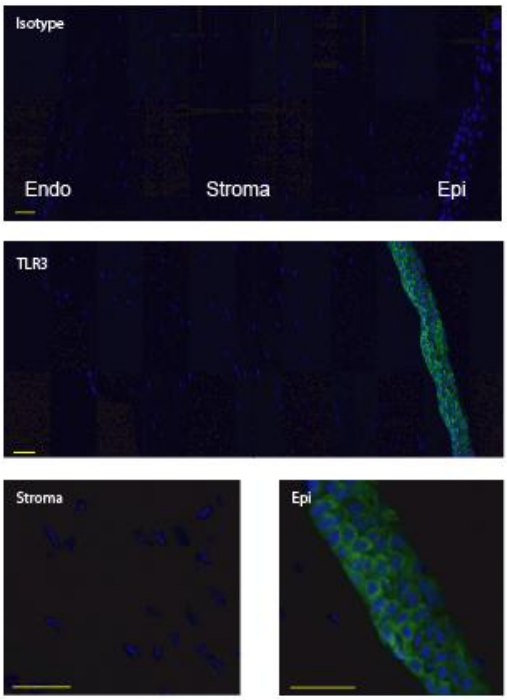
TLR mRNA Expression										
Cell Type	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Scraped Epi	+	+	+		+	+			+	
Primary HCEC	+	+	+		+	+	+		+	
SV40 HCEC	+	+	+	+	+	+	+		+	vary
CIC	+	+	+	+	+	+	+		+	+
IOBA NHC	+	+	+	+		+	+		+	
Keratocytes	+	+	+	+	+	+	+		+	+
Fibroblasts	+		+	+		+	+		+	+

Table 2.1 TLR 1-10 mRNA expression in various ocular surface cells.

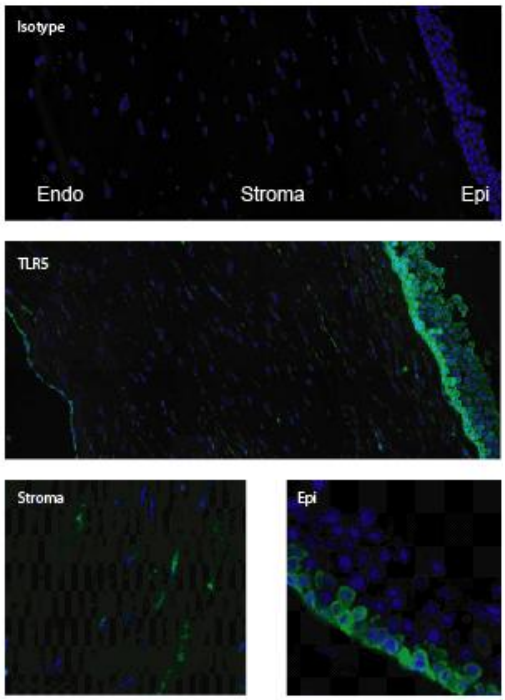
Positive mRNA expression is denoted by a plus (+) sign while negative mRNA expression was intentionally left blank. The table is representative of samples from 3-5 donors or 3-5 passages for cell lines. Vary= two of the five SV40 HCEC cultures were positive (for the other entire cell types, all of the samples tested were either positive or negative). HCEC= human corneal epithelial cells, CIC=conjunctival impression cytology, IOBA NHC= Normal human conjunctival epithelial cells.

As previous studies have shown that TLR mRNA expression is reflective of protein expression, the latter was "spot checked" in a limited number of samples. TLR3, 5 and 9 protein expression was examined in human corneas and in cultured cells by immunostaining and for TLR3 and 9 by flow cytometry. In the human corneal epithelium, TLR3 (Figure 2.1A) and 9 (Figure 2.1C) were expressed throughout the entire corneal epithelium, whereas TLR5 (Figure 2.1B) was expressed in basal and some wing cells but not in the superficial layers.

A TLR3



B TLR5



C TLR9

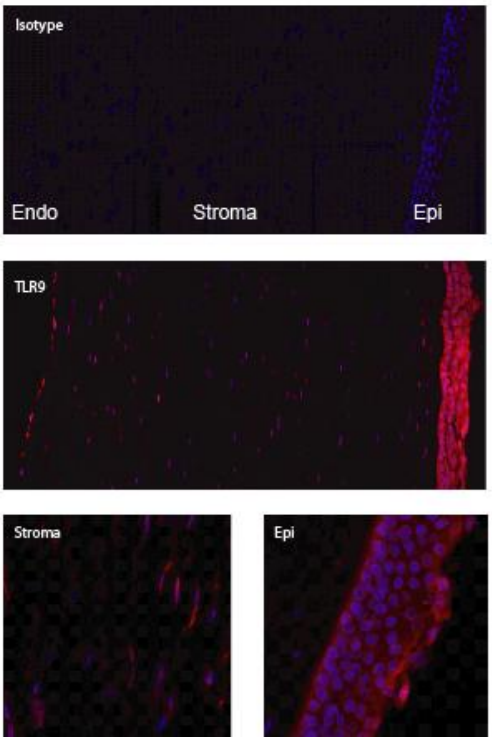


Figure 2.1 TLR expression in the human central cornea, stroma and epithelium. TLR3 (A), TLR5 (B), and TLR9 (C) expression in the human cornea. Montages encompassing theepithelium (epi), stroma and endothelium (endo) were prepared from central cornea cryosections stained for TLRs. In each section the top panel is the isotype control, the middle panel is tissue stained for the specific TLR and the bottom two images are enlarged to show detail for the epithelium and stroma. Blue fluorescent DAPI was used to stain the nuclei**.** All images were taken at 200X magnification. Scale bars (shown only for TLR3) represent 40 microns in all panels. Results are representative of 3-4 corneas.

TLR5 and 9, but not TLR3, were also expressed by the stromal keratocytes and the endothelium. TLR3 and 9 protein expression was confirmed in HCEC SV40 cells by flow cytometry and therefore was not further examined by immunostaining (Figure 2.2A). Cultured fibroblasts did not significantly express TLR3 and weakly expressed TLR9 as determined by flow cytometry (Figure 2.2B). Since TLR3 was detected by RT-PCR but not by flow cytometry, TLR3 and 9 expression was further examined by immunostaining (Figure 2.2C). This confirmed TLR9 expression and also showed there was no significant expression of TLR3 suggesting that the protein is either being degraded, its production is inhibited, or that it is not expressed in sufficient amounts to be detected by these methodologies.

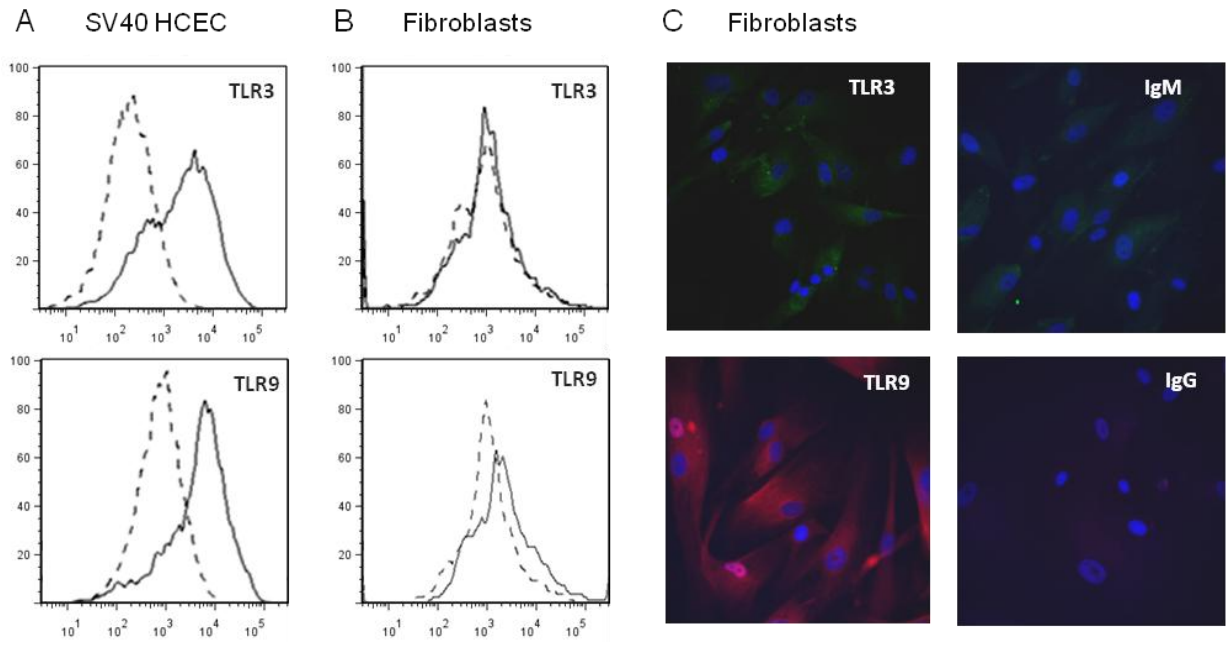


Figure 2.2 TLR3 and 9 protein expression in either SV40 HCEC or in primary cultured fibroblasts by flow cytometry and immunostaining.

TLR3 and 9 protein expression in SV40 HCEC by flow cytometry (A) or in primary cultured fibroblasts by flow cytometry (B) and immunostaining (C). Flow cytometry histograms show the relative fluorescence on the x-axis and the number of events (cell count) on the y-axis. Dashed line represents the isotype control antibody while the solid line represents the specific TLR. Fibroblasts were immunostained for TLR3 (green) and TLR9 (red) with DAPI (blue) being used to stain the nuclei (C). Isotype controls were IgM and IgG for TLR3 and 9 respectively. Images were taken at 200X magnification. The data are representative of 2-3 experiments.

TLR agonists modulate hBD-2 and hCAP-18 mRNA expression in ocular surface cells

Ocular surface cells were exposed to various TLR agonists and hBD-2 and hCAP-18, the precursor to LL-37, and mRNA expression was determined by RT-PCR. As shown in Figure 2.3A, TLR1/2, 3, 4, 5 and 6/2 agonists upregulated the expression of hBD-2 in SV40 HCEC. In IOBA-NHC cells only TLR1/2 and 6/2 agonists upregulated the expression of hBD-2 above baseline, with the activation of TLR6/2 stimulating the most robust response. In all samples tested, the upregulation of hBD-2 with TLR agonists was as effective as the positive control, IL-1β, with the exception of LPS which only modestly upregulated hBD-2 in SV40 HCEC. IL-1β and TLR agonists had no effect on hBD1 and hBD3 mRNA expression in any of the cell types tested (data not shown).

Since many primary HCEC show a baseline expression of hBD-2 and hCAP-18 mRNA, quantitative RT-PCR was performed to better quantitate any change in expression above baseline following TLR activation in these cells. Agonists for TLR3, 5 and 6/2 upregulated hBD-2 mRNA expression (Figure 2.3B) whereas only the TLR3 agonist was able to upregulate hCAP-18 expression in primary HCEC (Figure 2.3C). hCAP-18 mRNA expression was not modulated by TLR agonists as determined by quantitative RT-PCR in either IOBA-NHC or SV40 HCEC under the conditions tested (data not shown, n=2).

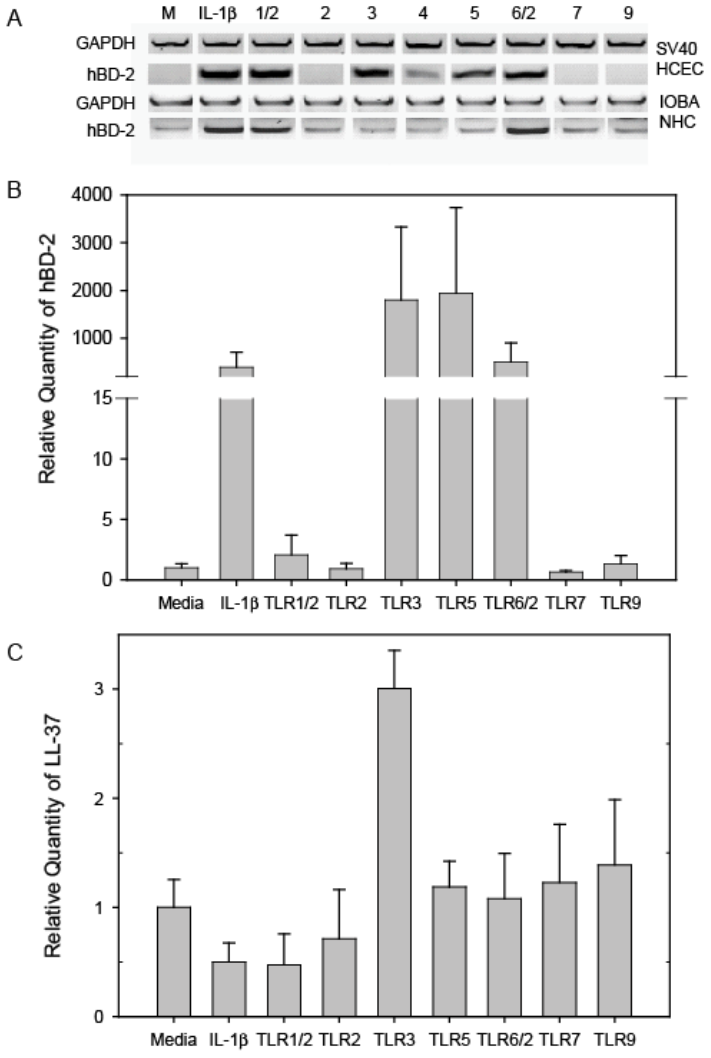


Figure 2.3 hBD-2 and hCAP-18 mRNA expression in response to TLR agonists in ocular surface cells.

SV40 HCEC and IOBA-NHC cells were treated with various TLR agonists: Pam3CSK4 (TLR 1/2), HKLM (TLR 2), PolyI:C (TLR 3), LPS E. coli K12 (TLR 4), Flagellin S. typhimurium (TLR 5), FSL1 (TLR 6/2), Imiquimod (TLR 7), ODN2006 (TLR 9), or 10ng/ml of IL-1β or serum free media (M) for 24 hours. (A) Ethidium bromide stained agarose gel for hBD-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Images

were inverted to improve contrast. Quantitative RT-PCR was performed to determine the relative quantity of hBD-2 (B) and hCAP-18 (C) mRNA in primary HCEC. The data are representative of 2-4 independent experiments.

TLR agonists modulate hBD-2 and LL-37 protein expression in primary HCEC

Immunoblot analysis was performed to determine hBD-2 and LL-37 peptide secretion by primary HCEC treated with a combination of TLR 3, 5 and 6/2 agonists. Levels of LL-37 secreted in to the culture media were estimated by semiquantitative immunoblotting (n=2). As depicted in Figure 2.4A, TLR agonist treatment increased the level of LL-37 and after accounting for the dilution factor, the concentration increased from 1.86 ± 0.07 to 3.02 ± 1.02 ng/ml or by 1.63 fold (Figure 2.4B). In regards to hBD-2 peptide, TLR agonist treatment modestly increased hBD-2 peptide in the culture supernatant and cell lysate compared to the untreated control as determined by immunoblot (data not shown). To quantify the protein levels, hBD-2 protein was detected by ELISA in both the culture supernatant and cell lysate. In the culture supernatant, there was a significant ($P<0.05$) increase in hBD-2 peptide concentration from 0.102 ± 0.011 ng/ml to 1.47 ± 0.66 ng/ml (14.4 fold increase) following TLR agonist treatment (Figure 2.4C). While in the cell lysate, TLR agonist treatment significantly increased hBD-2 levels by 15.2 ± 5.3 fold and hBD-2 concentration increased from 0.138 ± 0.06 to 2.23 ± 1.51 ng/ml (Figure 2.4C).

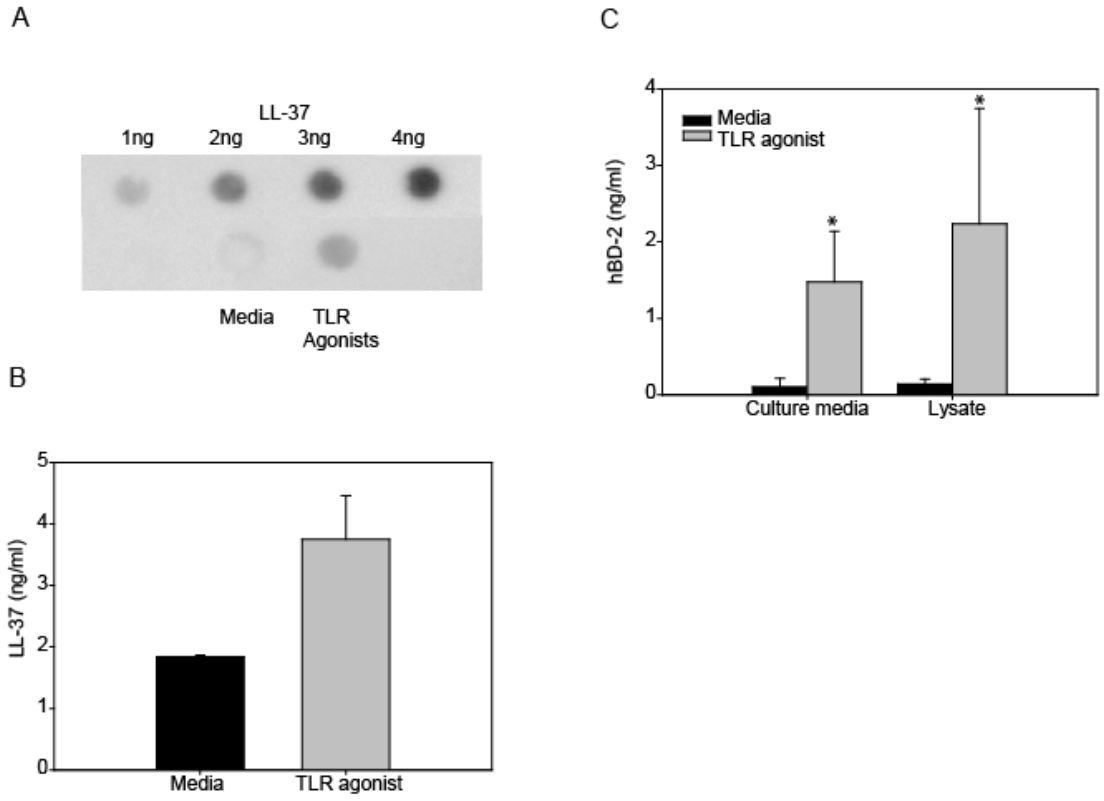


Figure 2.4 TLR agonists upregulate LL-37 and hBD-2 peptide production as detected by immunoblotting and ELISA respectively.

Primary HCEC were treated for 20 hours with either media alone or a combination of TLR 3, 5 and 6/2 agonist which upregulate hBD-2 or LL-37 mRNA. (A) Representative immunoblot for LL-37 secreted in to the culture media (n=2). (B) The pixel intensity of each dot was used for semi-quantitative analysis. (C) hBD-2 protein production was quantitated in the culture media and cell lysate by an ELISA, n=3. A P-value of <0.05 (*) was considered to be statistically significant by Student's t-test.

Modulation of TLR mRNA expression in ocular surface cells

Previous studies have shown that the activation of TLR2 (Kumar *et al.,* 2006) and TLR3 (Ueta *et al.,* 2005) modulates their own expression in human corneal epithelial cells. Therefore, this study examined if a similar effect would occur following the activation of TLR4, 5, and 9 in various ocular surface cells. Since primary HCEC and IOBA-NHC did not express detectable amounts of TLR4 and TLR5 respectively, a change in their expression following agonist treatment for these specific TLRs was not examined. Here, TLR activation by various TLR agonists did not modulate TLR mRNA expression (data not shown). More specifically in primary HCEC, flagellin and ODN2006 were not able to modulate TLR5 and TLR9 mRNA expression respectively (n=2) and LPS and ODN2006 did not modulate TLR4 and TLR9 mRNA expression in IOBA-NHC cells (n=2). Furthermore activation by LPS, flagellin, and ODN2006 did not modulate TLR4, 5, and 9 mRNA expression respectively in SV40 HCEC (n=3).

To determine if antimicrobial peptides modulate TLR expression, ocular surface cells were cultured with 3 μg/ml hBD-2 or 5 μg/ml LL-37, concentrations comparable to those used in previously published studies examining the functional activity of these peptides (Huang *et al.,* 2006, 2007; Li *et al.,* 2009). RT-PCR was performed to determine TLR 1-10 mRNA expression and the results are represented in Table 2.2. The general trend was for no change or a downregulation of TLRs. However, due to variability among the samples, statistical significance was achieved in only three instances. In IOBA-NHC cells, TLR9 mRNA was significantly downregulated by LL-37 by 12.5 ± 4.3% (P-value $\leq$ 0.05), in SV40 HCEC, TLR7 mRNA was downregulated by hBD2 by 11 ± 1.40% (P-value $\leq$

0.05) and in primary HCEC, TLR5 mRNA was downregulated by LL-37 by 34 ± 6.5% (P-value ≤ 0.05), n=3.

Cell Type / Treatment	Percent Change in TLR mRNA Expression								
	TLR 1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR9	TLR10
IOBA NHC									
a.) hBD-2	-3.7 ± 8.2%	11 ± 29%	-7.6 ± 2.6%	-6.0 ± 2.8%	NA	-3.6 ± 7.9%	3 ± 3.5%	-16 ± 12%	NA
b.) LL-37	-7.6 ± 17%	-9.0 ± 25%	-18 ± 12%	0.3 ± 23%	NA	-11 ± 7.3%	-25 ± 21%	***-12.5 ±4.3%**	NA
SV40 HCEC									
a.) hBD-2	3.0 ± 4.2%	17 ± 22%	-2.0 ± 5.1%	-23 ± 43%	21 ± 15%	2.0 ± 0.9%	***-11 ±1.40%**	-13 ± 13%	24 ± 4.8%
b.) LL-37	0.0 ± 4.6%	-9.0 ± 14%	-1.0 ± 1.6%	-4.0 ± 23%	-5.0 ± 6.3%	-28 ± 44%	-22 ± 15%	-7.0 ± 7.7%	-14 ± 22%
Primary HCEC									
a.) hBD-2	0.0 ± 6.4%	-7.0 ± 2.6%	-1.0± 18%	NA	-5.0 ± 20%	-8.0 ± 7.2%	-13 ± 12%	-3.0 ± 7.5%	NA
b.) LL-37	-24 ± 23%	-18 ± 17%	29 ± 27%	NA	***-34 ± 6.5%**	-32 ± 38%	-20 ± 20%	19 ± 8.3%	NA

Table 2.2 Percent change in TLR mRNA in response to antimicrobial peptide treatment in ocular surface cells

TLR 1-10 mRNA expression was determined in various ocular surface cells in response to treatment with hBD-2 and LL-37. Table is representative of three independent experiments, with the exception of TLR10 which was done in duplicate. HCEC= human corneal epithelial cells, IOBA NHC= Normal human conjunctival epithelial cells. Samples with an asterisk indicate a P-value ≤ 0.05 by Student T-test.

Conditioned media from TLR agonist challenged HCEC possesses antimicrobial activity that is mediated by LL-37

Since a combination of TLR3, 5 and 6/2 agonists upregulated hBD-2 and LL-37 in primary HCEC, The antimicrobial activity of the culture media against PA was next determined. As shown in Figure 2.5A, the culture media from agonist treated cells was able to significantly kill PA as determined by a reduction in PA colonies compared to the media treated control (P-value < 0.01, n=4). As noted above, previous studies have shown reduced antimicrobial activity in the presence of salt (Huang *et al.*, 2006; 2007b), therefore the antimicrobial effect of synthetic LL-37 and recombinant hBD-2 in the presence of EpiLife culture media which contains 130mM NaCl was examined (Figure 2.5B). Under these conditions 3 μg/ml LL-37 was able to significantly kill over 75.5 ± 22.5% of the bacteria, whereas at over three times the concentration, hBD-2 was only able to kill 19.9 ± 6.2% of the bacteria. Based on these findings, this study then determined if LL-37 secreted in to the culture media following TLR agonist treatment was the key component responsible for killing PA. The antimicrobial activity of LL-37 was examined at 3 ng/ml, to determine if there was any activity at the concentration found in the media following TLR agonist stimulation. At this concentration, LL-37 was able to modestly kill PA by 9.1 ± 6.11% (Figure 2.5C). Furthermore, following TLR agonist treatment, blocking LL-37 with a C-terminal antibody significantly reduced the bacterial count beyond baseline (-11.5 ± 11.0%) (Figure 2.5C). In contrast, significant killing (50 ± 11.6%) of PA was still achieved when the culture media was treated with pre-immune rabbit serum prior to antimicrobial assays compared to media alone and 3 ng/ml of LL-37.

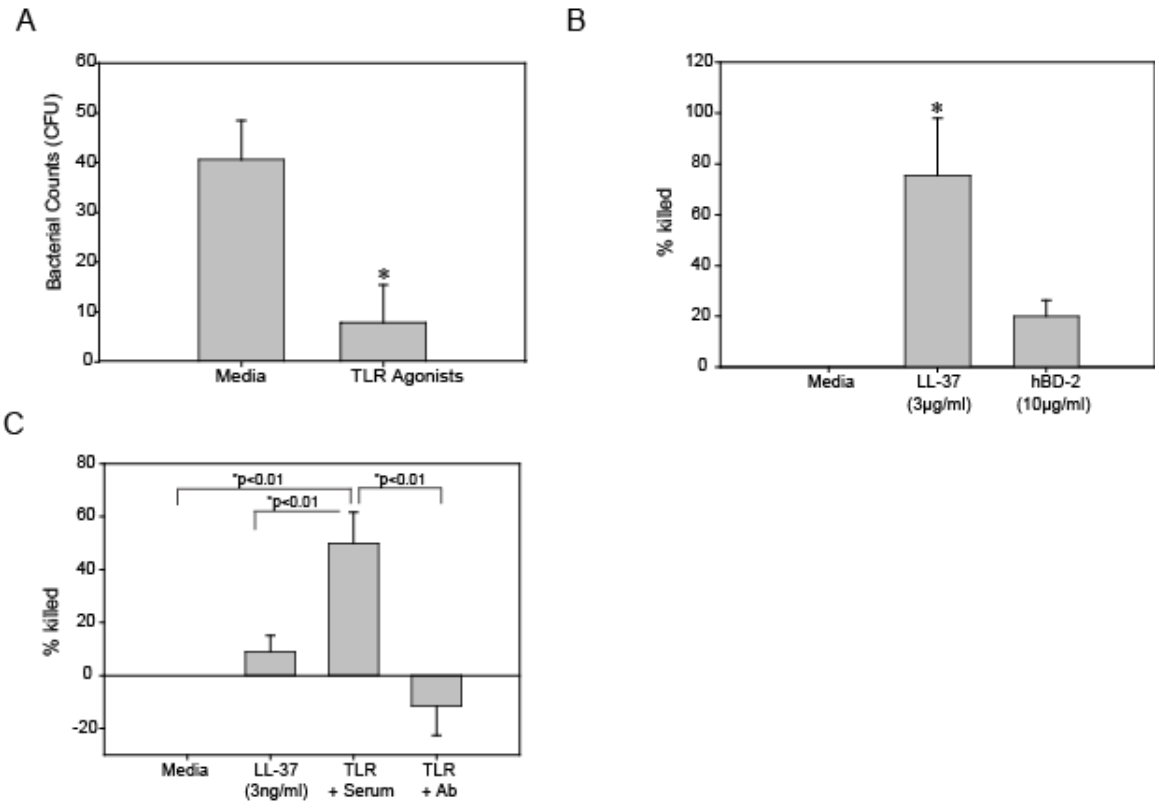


Figure 2.5 Antimicrobial activity of HCEC treated with TLR agonist is dependent on LL-37.

(A) The antimicrobial activity of culture media from primary HCEC treated with TLR 3, 5 and 6/2 agonists was determined by a colony count assay. (B) To determine the activity of AMPs in culture media, synthetic LL-37 (3 µg/ml) or recombinant hBD-2 (10 µg/ml) peptides were incubated with culture media and PA and the percent bacteria killed was calculated with media treated representing no killing. (C) PA were incubated with culture media alone, synthetic LL-37 (3 ng/ml) or with media from primary HCEC treated with TLR3, 5 and 6/2 agonists which had then been incubated with preimmune rabbit serum (serum) or a LL-37 blocking antibody (Ab) and the percent bacteria killed was calculated. The Figures are representative of 2-6 independent experiments. When comparing two

groups, an unpaired Student's *t* test was used and a P-value of <0.05 (*) was considered to be statistically significant. For all others, a P-value <0.01 was considered to be significant by ANOVA with Bonferroni's correction for multiple comparisons.

2.4 Discussion

The results from the present study show that TLRs are expressed not only by cultured (cell lines and primary) HCEC but also by freshly isolated HCECs from normal human cadaver corneas, stromal keratocytes and fibroblasts, conjunctival impression cytology samples and IOBA-NHC conjunctival epithelial cells. The activation of specific TLRs in primary HCECs upregulates hBD-2 and LL-37 mRNA and peptide expression and that LL-37 is more effective than hBD-2 in killing PA. Furthermore the activation of specific TLRs does not modulate their own expression whereas hBD-2 and LL-37 peptides are able to modestly downregulate the expression of some TLRs in human corneal and conjunctival epithelial cells.

Although TLR expression has been examined in ocular surface cells by others, this is the first study to comprehensively examine TLR expression simultaneously in several ocular surface and corneal stromal cells. Here, TLR expression was examined in three sources of HCEC, cells scraped from donor corneas, primary cultured, and a SV40 transformed cell line. As stated previously, TLR1, 2, 3, 5, 6, and 9 were expressed by all three sources of cells tested while only SV40 HCEC expressed TLR4 and TLR7, however, using quantitative RT-PCR, low levels of TLR4 were detected in primary HCECs by quantitative RT-PCR (data not shown). As discussed in the results section, a few samples were selected to correlate protein production with mRNA expression. TLR3, 5 and 9 expression was confirmed in human corneal epithelium by immunostaining tissue sections, and that of TLR3 and 9 in SV40 HCEC by flow cytometry. Notably TLR5 was only expressed by basal and wing epithelial cells, which is in agreement with Hozono *et al.* 2006 who suggested that this distribution may contribute to the immunosilent nature of

the normal cornea. Unlike most other TLRs, TLR10 was not reproducibly expressed by HCECs from any source in our study. In general, these data are in agreement with previously published studies. Wu *et al.* (2007) found that human corneal epithelial samples collected from patients undergoing photorefractive keratotomy, in general, strongly expressed TLR1, 2, 3, 5, 6 and 9 and weakly expressed TLR4, 7 and 8, if at all. Jin *et al.* (2007) observed expression of all ten TLRs in donor cornea biopsies, with TLR1, 2, 3, 4 and 6 having the highest and TLR 7, 8 and 9 the lowest levels. These previously published observations indicate a variable expression pattern for several TLRs which may account for our results with TLR4 and 7. The SV40 HCEC cell line consistently expressed TLR1-7 and 9, and all of these TLRs have been detected in studies of normal corneal epithelial tissue, suggesting that this cell line is a suitable model for studying the role of these TLRs at the ocular surface.

Few studies have specifically addressed TLR expression in corneal layers other than the epithelium. Here, TLR expression was examined in corneal stromal cells from cadaver donors, freshly isolated keratocytes and cultured stromal fibroblasts (repair phenotype keratocytes). Such a comprehensive analysis of keratocyte TLR expression has not been published. Previously, Ebihara *et al.* (2007) reported that keratocytes and cells they referred to as corneal myofibroblasts expressed TLR2 and 4 mRNA, but only the myofibroblasts expressed TLR3 and 9. Furthermore functional studies have shown that TLR4 (Kumagai *et al.*, 2005) activation in corneal fibroblasts results in cytokine secretion. Here, keratocytes expressed TLR1-7, 9, and 10 mRNA, whereas cells that had transformed into the fibroblast phenotype no longer expressed TLR2 and TLR5 mRNA. The lack of TLR2 expression in corneal fibroblasts was unexpected as previous studies

have shown TLR2 and TLR 4 mRNA expression in primary cultured corneal fibroblasts by quantitative RT-PCR (Gao *et al.,* 2009; Jin *et al.,* 2009). As examined here, trace amounts of mRNA expression may not have been detected by the current methods (RT-PCR) that may have been detectable by the quantitative RT-PCR method or the lack of expression may be reflective of donor variability. In human cornea sections, immunostaining revealed that TLR5 and TLR9 were expressed by what appear to be corneal keratocytes based on the number and distribution of cells stained. However, additional immunostaining was not performed to distinguish them from resident immune cells. This experiment also showed that corneal endothelial cells express TLR5 and 9. Although both keratocytes and cultured fibroblasts expressed TLR3 mRNA, immunostaining and flow cytometry could not confirm actual protein expression for this TLR by these cells. As stromal fibroblasts are only present at times when the cornea is compromised due to injury and inflammation, a change in TLR expression may be beneficial or destructive to the ocular surface in modulating inflammation. A reduction in expression of individual TLRs accompanying the transition from keratocyte to fibroblast observed here may help prevent further undue inflammation in the stroma. However, it may also leave the stroma with a reduced ability to detect and remove pathogens rendering it more susceptible to microbial keratitis.

Unlike the other TLRs, little is known regarding TLR10 expression and function. A recent study found that TLR10 requires TLR2 to recognize microbial lipopeptide but it lacks the downstream signaling that is shared with other TLR2 family members (Guan *et al.,* 2010) which call in to question its function. To our knowledge, this is the first study to examine TLR10 expression in various ocular surface cells. Previous studies (Jin *et al.,*

2007; 2008) have found TLR10 expression in full thickness penetrating keratoplasty biopsies, but attempts were not made to localize its expression to specific cell types. Here, TLR10 expression was unique to freshly isolated stromal keratocytes and fibroblasts. While keratocytes form the majority of stromal cells, several immune cells, such as dendritic cells and monocytes have recently been found in the stroma (Hamrah and Dana, 2007; Mayer *et al.,* 2007) and may serve as the source of TLR10 mRNA detected. However, the likelihood of this being the case is small as immune cells constitute only a small portion of the total cells in the normal cornea and of these, TLR10 is known to be expressed on dendritic cells (Hasan *et al.,* 2005) but not on monocytes (Hornung *et al.,* 2002). Further, immune cells are often depleted from cadaver corneas when placed into storage media (Jeng, 2006) and would most likely not survive or would be washed away when placed in culture with the keratocytes. Thus, this study suggests that TLR10 expression in the cornea is unique to keratocytes and fibroblasts, but its exact role and function remains unknown.

In regards to TLR expression in the conjunctiva, Bonini *et al.* (2005) have found that the healthy conjunctival epithelium and stroma expresses TLR2, 4 and 9 mRNA and protein, while Cook *et al.* (2005) observed cell surface expression of TLR2 in cultured conjunctival epithelial cells and in CIC samples from human subjects with atopic keratitis and allergic conjunctivitis, but not in patients without ocular allergies. Li *et al.* (2007) have reported that TLR1, 2, 3, 5 and 6 mRNA were expressed in conjunctival and limbal epithelial samples, whereas the expression of TLR4 and 9 mRNA was variable and TLR7, 8 and 10 mRNA were not detected. In addition, they also reported TLR1-6 and TLR 9 protein to be expressed in human limbal and conjunctival epithelial cells by western blot.

In agreement with the general findings of these studies, TLR 1-6 and 9 mRNA was detected in conjunctival impression cytology samples as well as TLR7 and TLR10 expression. The IOBA-NHC cell line showed a similar pattern of expression with the exception that it did not express TLR5 and TLR10. Therefore this cell line might serve as a suitable model for investigating some TLRs in the conjunctiva in future studies. Interestingly, a principal component analysis has shown that IOBA-NHC are most similar to primary human conjunctival epithelial (PCEC) cells compared to another conjunctival epithelial cell line (Tong *et al*., 2009). However, TLR4 protein levels were reduced in the IOBA-NHC cells compared to PCEC which should be considered when using this cell-line to investigate TLR4 in future studies.

In addition to the known role in surveillance for microbial pathogens, TLRs at the ocular surface modulate the expression of antimicrobial peptides providing additional protection to ward off microorganisms. Although some studies have examined the relationship between TLR activation and antimicrobial peptide expression in human corneal epithelial cells, very little is known about this relationship in the conjunctiva. Further, this is the first study to report a concurrent comparison of antimicrobial peptide expression across the whole range of TLR activation in ocular surface epithelial cells. With the exception of TLR2, 7 and TLR9, when using the manufacturer's recommended concentrations all the TLR agonists were able to modulate the expression of antimicrobial peptides depending on the cell type. A previous study has shown that TLR2 and 9 agonists, when used at the same concentrations in this study, can modulate functions of other cell types (Rowlett *et al*., 2008). However, the possibility remains that the concentrations/treatment times of the TLR2, 7 and 9 agonists were not optimal for the

cells examined in this study. Here, fewer agonists were found to stimulate hBD-2 expression in the IOBA-NHC cells than in the SV40-HCECs. At least in the case of TLR4, this may be attributed to the lack of essential costimulatory molecules required for activation in IOBA-NHC. TLR4 activation is dependent on complex formation with LBP, CD14 and MD-2 and TLR4 unresponsiveness to LPS has been attributed to lack of these in some studies (Blais *et al.*, 2005). Alternatively, Li *et al.* (2007) detected TLR4 in the conjunctival epithelium by immunohistochemistry, but these cells did not demonstrate secretion of proinflammatory cytokines in response to LPS even in the presence of MD-2 and exogenous LBP and CD14. The authors suggested that ocular surface cells may require priming with IFNγ or TNFα which is required by other mucosal epithelia cells to render them responsive to LPS treatment.

Activation of TLR3, 5 and 6/2 stimulated hBD-2 mRNA expression in both primary and SV40 HCEC and TLR1/2 and 4 activation also stimulated hBD-2 mRNA expression in SV40 HCEC. However, TLR4 agonist stimulation was not examined in primary HCEC as these cells lacked significant TLR4 mRNA expression. Previously, Kumar *et al.*, (2007) found that PA, a TLR4 and 5 agonist, induced the upregulation of hBD-2 in a human corneal-limbal cell line while Kumar *et al.* (2006) and Li *et al.* (2008) reported that a human corneal-limbal cell line and primary HCEC respond to TLR1/2 and TLR2 agonist by expressing hBD-2 mRNA and secreting hBD-2 into the culture media. Although data with SV40 HCEC line is in agreement with this, primary HCEC were unresponsive to TLR1/2 agonist stimulation. This discrepancy could result from donor variation or variations in experimental protocols, as this study used freshly isolated primary HCEC at passage one and a lower concentration of Pam3CSK4 (1µg/ml) whereas

they used primary HCEC at passage three and a higher concentration of Pam3Cys (10 μg/ml), a similar agonist but from a different manufacturer.

hCAP 18, the precursor to LL-37, was found to only be upregulated in response to TLR3 agonist treatment in primary HCECs after 24 hours and as early as 30 minutes following treatment (unpublished observation). However, in all other cell types tested there was no significant change in hCAP 18 mRNA expression following TLR agonist treatment. This was a surprising result as Kumar *et al.* (2007) and Li *et al.* (2008) found that freshly isolated PA flagellin or SA extract were able to increase LL-37 mRNA and protein expression in a cornea-limbal epithelial cell line. However in this study, freshly isolated primary HCEC were stimulated with commercially available flagellin from S. typhimurium, a common pathogen of the intestine, and it is possible that this ligand, which does upregulate hBD-2, was not optimal for upregulating LL-37.

In addition, hBD-2 and LL-37 protein levels and the antimicrobial activity of the culture media supernatant following cell stimulation with a cocktail of TLR3, 5 or TLR6/2 agonist was also examined. Activation of these TLRs stimulated the secretion of low levels (in the nanogram range) of both hBD-2 and LL-37 into the culture media. Such concentrations are in keeping with hBD-2 levels in tears (329 ± 154 pg/ml, Redfern & McDermott unpublished observation) and cornea (Garreis *et al.*, 2010). Notably, the culture media for agonist treated cells had significant antimicrobial activity against PA and additional experiments suggest that this antimicrobial activity is primarily attributable to LL-37.

Previous studies have shown that hBD-2 is more salt sensitive than LL-37 (Huang *et al.*, 2006; 2007b, Starner *et al.*, 2005) implying greater activity of the latter in a

physiological environment. In culture media, LL-37 was significantly more effective at killing PA than hBD-2 even when used at a using a third of the concentration of hBD-2. Furthermore, the antimicrobial activity of the culture media following TLR agonist treatment was completely abolished in the presence of an antibody that blocks LL-37. It is interesting to consider that 3 ng/ml of synthetic LL-37 was significantly less effective at killing PA compared to the culture media from primary HCEC that were stimulated with the TLR agonist cocktail. An obvious explanation for this discrepancy would be that LL-37 is not the only factor secreted with antimicrobial activity. However the experiments with the LL-37 antibody, which were properly controlled with pre-immune serum, argue against this. Also as discussed, while hBD-2 is present, it does not have significant antimicrobial activity under the assay conditions. One possibility is that, as has been shown for skin, LL-37 is further processed to smaller fragments which also have potent antibacterial activity and which would be blocked by the antibody (Murakami *et al.*, 2004). Taken together, these results suggest that LL-37 is responsible for killing PA and may be a novel therapeutic option to reduce the risk of microbial keratitis. In support of this, LL-37 has antimicrobial activity against Gram-positive (*S. aureus and S. Epidermidis*) and Gram-negative (PA) organisms that are often associated with bacterial keratitis and contact lens associated keratitis. Contact lens wear reduces PA induced upregulation of hBD-2 in HCEC *in vitro* (Maltseva *et al.*, 2007) and the antimicrobial activity of hBD-2 against PA is reduced by human tears (Huang *et al.*, 2007b) by up to 90%, whereas that of LL-37 is not impacted nearly as much (Huang *et al.*, 2007b).

Previous studies have shown that the activation of TLR2 (Kumar *et al.*, 2006), TLR3 (Ueta *et al.*, 2005) and TLR4 (Zhao and Wu, 2008) modulates their own mRNA

expression in human corneal epithelial cells after a short time period of up to six hours although later time points were not examined, whereas this study examined TLR4, 5 and 9 mRNA expression was not modulated by agonist activation after 24 hours. These observations suggest that TLR activation may lead to an early transient increase in TLR expression after six hours that returns to baseline by 24 hours.

As TLR activation results in the production of AMPs, this study then sought to determine if AMPs can then modulate TLR expression suggesting either a negative or positive feedback loop. In this study, 24 hours exposure to hBD-2 and LL-37 modestly downregulated the expression of some TLRs in human corneal and conjunctival epithelial cells. A previous study has also suggested that TLR expression can be modulated by some defensins, although as this was conducted *in vivo* it is unclear if this was a direct or indirect effect (Wu *et al.* 2009a). Other studies have shown that some defensins use TLRs to modulate mammalian cell activities (Biragyn *et al.,* 2002; Funderburg *et al.,* 2007). These findings suggest that the activation of TLRs stimulates an antimicrobial response by the upregulation of antimicrobial peptides which in turn may serve as endogenous ligands for TLRs to modulate their own expression. Since hBD-2 and LL-37 are expressed at the ocular surface in response to injury and proinflammatory cytokines, a downregulation of TLRs in response to AMPs might provide the ocular surface with a reduced risk for severe inflammation. However, the small changes of TLR mRNA expression observed here may not be physiologically relevant on the ocular surface due to the redundant nature of these receptors and the high concentrations (relative to the levels secreted in to the culture media) of hBD-2 and LL-37 used in these studies.

Overall, these results show that the ocular surface expresses a variety of TLRs which allows the rapid detection and killing of PA and potentially other pathogens. TLR activation by their respective agonists resulted in the production of antimicrobial peptides, in particular LL-37, which in turn may increase the spectrum of antimicrobial activity to ward off invading pathogens.

Chapter 3 - Dry Eye Associated Factors Modulate the Expression of Toll-Like Receptors on the Ocular Surface

3.1 Introduction

Dry eye syndrome, an ocular surface condition that affects millions of individuals every year, is largely under diagnosed and is one of the leading causes for visits to the eye doctor (Brewitt and Sistani, 2001). Patients with severe dry eye have an increased risk for corneal ulceration, melting (Vivino *et al.,* 2001) and ocular infection (Derk and Vivino, 2004), which may result in vision loss. Although dry eye typically does not result in blindness, patients often report a decreased quality of life and reduced ability to perform professional work and daily tasks such as reading and driving (Miljanović *et al.,* 2007). The etiology behind dry eye is often elusive and multifactorial. Treatment is frequently palliative and it can be challenging to obtain efficacious therapy. Despite its common occurrence, the pathogenesis of dry eye is poorly understood.

As discussed in Sections 1.3.2.2 and 1.5, the core mechanisms behind dry eye inflammation are driven by tear film hyperosmolarity (Gilbard *et al.,* 1978; Farris 1994; Bron *et al*., 2002) and instability which stimulate an increase in proinflammatory cytokines (Afonso *et al.,* 1999; Pflugfelder *et al.,* 1999; Solomon *et al.,* 2001) at the ocular surface. The resulting inflammation disrupts the ocular surface epithelium and exacerbates the dry eye creating a vicious cycle (2007 Report of the Dry Eye Workshop, DEWS). The range for normal tear film osmolality is 296-308 mOsm/L (Gilbart, 1978), while the peak value in dry eye patients has been documented to reach as high as 440 mOsm/L (Farris, 1994). One recent study suggests that tear film osmolality has transient spikes ranging from 800 to 900 mOsm/kg and these spikes are responsible for some of the symptoms

(ocular burning and discomfort) that many dry eye patients experience (Lui *et al.*, 2009). Luo *et al.* (2005) demonstrated that hyperosmolar stress stimulated the expression of pro-inflammatory cytokines IL-1β and TNF-α, and also matrix metalloproteinase (MMP)-9 on the mouse ocular surface *in vivo*. This raises an interesting possibility that hyperosmolar stress as seen in dry eye may regulate the expression of other inflammation associated molecules such as TLRs on the ocular surface

Toll-like receptors are a family of highly conserved glycoprotein receptors that recognize conserved motifs on pathogen associated molecular patterns on protozoa, bacteria, and viruses and, as suggested by some studies, host endogenous ligands (Takeda *et al.*, 2003; Medzhitov *et al.*, 1997). The activation of TLRs leads to the production of various cytokines and chemokines (Akira *et al.*, 2004; Takeda *et al.*, 2003). Ten functional human TLRs have been identified, each binding a distinct microbial ligand. For a review on TLR ligands see Section 1.1.

Recent studies have shown that the activation of TLRs on the corneal epithelium can produce extensive ocular surface inflammation (Adhikary *et al.*, 2008; Johnson *et al.*, 2008; Zhang *et al.*, 2003; Kumar *et al.*, 2006a and 2006b). In particular, the activation of TLR2, 4 and 9 in the murine corneal epithelium has been shown to induce potentially sight-threatening keratitis (Johnson *et al.*, 2005), whereas the application of eritoran tetrasodium, a TLR4 antagonist can significantly inhibit corneal inflammation in response to stimulation with LPS, suggesting a potential therapeutic role for TLR antagonists in modulating corneal inflammation.

Other studies have reported an increase in TLR expression in patients with dry eye and in its most severe form, Sjögren's syndrome (SS) as previously discussed in detail in

Section 1.3.2.2. In particular, in the parotid gland in patients with SS, TLR7 and TLR9 expression was found to be throughout the gland while in control patients, TLR7 and TLR9 expression was limited to the ductal epithelial cells (Zheng *et al.*, 2010). Using a SS mouse model, Christopherson *et al.,* (2005) found that TLR4 and TLR5 mRNA was upregulated in the mouse cornea and TLR4 was upregulated in the lacrimal gland. Together these studies suggest that TLR may be involved in the pathogenesis of dry eye inflammation. The pathophysiology behind dry eye inflammation is still not fully understood, but one potential source of inflammation may result from an upregulation or undesired activation of TLRs on the ocular surface. Considering this, TLR expression was examined in dry eye subjects by conjunctival impression cytology and various ocular surface cells in response to dry eye associated conditions, such as hyperosmolar stress, desiccation and cytokines. This study focuses on TLR4, 5, and 9 which are known to be expressed by ocular surface cells and have been implicated in ocular surface inflammation.

3.2 Methods

Primary human corneal epithelial cell (HCEC) cultures were prepared from human corneas unsuitable for transplantation which were obtained from eye banks within 3 to 5 days of death and the mean donor age was 71.5 ± 8.9 years. The tissue was obtained in accordance with the guidelines of the Declaration of Helsinki regarding research involving human tissue. HCEC were isolated as previously described (McDermott *et al.,* 2003) and were maintained in EpiLife medium (Invitrogen; Portland, OR).

A principal component analysis has shown that normal human conjunctival (IOBA-NHC) epithelial cells are most similar to primary human conjunctival epithelial

cells compared to another conjunctival epithelial cell line (Tong *et al.*, 2009). Therefore these cells were used in this study since it is difficult to reliably generate primary conjunctival epithelial cell cultures. IOBA-NHC cells (Diebold Y, *et al.*, 2003) were cultured in DMEM-F12 (1:1 vol/vol), containing 10% FBS, 2 ng/mL mouse epidermal growth factor (EGF), bovine insulin (1 µg/ml), cholera toxin (0.1 µg/ml), hydrocortisone (5 µg/ml), amphotericin B (2.5 µg/ml), and a penicillin streptomycin mixture (5000 U/ml and 5000 µg/ml, respectively). SV40-transformed HCEC were a gift from Dr. Kaoru Araki-Sasaki (Tane Memorial Eye Hospital, Osaka, Japan). The cells were maintained in SHEM (DMEM-Ham's F12, 1:1 by volume) supplemented with 10% FBS, mouse epidermal growth factor (EGF) (0.01 µg/ml), bovine insulin (5 ng/ml), cholera toxin (0.1 µg/ml) and a penicillin streptomycin mixture (5000 U/ml and 5000 µg/ml respectively). All cultured cells were maintained at 37^{o}C in 5% CO_2.

Hyperosmolar Stress Treatment

Cells were cultured to 60-70% confluency, washed three times to remove residual serum and growth factors and placed in supplement free (primary HCEC) or serum-free (cell lines) media overnight. Cells were then cultured for an additional 24 hours in serum-free media or serum-free media with an osmolality ranging from 400 to 500 mOsm/kg which was achieved by adding various amounts of NaCl as previously described (Li *et al.*, 2006). Osmolality of the media was confirmed with a vapor pressure osmometer (Vapro 5520; Wescor, Logan, UT) prior to each experiment. In some samples, at the end of the 24 hour incubation the hyperosmolar media was removed; the cells were washed three times with PBS, and cultured with normal growth media for an additional 6 or 24 hours. At the end of

the incubations the cells were either harvested in RLT lysis buffer (Qiagen; Valencia, CA) or pelleted, then snap frozen and stored at -80^{o}C until RNA extraction or western blotting to analyze TLR mRNA and protein expression respectively.

Cytokine Treatment

Recombinant human cytokines were obtained from R&D Systems (Minneapolis, MN). Cells were cultured to 60-70% confluency, washed three times and placed in extract or serum-free media overnight. Cultures were exposed to 1, 10, 100, or 1000 ng/ml of IL-1α, IL-1β, TNFα or TGFβ for 3, 9, 12 or 24 hours or serum-free media alone. At the end of the incubation period, the cells were either harvested in Qiagen RLT lysis buffer or pelleted, snap frozen and stored at -80^{o}C until RNA extraction or western blotting to analyze TLR mRNA and protein expression respectively.

Desiccation Organ Culture Model

Human corneas were obtained from eye banks within 3-5 days of death. The mean age of the donors was 59 ± 1.7 years of age. The endothelial cavity was filled with M199 containing 0.5% agar which was allowed to set. The corneas were then placed epithelial side up into 35 mm culture dishes which were filled with M199 to the level of the limbal conjunctiva (desiccation model) or completely submerged (control). The corneas were maintained in a humidified atmosphere at 37°C and the exposed surface of the desiccation model was moistened by drop-wise (100 µl) application of M199 after 12 hours. Following 24 hours the corneal epithelium was collected and either placed in Qiagen RLT

lysis buffer or pelleted, then snap frozen for analysis of TLR mRNA and protein expression.

Human Subjects

All procedures involving human subjects were in accordance with the tenets of the Declaration of Helsinki and were approved by the University of Houston's Institutional Review Board. Written informed consent was obtained from all subjects before participation in the study. Subjects were screened and categorized as normal or dry eye by their subjective responses to the ocular surface disease index (OSDI) questionnaire (Schiffman *et al.,* 2000) and the presence of objective clinical signs. Subjects were excluded if they were currently using topical anti-inflammatory drops or had any other ocular surface disease other than dry eye.

Objective Clinical Assessments

General ocular surface health of all subjects was assessed with a slit-lamp biomicroscope. Vital staining of the corneal and conjunctival epithelia using fluorescein and lissamine green strips respectively were graded on a scale from 1-4 (Cornea and Contact Lens Research Unit [CCLRU] grading scale; School of Optometry, University of New South Wales, Sydney, NSW, Australia), tear secretion was measured by the phenol red thread test; tear film osmolality was measured using a vapor pressure osmometer (Vapro 5520); and tear stability was by measured by fluorescein tear break-up time (Dry Eye Test; Akorn, Chicago, IL).

Conjunctival Impression Cytology (CIC)

Following the completion of all of the objective clinical assessments, a single drop of 0.5% proparacaine hydrochloride anesthetic (Bausch and Lomb; Rochester, NY) was instilled onto each eye. Two to three 6.5 x 13 mm sterile polyether sulfone membranes (Supor; Pall Gellman Sciences; East Hills, NY) were placed on the superior or inferior bulbar conjunctiva without applying pressure on each eye. The membranes from both eyes of one subject were removed and placed directly into one tube containing 350 µl of lysis buffer (Qiagen) and stored at -80°C until RT-PCR analysis for TLR4, 5, and 9 mRNA expression.

Real-time RT-PCR

Total RNA from CIC samples was extracted using a RNeasy Micro Kit (Qiagen) and all other samples were extracted using a RNeasy Mini Kit (Qiagen). RNA elution columns were DNAse treated prior to RNA elution to avoid genomic DNA contamination. Real-time RT-PCR was used to quantitate relative mRNA expression of TLR4, 5 and 9. cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Reverse transcription was performed at 50°C for 60 minutes. Samples containing no reverse transcriptase or water in place of RNA (no template control) served as negative controls. PCR amplification of cDNA was performed with Brilliant SYBR Green QPCR Master Mix (Stratagene; La Jolla, CA) using specific primers for TLR4 Forward: 5'AATCCCCTGAGGCATTTAGG3', TLR4 Reverse: 5'AAACTCTGGATGGGGTTTCC3', TLR5 Forward: 5'ACTGACAACGTGGCTTCTCC3' TLR5 Reverse:

5’GTCAATTGCCAGGAAAGCTG3’, TLR9 Forward:
5’CCTTCCCTGTAGCTGCTGTC3’, TLR9 Reverse:
5’GACTTCAGGAACAGCCAGTTG3’ and GAPDH Forward,
5’GACCACAGTCCATGCCATCA3’, GAPDH Reverse,
5’CATCACGCCACAGTTTCCC3’at optimized concentrations. Thermocycler parameters were 95℃ for 10 minutes, followed by amplification of cDNA for 40 cycles of denaturation at 95℃ for 30 seconds, annealing at 60℃ for 60 seconds, and extension at 72℃ for 30 seconds. Reactions were done in triplicate using an Mx3005P QPCR System (Stratagene). Amplified gene products were normalized to GAPDH, the internal control, and calibrated to age-and gender-matched control (CIC samples) or non-treated culture samples. The relative change of patient/treated versus control samples was then determined with the value of control samples being normalized to one. Disassociation melt curves were analyzed to ensure reaction specificity. For each experiment, the samples were analyzed in triplicate and the mean relative quantity of TLR expression was calculated. Data are representative of a minimum of two-three experiments and were analyzed using an unpaired Student’s *t*-test where $P \leq 0.05$ was considered a significant difference.

Western Blotting

Cell pellets were lysed in 100ul of RIPA lysis buffer containing a protease inhibitor cocktail (Roche; Nutley, NJ) for 5 minutes. The lysates were sonicated by three bursts at level 3-4 (60 Sonic Dismembrator, Fisher Scientific; Pittsburgh, PA) for 10 seconds on ice. The cell lysates were then centrifuged at 4°C for 15 minutes at 10,000 rpm. Equal

amounts of total protein (30-40 µg) were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were then transferred to membranes and blocked with TBS containing 0.1% Tween-20 (TTBS) and 5% nonfat dry milk. The membranes were then incubated for 2 hours at room temperature or 1 hour at 37°C in blocking buffer with anti-TLR4 (1:1000, Santa Cruz Biotechnology; Santa Cruz, CA) or anti-TLR9 (1 µg/ml, AbCam; Cambridge, MA) respectively. Membranes probed with anti-TLR4 or anti-TLR9 antibodies were then incubated with goat anti-rabbit horseradish peroxidase (HRP) or goat anti-mouse HRP conjugated secondary antibodies for 1 hour at room temperature respectively and visualized with ECL Plus Western Blot Detection kit (GE Healthcare; Piscataway, NJ). The membranes were stripped, then reblocked for 1 hour at room temperature in blocking buffer, and reprobed using a GAPDH antibody (1:1000, Chemicon; Billerica, MA) for 1 hour at room temperature which served as an internal control. The membrane was then probed with anti-mouse HRP conjugated secondary antibody for 1 hour at room temperature and visualized with an ECL Plus Western Blot detection system. A digital image was captured using an Alpha Imager gel documentation system (Alpha Innotec; San Leandro, CA). The pixel intensity of the bands was determined and normalized to GAPDH, the internal control, and calibrated to non-treated samples. Data are representative of a minimum of three experiments and were analyzed using an unpaired Student's *t*-test where $P \leq 0.05$ was considered to statically significant.

3.3 Results

Hyperosmolar Stress Modulates the Expression of TLR4 and TLR9 but not TLR5

To determine if hyperosmolar stress (HOS) modulates TLR expression by ocular surface cells, TLR4, 5, and 9 mRNA expression was investigated by quantitative RT-PCR in

SV40 HCEC (Figure 3.1A) after 24 hours exposure to hyperosmolar conditions. In response to 400, 450, and 500 mOsm/kg stress, TLR4 mRNA was upregulated by 1.40, 2.72, 8.18 fold in SV40 HCEC, and TLR9 mRNA was downregulated by 0.38, 0.58 and 0.16 fold compared to the control (P–value <0.05 Student's t-test, n=3). Unlike TLR4 and TLR9, TLR5 mRNA expression was not significantly modulated in response to HOS (data not shown for TLR5). To determine if the change in TLR4 and TLR9 mRNA expression would return to baseline upon withdrawal of HOS, SV40 HCEC were allowed to recover for 6 and 24 hours in normal growth media following 24 hours treatment with 500mOsm/kg media then TLR4 and TLR9 mRNA expression was examined (Figure 3.1A). TLR4 expression returned to baseline after a 6 hour incubation in normal growth media (n=4) while TLR9 mRNA expression remained downregulated at 6 hours (n=3) and returned to baseline compared to the untreated control after 24 hours (n=2).

Since HOS dramatically increased TLR4 mRNA expression more so than TLR5 or TLR9, TLR4 mRNA expression was then examined in other ocular surface cells (Figure 3.1B). As with the SV40 HCEC, HOS significantly increased the expression of TLR4 by 1.35, 3.56, and 9.70 fold in primary HCEC and in IOBA NHC cells by 2.41, 3.55, 3.36 fold in response to 400, 450, and 500 mOsm/kg stress respectively, n=3. To confirm a potential change in TLR4 and TLR9 protein expression in response to HOS, semi-quantitative western blotting was performed on SV40 HCEC cells treated with 400, 450, and 500 mOsm/kg media (Figure 3.1C). TLR9 protein expression was decreased in response to HOS but this only reached statistical significance at 500 mOsm/kg under which condition TLR9 expression was reduced by 72% (n=3). Unexpectedly, TLR4

protein levels also decreased by 33.5%, 42.8%, and 67.7% in response to 400, 450, and 500mOsm stress, (n=3).

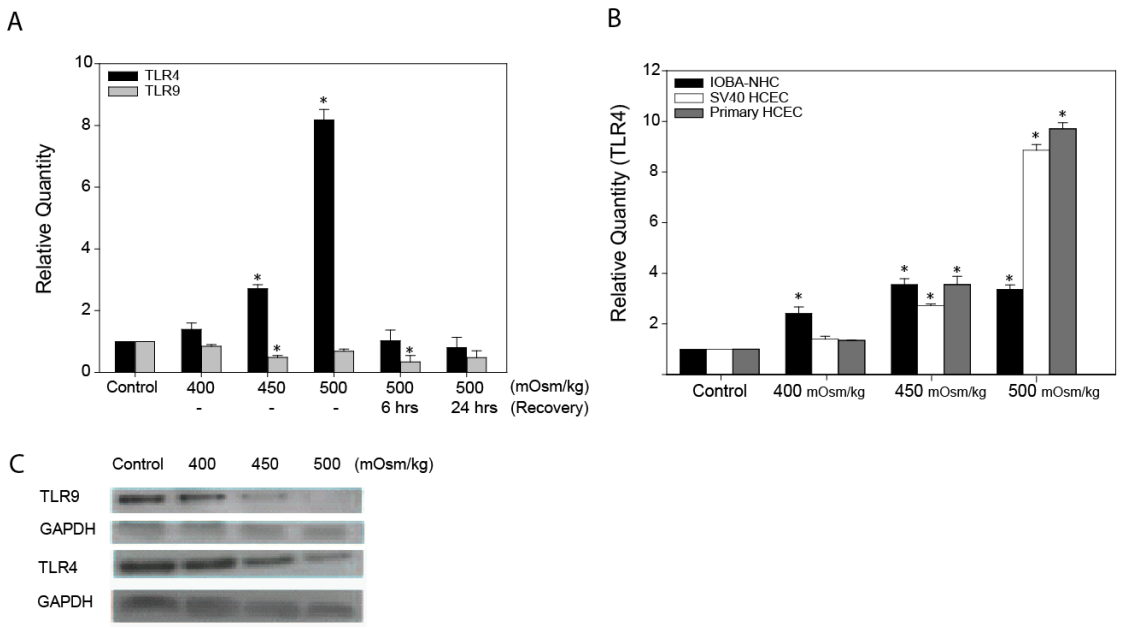


Figure 3.1 TLR mRNA and protein expression is modulated in response to hyperosmolar stress (HOS) in ocular surface cells.

SV40 HCEC were cultured under HOS (400-500 mOsm/kg) or media alone (control) for 24 hours and TLR4 and 9 mRNA expression was determined by quantitative RT-PCR. In some experiments, following HOS of 500 mOsm/kg, the cells were allowed to recover for 6 and 24 hours in normal growth media, n=4 for TLR4 and n=2 for TLR9 (A). Corneal (primary and SV40 HCEC) and conjunctival epithelial cells (IOBA-NHC) were cultured under HOS then TLR4 mRNA expression was determined (B). To confirm a change in protein expression, cell lysates from HCEC cultured under HOS were analyzed by western blotting for TLR4, 9 and GAPDH (C). Unless otherwise stated, data are representative of a minimum of three experiments and were analyzed using an unpaired Student's *t*-test where $P \leq 0.05$ was considered to be statistically significant compared to control (*).

Dry Eye Associated Cytokines Do Not Modulate the Expression of TLR4, 5, and 9

Since various cytokines have been found in the tear film of patients with dry eye, ocular surface cells were cultured with dry eye associated cytokines and TLR4, 5, and 9 mRNA expression was determined by quantitative RT-PCR and semi-quantitative western blotting. Using 10 ng/ml for 24 hours, IL-1α, IL-1β, TNFα, and TGFβ, did not modulate the expression of TLR4 mRNA (Figure 3.2A) or protein (Figure 3.2B) or TLR9 protein in SV40 HCEC (Figure 3.2B), n=3. Additional concentrations and time points were then examined to ensure that a more optimal testing condition was not overlooked. Again, IL-1β did not significantly modulate the expression of TLR4, 5, and 9 mRNA in SV40 HCEC at concentrations ranging from 1,10, 100, and 1000 ng/ml (n=2) after 24 hours or after 3, 9, and 12 hours of treatment using 10 ng/ml (Table 3.1).

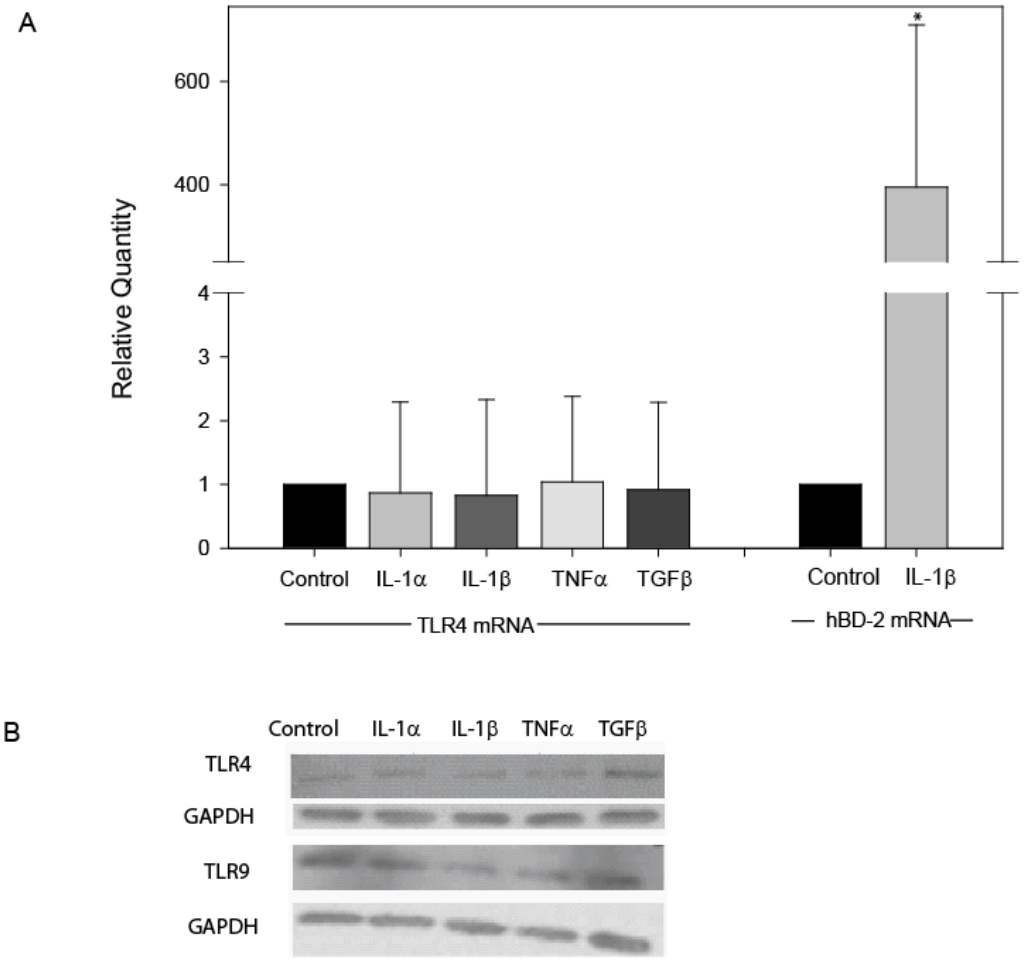


Figure 3.2 Various cytokines do not modulate TLR4 and TLR9 mRNA expression. SV40 HCEC were cultured with dry eye associated cytokines, IL-1α, IL-1β, TNFα, and TGFβ (10 ng/ml for 24 hours) and either quantitative RT-PCR was performed to determine TLR4 or hBD-2 mRNA expression, n=3 (A) or western blotting was performed to determine TLR4 and TLR9 protein levels, n=3 (B). Data were analyzed using an unpaired Student's *t*-test, where an (*) represented statistically significance (P=0.0465).

Relative Quantity (RQ) of TLR mRNA expression in response to IL-1β

24 hours (ng/ml) IL-1β	**TLR4** RQ ± std. dev.	**TLR5** RQ ± std. dev.	**TLR9** RQ ± std. dev.
1	1.10 ± 0.22	1.46 ± 0.31	1.01 ± 0.07
10	1.66 ± 0.38	1.86 ± 0.26	1.12 ± 0.15
100	1.48 ± 0.25	1.62 ± 0.42	1.21 ± 0.18
1000	1.09 ± 0.03	1.93 ± 0.57	1.31 ± 0.20
10ng/ml IL-1β (time)	TLR4 RQ ± std. dev.	TLR5 RQ ± std. dev.	TLR9 RQ ± std. dev.
3hr	1.25 ± 1.24	1.24 ± 0.20	0.61 ± 0.75
9hr	2.06 ± 0.74	0.94 ± 0.28	1.31 ± 0.23
12hr	1.52 ± 0.71	1.15 ± 0.09	1.55 ± 0.54

Table 3.1 IL-1β did not significantly modulate the expression of TLR4, 5, and 9 mRNA in SV40 HCEC at various time points and concentrations.

SV40 HCEC were cultured with IL-1β at concentrations ranging from 1, 10, 100, and 1000 ng/ml for 24 hours or with 10 ng/ml of IL-1β for 3, 9, and 12 hours. Quantitative RT-PCR for TLR4, 5 and 9 mRNA expression was then performed using the cell lysates. Data are given as the relative quantity (RQ) of TLR mRNA expression compared to the untreated control when normalized to the housekeeping gene, GAPDH. Data are representative of two independent experiments.

Desiccation Modulates the Expression of TLR4, 5 and 9 in Human Corneal Epithelial Cells

TLR expression in response to desiccation in a human cornea organ culture model (Figure 3.3), was also examined. Under these conditions, TLR4 and 5 mRNA were upregulated in human corneal epithelial cells by 4.81 (P-value <0.0001) fold and 2.51 (P =0.0304) fold respectively whereas TLR9 was downregulated by 0.86 (P-value <0.0001) fold of the control, n=3 (Figure 3.3A). Semi-quantitative western blotting demonstrated an upregulation of TLR4 protein by approximately 10% and a downregulation of TLR9 protein by 20.47% in response to desiccation in two of the three samples tested compared to the submerged control, n=3 (Figure 3.3B).

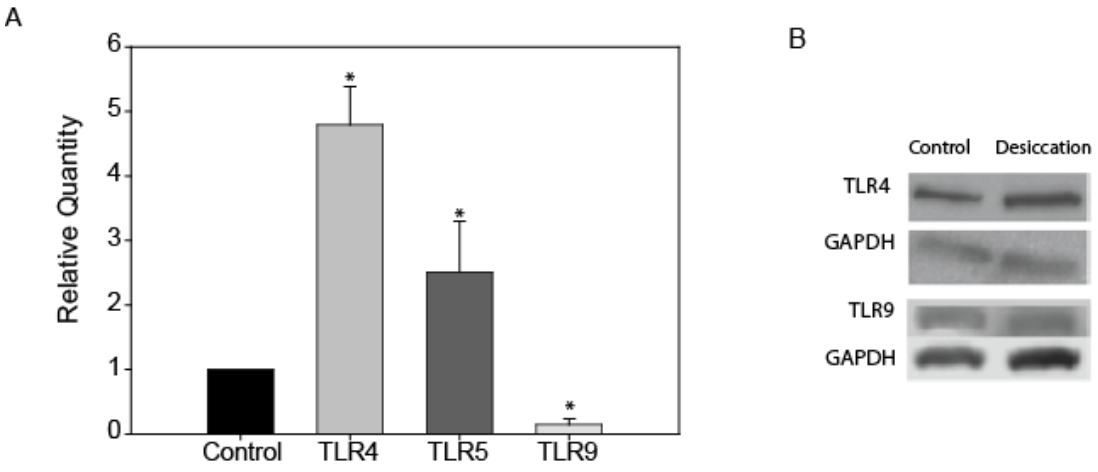


Figure 3.3 Desiccation modulates the expression of TLR4, TLR5 and TLR9 in human corneal epithelial cells in an organ culture model.

TLR mRNA expression by quantitative RT-PCR (A) or protein expression by western blotting (B) were determined in epithelial cells harvested from human corneas in organ culture for 24 hours. Data are representative of a minimum of three experiments and were analyzed using a one-way ANOVA where $P \leq 0.05$ was considered to be statistically significant (*).

TLR mRNA expression in Conjunctival Impression Cytology Samples from Dry Eye Subjects and Age-Matched Normal Subjects

Thirty two subjects (10 males, 22 females) enrolled in the study but of these, 8 dry eye subjects were excluded due to either low RNA yield from CIC samples or low tear volume collection. Clinical objective measurements are listed in Figure 3.4A for subjects that were included in the study (n=24). When comparing dry eye subjects to age and gender-matched normal subjects, there was a significant decrease in tear film stability (TBUT) and in tear production (phenol red thread test). Dry eye subjects also had a significant increase in OSDI score, tear film osmolality, corneal and conjunctival staining.

Conjunctival impression cytology samples were collected after all the objective measurements were made to compare TLR4, 5 and 9 mRNA expression between dry eye subjects and normal age and gender-matched controls (Figure 3.4B). TLR5 mRNA was downregulated to 0.67 ± 0.53 fold (P = 0.0844) of that of the normal subjects but this was not statistically significant. There was a significant (P = 0.006) downregulation of TLR9 mRNA to 0.415 ± 0.515 fold or by almost 59.5% on average in the dry eye subjects compared to the normal subjects. TLR4 was upregulated by 1.9 ± 3.2 fold, but as with TLR5, this was not statistically significant (P = 0.337). However, it is worth noting that in dry eye subjects, the greatest increase in TLR4 expression was in patients with a high ODSI score (>65) as shown in the scatter plot in Figure 3.4C. However across the entire data set the correlation was not statistically significant.

A

Subject	Age (years)	OSDI (score)	Phenol Red (mm)	TBUT (sec)	Osmolality (mOsM/kg)	Corneal Staining (grade)	Conjunctival Staining (grade)
Normal	44.2 ± 13.3	5.32 ± 5.28	28.8 ± 6.45	13.2± 6.44	305.4 ± 2.40	1 ± 0	1 ± 0
DES	45.1 ± 15.6	47.23 ± 18.46	21.7 ± 5.43	5.54 ± 3.01	324.1 ± 19.0	1.98 ± 0.944	2.31 ± 0.833
P-Value	NS	<0.0001	0.0002	<0.0001	0.044	0.007	0.0045

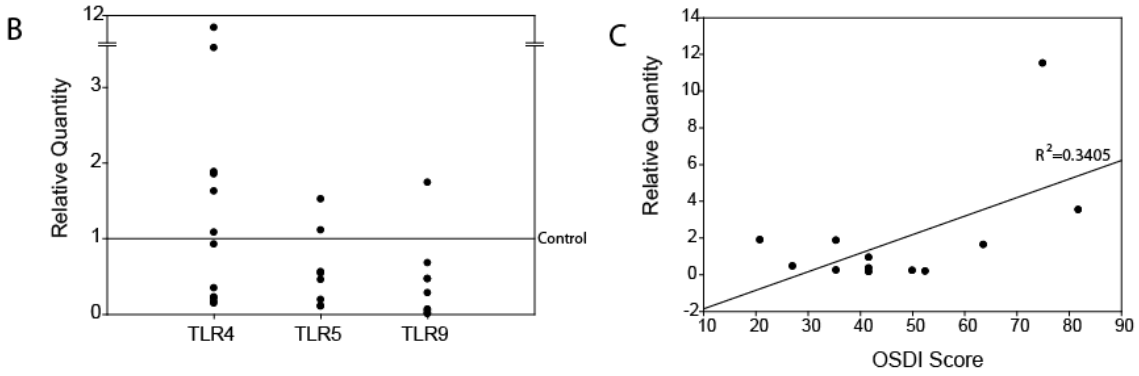


Figure 3.4 Clinical Characteristics and TLR mRNA Expression in Dry Eye Patients. Compared to age-and gender-matched controls, dry eye patients had significantly less tear production (phenol red), a less stable tear film (TBUT), and increased corneal and conjunctival staining and tear film osmolality (A). The relative quantity of TLR4, 5 and 9 mRNA expression in CIC samples from dry eye and age- and gender-matched controls in which the latter were normalized to one. Data points falling above or below the control line represent an upregulation or downregulation in TLR expression respectively. There was a statistically significant decrease in TLR9 mRNA expression. (B). Correlation between TLR4 mRNA expression and increasing severity of dry eye as measured by the OSDI, n=12 (C).

3.4 Discussion

The results from this study have shown that TLRs are modulated in dry eye and dry eye associated conditions (HOS and desiccation), suggesting their involvement in the pathogenesis of the disease. Overall it was found that in the dry eye environment TLR9 was downregulated, TLR 4 was upregulated and TLR5 was upregulated or unchanged.

To investigate the effects of HOS, primary and SV40 HCEC and the IOBA-NHC, conjunctival epithelial cell line were cultured with hyperosmolar media ranging from 400-500 mOsm/kg. The average osmolality of the culture media was 330 mOsm/kg therefore HOS was induced by raising the osmolality by 70-170 mOsm/kg through the addition of NaCl. In response to HOS, TLR4 was upregulated in a concentration-dependent manner, TLR9 was downregulated, but there was no significant change in TLR5 in SV40 HCEC. Since TLR4 demonstrated the most substantial change in expression in SV40 HCEC, TLR4 was also examined in IOBA-NHC and primary HCEC. Here, TLR4 mRNA was upregulated in the conjunctival epithelial cell-line and most significantly in the primary HCEC, which gave comparable results to those found with SV40 HCEC. However, unlike the mRNA expression, TLR4 protein was significantly downregulated in response to HOS. One possible explanation is that the upregulation of TLR4 mRNA is a compensatory mechanism in an attempt to increase protein levels that are diminishing with increasing HOS however it is unclear what causes the decrease in TLR protein with increasing HOS.

Hyperosmolar stress has been suggested to be the gold standard for diagnosing dry eye and the accepted range for normal tear film is 296-308 mOsm/L (Gilbart *et al.*, 1978). Tear film osmolarity in dry eye patients has been documented to reach as high as 440

mOsm/L (Farris, 1994) but is thought to reach even higher (800-900 mOsm/kg) transient levels and induce symptoms of dry eye (Lui *et al.*, 2009). However, transient spikes in tear film osmolality are difficult to detect as evident in this study, since the dry eye subjects tear film osmolality was only elevated by ~20 mOsm/kg compared to the normal subjects. When considering HOS alone, the osmolality that was detected in dry eye subjects was not as high as that which altered TLR4 expression *in vitro* which may be responsible for the lack of change in TLR expression *in vivo*. Furthermore, although this study attempted to isolated the effects of HOS, previous studies have shown that HOS increases the expression of several cytokines such as IL-1α, TNFα and IL-8 (Li *et al.*, 2006), which could act synergistically with HOS and be responsible for the change in TLR expression found in this study.

Previously, dry eye associated cytokines have been shown to modulate the expression of antimicrobial peptides (McDermott *et al.*, 2003). Therefore, this study examined if cytokines could have the same effect on TLR expression. Here, individual dry eye associated cytokines (IL-1α and β, TNFα and TGFβ) were not able to modulate the expression of TLR4, 5, and 9 mRNA and TLR4 and TLR9 protein expression. Very little is reported in the literature regarding the role of cytokines in modulating TLR expression. Cook *et al.* (2005) observed an upregulation of TLR2 in cultured conjunctival epithelial cells in response to interferon gamma (IFNγ). Begon *et al*. (2007), using longer time points than in the present study reported that IFNγ increased the expression of TLR2, 3, 4, 5, and 9, whereas TNFα increased the expression of all of these and TLR6 after 72 hours of stimulation in keratinocytes. The tear film in dry eye and SS is very complex and dynamic with an increase in numerous cytokines and chemokines, including those

examined in this study and others such as, IL-6, IL-8, IL-10 and IL-17 (Enríquez-de-Salamanca *et al.*, 2010). Although individual dry eye associated cytokines did not modulate the expression of TLRs, other cytokines not tested here may, alone or synergistically, modulate the expression of TLRs.

Currently there is no widely used technique for sampling the corneal epithelium in patients, so many studies utilize CIC samples or primary HCEC to investigate the ocular surface. Considering this, TLR expression was also examined in response to desiccation using an organ culture model. This model is beneficial as it may most mimic the dry eye environment (i.e. HOS and cytokines) that occurs *in vivo*, the corneal epithelium remains intact, stratified, and connected to the stroma and limbus and it is more representative of what occurs on the corneal epithelium *in vivo* compared to a monolayer culture. In the corneal epithelium of this model, there was an increase in TLR4 and TLR5 mRNA. Notably, there was also a significant decrease in TLR9 mRNA and protein in this model which is in agreement with the HOS and CIC samples. The exact stimuli that downregulate TLR9 expression in the desiccation model has yet to be determined. Therefore quantifying the HOS and cytokine production in this model would provide insight to the mechanism.

Unlike the corneal epithelium, sampling the conjunctival epithelium can be easily performed by impression cytology and provides the best representation of what occurs in the conjunctiva *in vivo* in dry eye patients. Therefore, TLR mRNA expression was examined in CIC samples from dry eye patients and age- and gender-matched normal subjects. TLR9 was significantly downregulated in patients with dry eye but there were no significant change in TLR5 and TLR4 mRNA expression among the subjects.

There are no published studies on ocular surface TLR expression in dry eye patients, although a preliminary study by Bonini *et al.* (2006) showed an upregulation in TLR2 mRNA but not protein. However, similar changes in TLR4 and TLR9 expression to those reported here have been observed in other ocular surface inflammatory conditions, such as allergic conjunctivitis and more specifically in seasonal vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). Bonini *et al.* (2005) found that TLR2, 4, 9 were expressed in conjunctival biopsy specimens from patients with a normal ocular surface undergoing cataract surgery and patients with VKC. In the VKC patients, there was an increase in TLR4 and a decrease in TLR9 which is similar to the dry eye data reported in this study. Cook *et al.* (2005) observed TLR2 expression in CIC samples from human subjects with AKC and allergic conjunctivitis, but not in patients without ocular allergies. It remains to be determined if changes in TLR expression are a "cause or effect" of ocular surface inflammation.

Of all the TLRs examined, TLR9 was consistently downregulated in the corneal and conjunctival epithelial cells in all the conditions tested with the exception of exposure to individual cytokines, which did not modulate the expression of any TLRs tested. TLR9 recognizes microbial DNA which is characterized by an abundance of unmethylated CpG dinucleotides, which can induce a strong inflammatory response (Hemmi *et al.*, 2000 and Bauer *et al.*, 2001). In the cornea, activation of TLR9 induces sight threatening keratitis (Johnson et al, 2009) while inhibiting TLR9 by siRNA in C57BL/6 mice infected with PA decreases corneal inflammation (Huang *et al.*, 2005). Thus downregulation of TLR9 may have an anti-inflammatory effect on the ocular surface. The ocular surface is the most exposed mucosal surface in the body and the epithelium is constantly exposed to a myriad

of pathogens. Previous studies have shown that TLR5 expression is limited to the basal and wing cells of the corneal epithelium (Hozono *et al.* 2006; Zhang et al, 2003) which may limit its responsiveness to pathogens on the ocular surface. In contrast, TLR9 is expressed throughout the corneal epithelium, even on superficial squamous epithelial cells. Considering this expression profile, downregulation in TLR9 may be beneficial in reducing undesired TLR activation to either commensal or pathogenic bacteria. Downregulation of TLR9 has been implicated to have pathogenic role in the development of disease in other mucosal tissue. TLR9 mRNA expression is downregulated in macrophages infected with human cytomegalovirus and Epstein-Barr virus (Lin and Li, 2009).

A change in TLR expression pattern may have both beneficial and detrimental effects. Increased TLR expression may enhance pathogen recognition, but may also lead to inappropriate and exacerbated inflammatory responses, thereby contributing to disease processes such as allergy and dry eye. Alternatively, reduced expression may lead to an inadequate recognition response and increased risk of infection. However, the latter may be compensated for by the fact that many pathogens are recognized in more than one way, including interactions with multiple TLRs and with other pattern recognition receptors. For example, mice deficient in TLR4 still respond to PA inoculation (Huang *et al.* 2006), presumably at least partially because flagellin PA can also be recognized via TLR5. In general, TLR activation by ocular surface epithelial cells stimulates an immune response through the production of cytokines and chemokines. Therefore, downregulation in TLR expression, such as seen here with TLR9, may represent a response to try to minimize hyper-responsiveness in dry eye.

Chapter 4 - Mice with Experimental Dry Eye are Resistant to Toll-Like Receptor Induced Inflammation

4.1 Introduction

Dry eye is a common multifactorial inflammatory condition that results in ocular discomfort and causes patients to be three times more likely to report problems with common daily activities (Miljanovic *et al.,* 2007). As discussed in Sections 1.3.2.2 and 1.5, dry eye patients have significant inflammation as evident by the production of various cytokines and MMPs, and by ocular surface epithelial cell apoptosis that increases the risk of corneal ulceration (Vivino *et al*., 2001). According to the 2007 DEWS Report, few data exist on the risk of infection in dry eye patients and future studies are needed to quantify the risk of ocular surface infection among patients with dry eye.

The ability of the ocular surface to mount an immune response to infectious pathogens is in part attributed TLRs and AMPs, and a change in their expression may alter the risk for infections. TLR activation triggers the production of various proinflammatory cytokines, chemokines, and AMPs. A number of AMPs have been identified in human corneal and conjunctival epithelial cells such as human β-defensin (hBD) -1-3 and cathelicidin (LL-37). hBD-1, hBD-3 and LL-37 are constitutively expressed by both corneal and conjunctival epithelia whereas hBD-2 expression is inducible by conditions mimicking injury, inflammation and in response to bacterial products (Gordon *et al.,* 2005; McDermott *et al.,* 2001, 2003; McNamara *et al.,* 1999).

TLRs have also been thought to be involved in dry eye inflammation. Kawakami *et al.* (2007) studied TLR expression by immunohistochemistry in labial salivary glands and observed increased expression of TLR2, 3 and 4 in samples from patients with SS.

Spachidou *et al.*, (2007) also reported that the expression of TLR1, 2 and 4 was significantly greater in salivary gland epithelial cells from SS patients, suggesting the active participation of TLRs in the pathophysiology of SS. The expression of TLR8 and 9 has been found to be significantly upregulated in the salivary glands of SS patients (Gottenberg *et al.*, 2006). Taken together, TLRs may play a role in mediating some of the events in dry eye inflammation. In such a scenario, TLR activation would, likely be via various endogenous ligands and/or normal flora bacteria rather than pathogens. For example, a sudden increase in the level of TLRs may lead to TLR overactivation, but also an increase in the production of AMPs to reduce the risk of microbial infections. Therefore, determining the potential role for TLRs and AMPs in the pathogenesis of dry eye may lead to the development of much needed novel therapeutic options. In this study, the expression of TLRs and/or antimicrobial peptides was examined in mice with experimental dry eye and in dry eye subjects. To determine if mice with experimental dry eye (EDE) are susceptible to TLR induced inflammation, mice with EDE were subjected to topical TLR agonist treatment and the corneal inflammatory response was examined by *in vivo* imaging and immunohistochemistry.

4.2 Methods

Experimental dry eye

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine or the Institutional Animal Care and Use Committee of the University of Houston and conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experimental dry eye (EDE) was induced in 6- to 12-week-old C57BL/6 mice of both genders (Jackson Laboratories, Bar

Harbor, ME) by subcutaneous injection of 0.5 mg/0.2 ml scopolamine hydrobromide (Sigma-Aldrich; St. Louis, MO) in the hindquarters four times a day, exposure to an air draft and <40% humidity, as previously reported (De Paiva *et al.*, 2007) for five days. Age- and gender-matched mice that did not receive any treatment to induce dry eye served as the untreated (UT) control. After 5 days of treatment, the animals were euthanised and the corneal epithelium was removed by scraping and the conjunctiva and lacrimal gland were removed by dissection and placed in respective tubes containing denaturation solution (ToTALLY RNA kit, Ambion; Austin, TX), snap frozen and stored at -80^0C until further analysis to determine TLR2, 3, 4, 5, 9, CRAMP, mBD-3 and mBD-4 mRNA expression (5 animals/group, n=3-4). In another set of animals, whole eyes were removed for immunohistochemistry to determine TLR2-5, CRAMP, mBD-3 and mBD-4 protein expression (n=2).

Epithelial Abrasion Model

Six- to 12-week-old C57BL/6 mice of both genders (Jackson Laboratories, Bar Harbor, ME) were anesthetized by intraperitoneal injection (ketamine, 60 mg/kg; xylazine 6 mg/kg; Vedco, Inc., St. Joseph, MO). Using a surgical microscope, three parallel 1 mm scratches penetrating the epithelial basal lamina into the superficial corneal stroma were made to the right and left eye of anesthetized mice using a sterile 27-gauge needle as previously described (Huang *et al.*, 2007).

Topical Application of TLR Agonist Cocktail

To evaluate the role of TLR agonists in dry eye inflammation, C57BL/6 mice were divided into three treatment groups: (1) UT control mice, n=6, (2) EDE mice, n=8 and (3) scratch model, n=3, which received three full-thickness epithelial abrasions (positive control). Upon recovering from general anesthesia,mice were manually restrained and a one-time topical application of 5 µl of the vehicle control (endotoxin-free physiological water) was applied to the left eye or 5 µl of TLR agonist cocktail was applied to the right eye. Individual mouse TLR agonists were purchased from Invivogen (San Deigo, CA), and were reconstituted in endotoxin-free physiological water to specific concentrations as previously described (Johnson et.al, 2005) and used as a cocktail containing TLR2 agonist: Pam3CSK3 (µg/µl), TLR3 agonist: PolyI:C (1 µg/µl), TLR5 agonist: Flagellin (1 µg/µl), and TLR9 agonist: ODN M362 (4 µg/µl). Following instillation of drops, the animals were returned to the vivarium (UT or scratch mice) or continued EDE for an additional day. Twenty four hours after agonist application, *in vivo* images using the Heidelberg Retinal Tomography III and Spectral Domain Optical Coherence Tomography were taken, the mouse was then euthanized and the entire eye was removed and processed for immunostaining for CRAMP, mBD-3 and mBD-4.

Human Subjects

All procedures involving human subjects were in accordance with the tenets of the Declaration of Helsinki and were approved by the University of Houston's Institutional Review Board. Written informed consent was obtained from all subjects before participating in the study. Subjects were screened and categorized as normal or dry eye by their subjective responses of the ocular surface disease index (OSDI) questionnaire

(Schiffman *et al.*, 2000) and the presence of objective clinical signs as follows. General ocular surface health of all subjects was assessed with a slit-lamp biomicroscope. The corneal and conjunctival epithelia integrity was visualized using fluorescein and lissamine green respectively were graded on a scale from 1-4 (Cornea and Contact Lens Research Unit [CCLRU] grading scale; School of Optometry, University of New South Wales, Sydney, NSW, Australia), tear secretion was measured by the phenol red thread test; tear film osmolality was measured using a vapor pressure osmometer (Vapro 5520; Wescor, Logan, UT); and tear stability by measured by fluorescein tear break-up time (Dry Eye Test; Akorn, Chicago, IL). Subjects were excluded if they were currently using topical anti-inflammatory drops or if they had any ocular infection or any other ocular surface disease other than dry eye.

Conjunctival Impression Cytology

Following the completion of all the objective clinical assessments, a single drop of 0.5% proparacaine hydrochloride anesthetic (Bausch and Lomb; Rochester, NY) was instilled onto the eyes. Two to three 6.5 x 13 mm sterile polyether sulfone membranes (Supor; Pall Gellman Sciences; East Hills, NY) were placed on the superior or inferior bulbar conjunctiva without applying pressure on each eye. The membranes were removed from both eyes and placed directly in one tube containing 350 µl of lysis buffer (Qiagen; Valencia, CA) and stored at -80°C until further RT-PCR analysis for hBD-2 and hCAP-18 (LL-37 precursor) mRNA expression. Total RNA from CIC samples was extracted using an RNeasy Micro Kit (Qiagen). RNA elution columns were DNAse treated prior to RNA extraction to avoid genomic DNA contamination

Real-time RT-PCR

Real-time RT-PCR was used to quantitate mRNA expression of TLRs and AMPs. cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen; Portland, OR). Reverse transcription was performed at 50°C for 60 minutes. Samples containing no reverse transcriptase or water in place of RNA (no template control) served as negative controls. PCR amplification of cDNA was performed with Brilliant SYBR Green QPCR Master Mix (La Jolla, CA) using specific primers listed in table 4.1. Thermocycler parameters for these primers were 95 °C for 10 minutes, followed by amplification of cDNA for 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 30 seconds. To examine mouse TLR4 mRNA expression, FAM labeled Taqman primers (Applied Biosystems Carlsbad, California) were used (mouse TLR4: Mn00445273_ml and mouse GAPDH: Mn03302249_ml) using thermocycler parameters of 95 °C for 10 minutes, followed by amplification of cDNA for 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at at 60°C for 60 seconds. All reactions were done in triplicate using a Mx3005P QPCR System (Stratagene). Amplified gene products were normalized to GAPDH or RPII, the internal control and calibrated to UT samples. The relative change of EDE versus UT samples was then determined with the value of control samples being normalized to one. Disassociation melt curves were analyzed to ensure reaction specificity. Data are representative of a minimum of two-three experiments and were analyzed using a Student's *t*-test where $P \leq 0.05$ was considered a significant difference.

Primer Name	Sequence (5'-3')	Tm (^{O}C)	Product Size
hGAPDH	Forward: GACCACAGTCCATGCCATCA	66.6	72bp
	Reverse: CATCACGCCACAGTTTCC	62.5	
hBD-2	Forward: GACTCAGCTCCTGGTGAAGC	60.6	831bp
	Reverse: TTTTGTTCCAGGGAGACCAC	60.9	
LL-37	Forward: GGACAGTGACCCTCAACCAG	60.2	1915bp
	Reverse: AGAAGCCTGAGCCAGGGTAG	60.8	
mTLR2	Forward: CGTTGTTCCCTGTGTTGCT	59.7	119bp
	Reverse: AAAGTGGTTGTCGCCTGCT	60.8	
mTLR3	Forward: TTGCGTTGCGAAGTGAAG	59.7	1829bp
	Reverse: TAAAAAGAGCGAGGGGACAG	59.4	
mTLR5	Forward: CAGGATGTTGGCTGGTTTCT	60.1	169bp
	Reverse: CGGATAAAGCGTGGAGAGTT	59.3	
mRPII	Forward: CTACACCACCTACAGCCTCCAG	61.1	656bp
	Reverse: TTCAGATGAGGTCCATGAGGAT	60.9	
CRAMP	Forward: GCCGCTGATTCTTTTGACAT	60.2	1006bp
	Reverse: GCCAAGGCAGGCCTACTACT	60.8	
mBD-3	Forward: GGATCCATTACCTTCTGTTTGC	59.8	1827bp
	Reverse: ATTYGAGGAAAGGAACTCCAC	55.7	
mBD-4	Forward: GCTTCAGTCATGAGGATCCAT	59.1	2611bp
	Reverse: CTTGCTGGTTCTTCGTCTTTTT	59.1	

Table 4.1 Human and Mouse TLR and AMP Primer Sequences used with SYBR Green Analysis

Immunohistochemistry

The eyes including the lids of mice were excised, embedded in optimal cutting temperature compound (VWR, Suwanee, GA), and frozen in liquid nitrogen. 10 μm sections were cut along the sagittal plane of the eye with a cryostat (Leica CM 1950; Leica, BannockBurn, IL) and placed on glass slides and stored at −80°C. Sections were then fixed with acetone and placed in blocking solution (0.1% either goat or rabbit serum, 0.1% Triton X-100, 1% fish gelatin and 5% BSA). After blocking for two hours, the sections were incubated with specific primary antibodies listed in table 4.2. Briefly, 1 μg/ml of either goat anti-CRAMP, rabbit anti-mBD-3, rabbit anti-mBD-4 (Santa Cruz Biotechnology; Santa Cruz, CA), 10 μg/ml anti-TLR2 (Abcam; Cambridge, MA), 10 μg/ml anti-TLR3 (Imgenex; San Diego, CA), 5 μg/ml anti-TLR4 (Abcam), or 10 μg/ml anti-TLR5 (Imgenex), at 4°C overnight. The following morning the slides were washed in PBS and blocked again for 30 minutes at room temperature and then incubated with the respective (5 μg/ml) Alexa 546- or (6.6 μg/ml) Alexa 488- conjugated second antibody (Invitrogen) in blocking solution for one hour at room temperature in the dark. As a negative control, some sections were incubated with the same concentration of the relevant isotype control instead of the primary antibody. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and the sections viewed and processed identically with a DeltaVision imaging system.

Company	Antibody	Product #	Isotype	Clone or Protein Accession #
Santa Cruz	goat polyclonal anti-cramp	SC34169	IgG	P51437
Santa Cruz	rabbit anti-mBD3	SC30116	IgG	Q9WTL0
Santa Cruz	rabbit anti-mBD4	SC30118	IgG	P82019
Abcam	mouse anti-mouse TLR2	ab16894	IgG1	t2.5
Imgenex	mouse anti-mouse TLR3	DDX0475	IgM	716G10.15
Abcam	rabbit polyclonal TLR4	ab47093	IgG	GLE ELQHLDFQHS TLKRV
Imgenex	mouse anti-mouse TLR5	IMG-664A	IgG2a	19D759.2

Table 4.2 Primary Antibodies used for Immunostaining

In vivo Imaging using Heidelberg Retinal Tomography (HRT) III

A HRT III Rostock Corneal Module (Heidelberg Engineering; Heidelberg, Germany) was used to investigate the corneal inflammatory response using enface and oblique-sectional images similar to that previously described (Labbé *et al*., 2006). The objective lens was covered with a TomoCap (Heidelberg Engineering; Heidelberg, Germany) and Genteel (Novartis, Basel, Switzerland) was placed on the tip of the objective lens and the cap to maintain immersion contact between the objective lens and the eye. Animals were positioned so that the corneal apex was perpendicular to the objective cap. Oblique-sections and enface images were taken with a 400 µm objective lens, covering an area of 400×400 µm and were acquired using an automatic-gain mode. Corneal images were taken from the apex of the cornea and oblique sections of the peripheral cornea were obtained by focusing up to a depth of 700 µm. Enface images, 5-10 µm beneath the basal epithelium were identified as previously described (Reichard et al, 2010) and evaluated using a volume scan.

Central Corneal Thickness

A Spectralis Spectral Domain Optical Coherence Tomography (SD-OCT) instrument (Heidelberg Engineering) was used for *in vivo* central corneal thickness (CCT) measurements as previously described (Hanlon *et al.*, 2010). Briefly, a 30D aspheric lens was attached to the front of the instrument to allow for corneal scanning. The optimal focal power and reference arm setting for image acquisition were determined and the same values used for all subsequent scans. Mice from the three groups (UT, EDE, and scratch)

were anesthetized and the cross-sectional measurement was obtained by selecting the scan which passed through the center of the pupil with the iris plane perpendicular to the scanning beam. To obtain CCT measurements, images were analyzed using ImageJ software and an appropriate pixel to length (μm) conversion factor was applied. Three CCT measurements were made for each scan, one at the center and one 50μm to the right and left of center. Data were analyzed using a Student's *t*-test where $P \leq 0.05$ was considered a significant difference compared to the UT control group.

Fluorescein staining

The SD-OCT was used to evaluate the corneal epithelial integrity in UT (n=6) mice and mice subjected to EDE (n=8). Following treatment, mice were anesthetized and a sodium fluorescein strip (Ful-Glo; Akorn, Lake Forest, IL) wet with preservative-free saline (Unisol; Alcon, Fort Worth, TX) was applied to the ocular surface corneal staining examined from photos taken under 488 nm blue-light illumination. To evaluate the fluorescein staining intensity, images were analyzed using ImageJ software. The fluorescein staining was graded in the central cornea using a 1 mm diameter circle, which was placed on the center of the cornea with approximately equal distances to the nasal/temporal aspects of the eye. The grayscale was converted to a black and white binary detection scale. To determine the baseline detection threshold to be used, the pixel intensity level was reduced until detection threshold was zero from UT mouse images. Next, the average pixel level that resulted in a threshold of zero for UT mice served as the detection threshold and was used to analyze all images (EDE and UT). The number of black dots (fluorescein stained areas of the cornea) were counted and averaged for the

right and left eye of each group. Data were analyzed using a Student's *t*-test where $P \leq 0.05$ was considered a significant difference compared to the UT control group.

4.3 Results

TLR mRNA expression is upregulated on the ocular surface and lacrimal gland in mice with dry eye.

C57BL/6 mice were subjected to experimental dry eye for 5 days and then expression of TLR2-5 and TLR9 mRNA was compared to that of UT control mice by quantitative RT-PCR (Figure 4.1A). In the corneal epithelium, there was a significant upregulation in TLR2, 3, 5 and TLR9 mRNA expression by 2.46 ± 0.27, 3.81 ± 1.44, 2.20 ± 0.19 and 2.23 ± 0.34 fold respectively. However, there was no significant change in TLR4 expression (1.05 ± 0.11 fold) compared to the UT control mice. In the conjunctiva all TLR mRNAs were upregulated with TLR2, 3, 4, 5 and 9 being upregulated by 2.47 ± 0.386, 8.98 ± 5.10, 8.26 ± 5.27, 3.81 ± 2.01, and 6.67 ± 4.12 fold respectively. Unlike the corneal epithelium and conjunctiva, in the lacrimal gland TLR4 and TLR9 were downregulated by 0.424 ± 0.02 and by 1.32 ± 0.45 fold respectively, while TLR2 was upregulated by 5.98 ± 1.35 fold and TLR5 by 6.11 ± 1.02 fold. There was no significant change in TLR3 (2.60 ± 2.08 fold change).

TLR protein is upregulated on the ocular surface and lacrimal gland in mice with dry eye.

To determine if there were corresponding changes in protein levels immunohistochemistry was performed to examine TLR2-5 protein in mice with EDE and UT controls. Attempts were made to examine TLR9 protein but there was no detectable signal above background

using this technique despite trying different concentrations and antibodies from multiple sources. In the corneal epithelium there was an increase in TLR2, 3 and 5 and no detectable change in TLR4 (Figure 4.1B). In the palpebral conjunctiva, there was a visible increase in TLR2 and 3 and a modest increase in TLR5 (Figure 4.1C). When comparing TLR2 and TLR5 mRNA, both of these were upregulated by almost similar amounts, however immunostaining revealed a striking increase in TLR5 protein in the lacrimal gland, and there was a modest increase in TLR2 and TLR3 (Figure 4.1D). There was no distinguishable difference in TLR4 in the EDE and UT mice. Data is representative of two experiments.

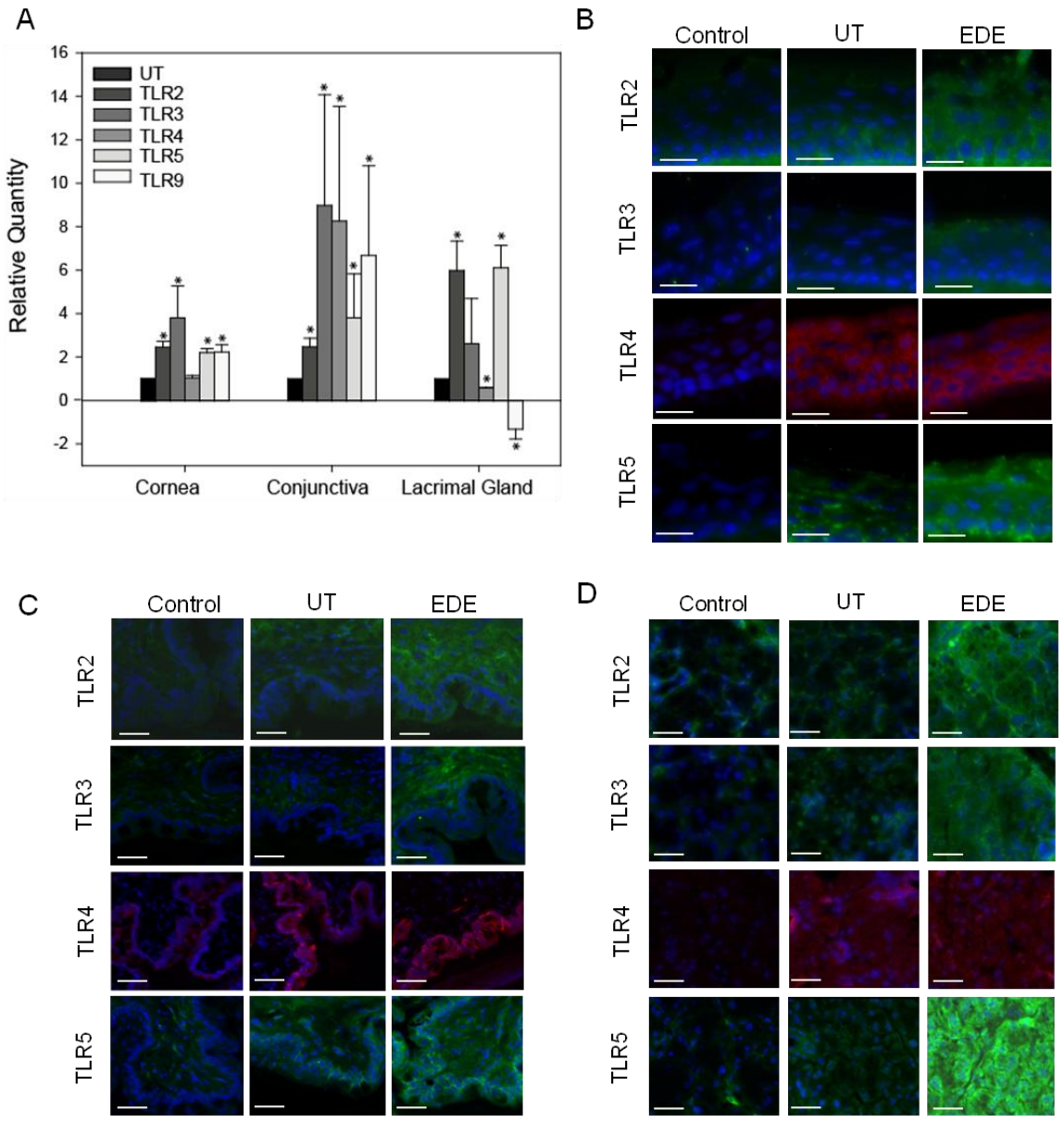


Figure 4.1 Toll-like receptor mRNA and protein expression on the ocular surface and lacrimal gland in mice with and without EDE.

Corneal epithelial, conjunctival and lacrimal gland TLR2, 3, 4, 5 and TLR9 mRNA expression was compared among mice subjected to EDE for 5 days and untreated (UT) controls, (n=3) (A). TLR2-5 protein was compared between mice with EDE and UT in the cornea (B), palpebral conjunctiva (C), and lacrimal gland (D). Some sections were

incubated with the respective isotype antibody (control). Data are representative of two independent experiments. Blue fluorescent DAPI (4,6'-diamidino-2-phenylindole) stained the cell nuclei**.** All images were taken at 200X magnification. Scale bars represent 20 (B) or 40 (C-D) microns. For each specific antibody and tissue, images were taken at the same exposure to compare TLR expression between the different treatment conditions. Quantitative RT-PCR data were analyzed using an unpaired Student's *t*-test where $P \leq 0.05$ was considered to be statistically significant (*).

EDE and TLR activation modulates the expression of antimicrobial peptides (AMPs) on the ocular surface.

Antimicrobial peptide mRNA and protein expression was compared among untreated (UT) mice and mice with EDE. Quantitative RT-PCR (Figure 4.2A) revealed that in the corneal epithelium CRAMP (LL-37 homolog) mRNA was significantly downregulated by 0.83 ± 0.29 fold ($P=0.007$), while mBD-3 and mBD-4 were upregulated although this did not reach statistical significance (n=3). Immunostaining (Figure 4.2B) revealed a decrease in CRAMP but no change in mBD-3 or -4 protein (n=2). In a separate experiment, mice were subjected to EDE and then a one-time topical application of a TLR agonist cocktail (to TLR2, 3, 5, 9) or the endotoxin-free vehicle control. The cocktail consisted of agonist for TLRs which were found to be upregulated in the corneal epithelium in mice with EDE (Figure 4.1). Immunostaining revealed that TLR agonist cocktail treatment decreased the expression of mBD-4 but had little effect on mBD-3 and CRAMP after 24 hours (Figure 4.2C).

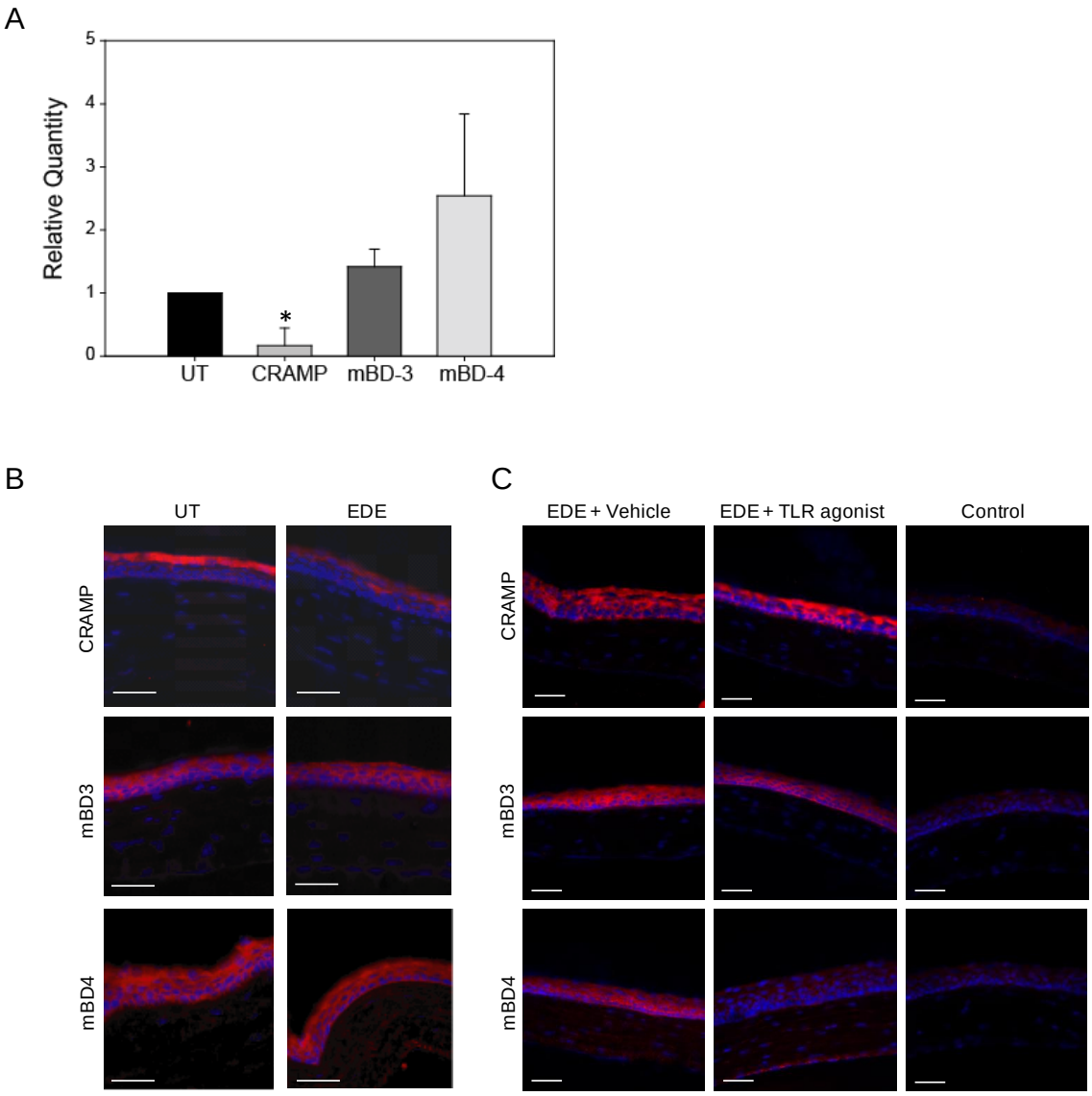


Figure 4.2 EDE and TLR activation modulates the expression of antimicrobial peptides (AMPs) in the corneal epithelium.

mRNA and protein expression of mBD-3, mBD-4 and CRAMP was compared among untreated (UT) mice and mice with EDE by quantitative RT-PCR, n=3 (A) and immunostaining, n=3 (B). A P-value ≤ 0.05 was considered to be significant (*) by Student's t-test. In a separate experiment, mice were subjected to EDE and then a one-

time topical application of TLR2, 3, 5, and 9 agonist cocktail or the endotoxin-free vehicle control. AMP expression was examined by immunostaining 24 hours later (C). Blue fluorescent DAPI stained the cell nuclei. Some sections were incubated with the respective isotype antibody (control). Scale bars represent approximately 40 microns. All images were taken at 200X magnification.

Clinical objective measurements and hCAP-18 and hBD-2 mRNA expression in dry eye and age- and gender-matched subjects.

All dry eye syndrome subjects (n=5) had significantly worse clinical objective findings than normal control subjects (n=4) except for corneal staining (Figure 4.3A). Dry eye subjects had a significant increase in hBD-2 mRNA expression by 19.8 ± 13.2 fold (P-value = 0.0131) while there was no change in hCAP-18 (LL-37) mRNA expression compared to age- and gender-matched normal subjects (Figure 4.3B).

A

Subject	Age	OSDI Score	Phenol Red (mm)	TBUT (sec)	Osmolality (mOsm/kg)	Corneal Staining (grade)	Conjunctival Staining (grade)
Normal	53.2 ± 8.55	1.63 ± 1.73	28.2 ± 4.32	12.6 ± 6.71	305.4 ± 2.40	1 ± 0	1 ± 0
DES	46.2 ±12.3	57.89 ± 21.2	19.2 ± 6.18	2.93 ± 0.51	314.6 ± 7.02	1.55 ± 0.758	1.9 ± 0.65
P-Value	0.327	0.0003	0.028	0.0121	0.02	0.143	0.014

B

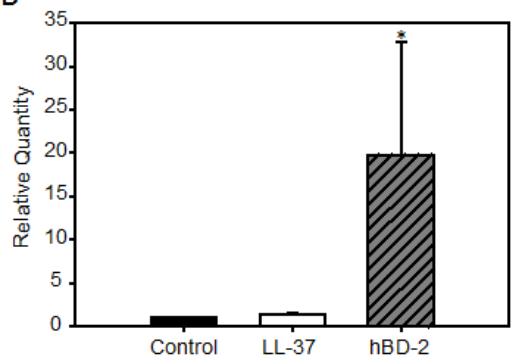


Figure 4.3 Clinical objective measurements and hCAP-18 and hBD-2 mRNA expression in subjects with dry eye syndrome and age/gender-matched subjects. Subjects were classified as having dry eye based on their subjective responses to the OSDI questionnaire and clinical objective measurements (A). CIC samples were analyzed to compare hCAP-18 (LL-37 precursor) and hBD-2 mRNA expression by quantitative RT-PCR (B). A P-value ≤ 0.05 was considered to be significant (*) by Student's t-test. The data are representative of 5 dry eye and 4 control subjects.

TLR agonists increase the recruitment of inflammatory cells into the anterior stroma in mice with a corneal scratch but not EDE

To evaluate the role of TLR agonists in dry eye inflammation, three groups of mice were studied (1) UT control mice (negative control), (2) EDE mice and (3) scratch model, which received three full-thickness epithelial abrasions (positive control). Each received a one-time topical application of TLR agonist cocktail to the right eye and vehicle control to the left eye. In the scratch model, enface images revealed TLR agonist treatment increased the recruitment of inflammatory cells into the anterior stroma compared to the fellow eye which received the scratch and vehicle control (Figure 4.4A). Oblique cross-sections confirmed that inflammatory cells were localized to the anterior half of the stroma (Figure 4.4B). However, in UT and EDE mice, TLR agonist topical treatment did not recruit significant numbers of inflammatory cells into the stroma.

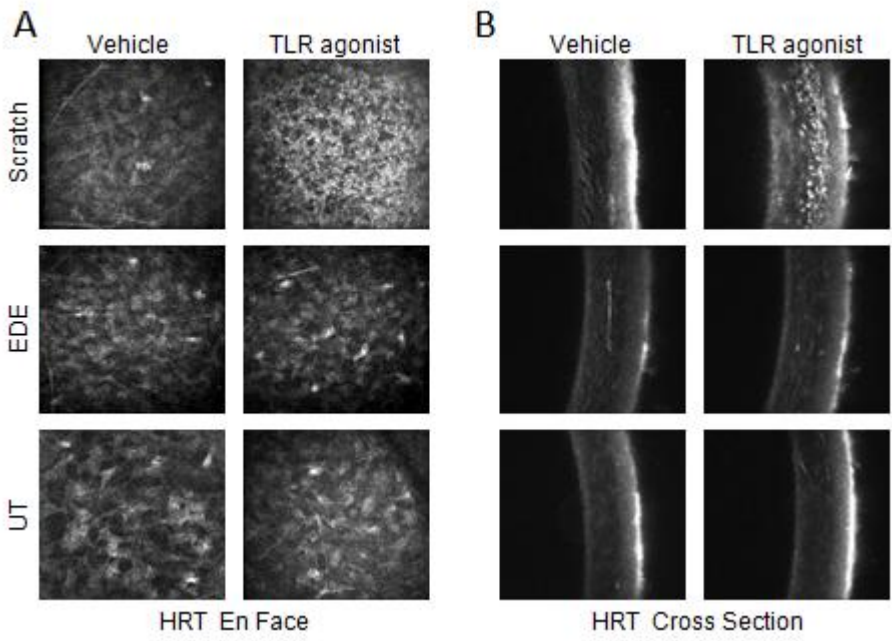


Figure 4.4 TLR agonists increase the recruitment of inflammatory cells into the anterior stroma in mice with a corneal scratch but not EDE.

TLR agonist cocktail was applied to the right eye or the vehicle control (endotoxin-free physiological water) was applied to the left eye of C57BL/6 mice in the following groups: (1) UT control mice (negative control), (2) EDE mice and (3) scratch model. 24 hours later, live *in vivo* enface (A) and oblique cross-sectional (B) images were taken with the Heidelberg Retinal Tomography III.

TLR agonists modulate the central corneal thickness (CCT) in mice with EDE and corneal scratch.

To further examine inflammation following TLR agonist treatment, the CTT was determined using the Spectralis. Cross-sectional images (Figure 4.5A) and measurements (Figure 4.5B) were obtained by selecting the scan which passed through the center of the pupil with the iris plane perpendicular to the scanning beam. Images and measurements were compared between the right (TLR agonist treated) and the left eye (vehicle control) for each group of mice. Central corneal cross-sectional images revealed that in EDE mice, six of eight (75%) eyes treated with the topical TLR agonist had an epithelial defect compared to two of the eight (25%) left eyes that were given the vehicle control. None of the untreated or the mice that were scratched 24 hours prior had central epithelial defects (Figure 4.5A). Measurements revealed no significant difference (P=0.4256) in CCT between the TLR agonist treated (OD) and the vehicle control (OS) in the UT mice. However, in mice with EDE, TLR agonist treatment significantly (P=0.0338) decreased CCT by almost 24% compared the vehicle control (87.15 ± 21.0 μm vs. 114.1 ± 10.3 μm). In the scratch model, TLR agonist treatment significantly (P=0.0484) increased CCT by 42.3 μm. In this model the TLR agonist treated eye CCT averaged 165.6 ± 13.86 μm, whereas vehicle control treated eye CCT averaged 122.3 ± 6.128 μm.

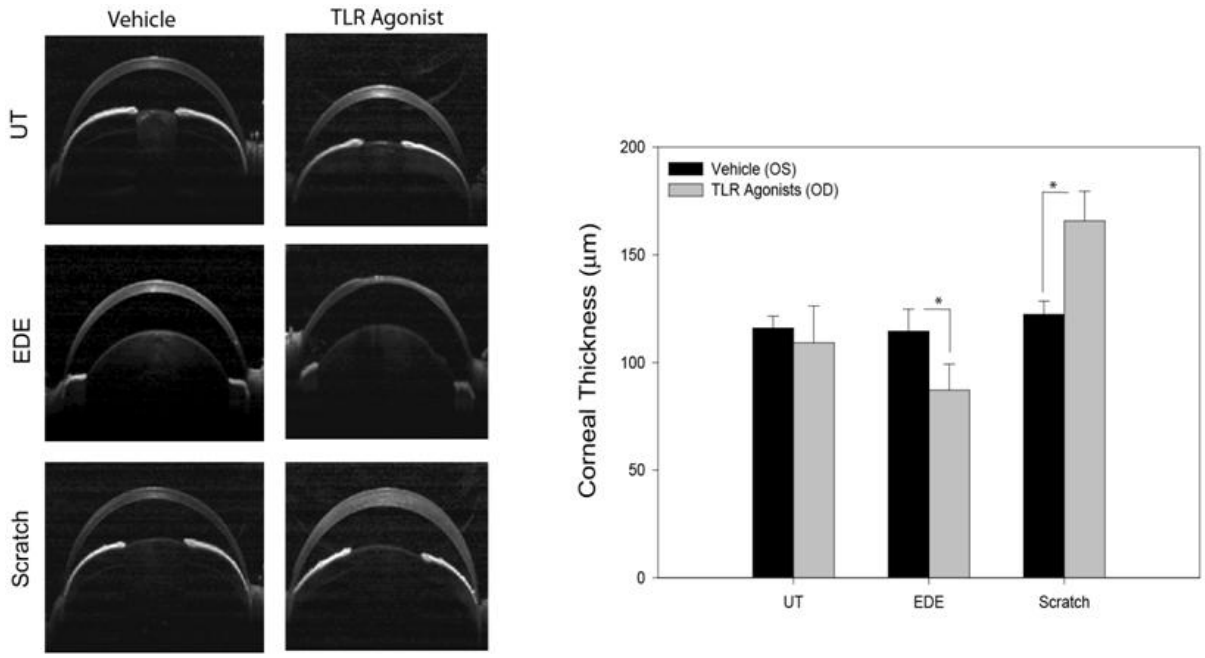


Figure 4.5 TLR agonists modulate the central corneal thickness in mice with EDE and corneal scratch.

Cross-sectional images (A) and measurements (B) were obtained from agonist treated mice by selecting the scan which passed through the center of the pupil with the iris plane perpendicular to the scanning beam. Using Image J three CCT measurements were made on each scan, one at the center and one 50µm to the right and left of center and averaged. A P-value ≤ 0.05 was considered to be significant (*) by Student's t-test.

Mice with EDE have significantly increased fluorescein staining compared to UT control mice.

To evaluate the corneal epithelium integrity following TLR agonist treatment, a sodium fluorescein strip (Ful-Glo) wet with preservative-free saline (Unisol) was applied to the ocular surface and corneal staining was examined on the right (TLR agonist treated) and left eye (vehicle). Images of untreated mice (n=6) revealed little corneal staining compared to the EDE mice (n=8, Figure 4.6A). To further quantify the staining pattern, ImageJ analysis was performed and revealed significantly more fluorescein staining i.e. pixels (OD: 8281.7 ± 3451.5, OS: 10458.5 ± 4735.5) in mice with 5D EDE compared to UT control mice (OD: 85.0 ± 156.4, OS: 280.8 ± 205.1) when comparing the same eye between the two groups (UT OD to EDE OD) as shown in Figure 4.6B.

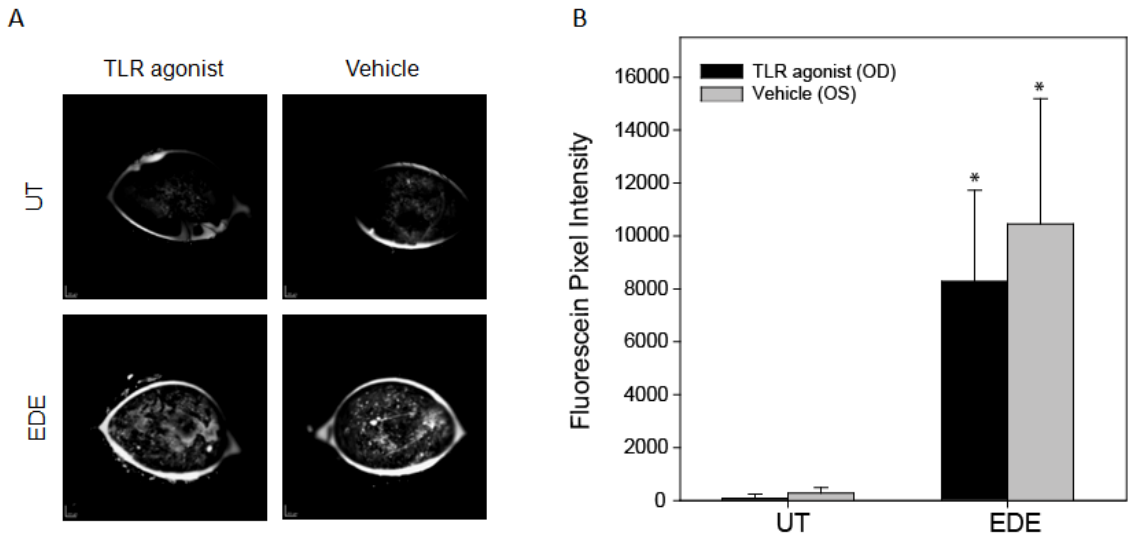


Figure 4.6 Mice with EDE have significantly increased fluorescein staining compared to UT control mice.

The Spectralis was used to evaluate the corneal epithelial integrity in UT (n=6) mice and mice subjected to EDE (n=8). Following treatment, mice were anesthetized, fluorescein was applied to the ocular surface and corneal staining was examined using a 488 nm blue-light illumination. Images were analyzed using ImageJ software to grade the fluorescein staining pattern in the central cornea. The pixel intensity was compared between UT and EDE mice with a P-value ≤ 0.05 considered significant (*) by Student's t-test.

4.4 Discussion

The results of this study demonstrate that EDE modulates the expression of several TLRs on the ocular surface and lacrimal gland, and the expression of AMPs on the ocular surface in humans and in the mouse model. Although, EDE increased TLR expression, topical application of TLR agonist did not stimulate an increase in corneal thickness or a recruitment of inflammatory cells to the cornea as shown in mice that received a corneal scratch. Rather, topical TLR agonist downregulated the expression of mBD-4 and resulted in a thinner cornea due to epithelial ulceration in EDE mice.

Previous studies have shown that TLRs are increased in SS and dry eye and it has been suggested this may contribute to the inflammatory environment. In this study, TLR2, 3, 5 and 9 were upregulated in the cornea, which is in partial agreement with Christopherson *et al.,* (2005) who found TLR4 and TLR5 mRNA expression is increased in the cornea of a SS mouse model. Aside from the data presented in chapter 3, only one other study has examined TLR expression on the ocular surface in patients with dry eye. Barabino *et al.* (2006) found that TLR2 mRNA expression is upregulated in conjunctival epithelial cells of patients with dry eye, but they did not find a corresponding increase in TLR2 protein, which the authors attributed to low sensitivity of their assay. Recently they have modified their technique using a new method of preservation of impression cytology samples that enhances flow cytometry analysis of epithelial and immune cells of the conjunctiva, which may allow them to detect changes in TLR protein expression that they could not previously (Barabino *et al.,* 2010).The activation of TLRs on the corneal epithelium can produce extensive ocular surface inflammation. In particular, the activation of TLR4 and 9 in the murine corneal epithelium has been shown to induce sight-

threatening keratitis (Johnson *et al.*, 2005) similar to that shown in this study. However in their model and the one used in this study, a scratch is required for inflammation to occur suggesting that disruption of epithelial basal lamina and superficial corneal stroma are required to initiate an inflammatory response. As shown in this study by corneal fluorescein staining, dry eye disrupts the corneal epithelium, but not to the extent required to permit TLR induced corneal edema and recruitment of inflammatory cells. Interestingly, in mice with EDE, topical TLR agonist treatment resulted in corneal thinning. A previous study has shown that the central and midperipheral cornea is significantly thinner in patients with dry eye using an Orbscan corneal topography system which the authors suggest is from a chronic state of desiccation, immune activation and corneal ulceration (Lui and Pflugfelder, 1999). Although here there was no difference in CCT between mice with EDE and UT mice, topical application of TLR agonist resulted in a significantly thinner central cornea by approximately 24% compared to the vehicle control. While the mechanism through which this occurs is unclear, considering that EDE stimulates the production of MMP-9 on the ocular surface (Corrales *et al.*, 2006), and TLR2 agonist can stimulate the production of MMP-9 in ocular surface cells (Li *et al.*, 2010), one scenario envisioned is that TLR agonists activate the corneal epithelium to produce additional MMP-9 on the ocular surface that further disrupts the corneal epithelium.

Although in humans, the corneal epithelium represents approximately only 10% of the total thickness of the cornea, the same relationship does not exist in the mouse. A recent study has shown that the epithelium contributes approximately 30% percent of the total central corneal thickness in the mouse (Tukler Henriksson *et al.*, 2009) suggesting

that a 24% reduction in CCT is plausible to be from epithelial cell loss alone and is in agreement with the *in vivo* images presented in this study. Since in this study, animals were exposed to a one-time topical dose of TLR agonist, additional exposure to TLR agonist may stimulate a significant inflammatory response, similar to that of the mice that received the scratch prior to exposure, putting these animals at risk for significant inflammation and visual impairment.

In this study, EDE significantly reduced the expression of CRAMP in the corneal epithelium which may predispose mice with EDE to microbial infections. In support of this, CRAMP knockout mice are more susceptible to PA keratitis, as these animals have significant delayed bacterial clearance and an increased number of infiltrating neutrophils in the cornea (Huang *et al.*, 2007a). Similar findings have also been observed in BALB/c mice when mBD-2 or mBD-3 expression is significantly reduced by siRNA. (Wu et. al, 2009a, 2009b). Conversely and most relevant to this study, a recent report has shown that C57BL/6 mice with EDE subjected to a high dose of either invasive (strain PA01) or cytotoxic (strain 6206) *P. aeruginosa* (PA) had normal clearance of PA and did not have an increase susceptibility to infection despite decreased tear volume (Heimer *et al.*, 2010). The lack of susceptibility to the PA infection may result from an insufficient disruption of epithelial basal lamina in EDE which occurs in the scratch model. Alternatively there may be an increase in other antimicrobial peptides to compensate for the reduced expression of CRAMP. In this current study using a small number of dry eye patients, there was a significant increases hBD-2 mRNA in conjunctival impression cytology samples, in mice with EDE, there was an increase in mBD-3 and-4 (ortholog to hBD-2) in the corneal epithelium but this was not statistically significant. Other antimicrobial peptides not

examined in this study may provide additional protection against infection during dry eye but little data exists in the literature regarding this area.

The human corneal epithelium normally expresses hBD-1 and -3, and low levels of LL-37, which may provide baseline protection against pathogens. A previous study has shown that hBD-2, but not hBD-1 and -3, were increased in patients with dry eye, but did not examine LL-37 expression (Narayanan *et al.,* 2003). Another study by the same group reported that mBD-2 was downregulated to undetectable levels in mice with EDE, whereas there was no change in mBD-1 (homolog to hBD-1) expression (Narayanan *et al.,* 2008). LL-37 is constitutively expressed on the ocular surface, and like hBD-2, its expression is upregulated during inflammatory conditions such as wound healing, whereas hBD-1 and -3 are not (Huang *et al.,* 2006; McDermott *et al.,* 2001). As LL-37 and hBD-2 are upregulated during inflammatory conditions, it seems logical that LL-37, in addition to hBD-2, would be upregulated in patients with dry eye when the ocular surface is compromised and inflamed. This could provide additional antimicrobial protection against potentially invading organisms and reduce the size of any epithelial defects (Huang *et al.,* 2006).

Interestingly, a new novel AMP, DEFB109 that is constitutively expressed on the ocular surface has been shown to be downregulated in patients with dry eye and also in patients with ocular microbial infections (Abedin *et al.,* 2008), this suggests that AMP expression may be downregulated at various levels of inflammation to maintain a balance of AMP expression. In regards to this present study, hBD-2 and mBD-4 expression were increased in response to inflammation that occurs in dry eye. However, upon additional

inflammation (i.e. TLR agonist stimulation), their expression maybe significantly reduced to prevent cytotoxic levels from accumulating on the ocular surface.

These findings demonstrate that dry eye modulates the expression of antimicrobial peptides and upregulates several TLRs on the ocular surface, however topical application of TLR agonist does not stimulate the robust inflammatory response in the stroma that occurs in mice with scratches that breach the epithelium and extend into the stroma. In mice with EDE, topical TLR agonists significantly decreased the CCT which was attributed by a marked thinning of the epithelium which may make the ocular surface vulnerable to additional inflammation.

Chapter 5 - Summary and Conclusion

Ocular surface inflammation is a major health concern and presents from various etiologies including dry eye and microbial infections. In particular, PA keratitis is the most common cause of contact lens related microbial keratitis. It is a very devastating condition that, if not treated properly, can result in permanent vision loss. In response to the organism, the immune response may reduce the microbial load. However the resulting inflammation may lead to the rapid destruction of the cornea beyond that resulting from the initial infection. Given that the major function of TLRs is pathogen recognition, it follows that these receptors play an important role in the ocular surface immune response to PA. Further understanding of TLR expression and function on the ocular surface would shed light on how a balance between microbial clearance and an appropriate inflammatory response can be achieved, allowing for the development of potential therapeutic paradigms to optimize antimicrobial effects while minimizing damaging inflammatory responses.

The ability of mucosal surfaces to kill and eliminate pathogens is partially attributed to TLRs and various antimicrobial peptides. To determine the potential role of TLRs in the response to pathogens at the ocular surface, this study examined baseline TLR expression in ocular surface cells and the functional consequence of TLR activation in primary HCEC (Chapter 2). In general, these studies have shown that all TLRs, with the exception of TLR8, are expressed throughout the cornea, by the conjunctival epithelium and by various ocular epithelial cell lines. In primary HCEC, TLRs are functionally active and upon activation by specific TLR agonist, small cationic antimicrobial peptides, (e.g., hBD-2 and LL-37), are secreted at low levels (~3ng/ml) into the growth media which is

able to kill PA. This antimicrobial activity against PA was attributed to LL-37 given that hBD-2 is not as effective as LL-37 under these conditions even at three times the concentration. Furthermore, when using a blocking antibody against LL-37, the antimicrobial activity of the growth media was abolished. It still remains to be determined how LL-37 secreted at low concentrations (~3 ng/ml) is effective at killing PA. One theory is that, LL-37 is further processed to smaller fragments which also have potent antibacterial activity as shown in skin and that the fragments act together in a synergistic fashion (Murakami *et al.*, 2004).

This study also sought to determine additional consequences of TLR activation. More specifically if TLR activation by TLR agonist or the products of TLR activation (hBD-2 and LL-37) can modulate TLR expression. As TLR activation most likely occurs when the ocular surface is being colonized or compromised by pathogens, an increase or decrease in TLR expression may further enhance or dampen the immune response. Here, TLR agonists did not modulate the respective TLR expression, however depending on the cell type examined; higher concentrations of LL-37 and hBD-2 (~µg/ml) downregulated TLR expression and additional studies are needed to examine the mechanism by which this occurs. This presents an interesting scenario since a downregulation of TLR expression may dampen the immune response to prevent additional stimulation and inflammation when the concentration of AMPs reaches the levels tested in this study. Given that the ocular surface is bathed by the tear film, further studies investigating the effect of other AMPs found in the tear film, presumed to be secreted by neutrophils (e.g., HNP-1-4) may provide additional insight in the ability of AMPs to modulate TLR expression. A previous study has shown that the concentration of HNP-1 and -2 can

increase to as high as 15µg/ml in the tear film following ocular surface trauma (Zhou *et al.,* 2004). These concentrations are well above the concentrations tested in this study for LL-37 and hBD-2 which alludes to their potential effectiveness in modulating TLR expression.

Like PA infection, dry eye syndrome is a significant health concern that reduces the quality of life of those affected. Despite the common occurrence and increasing prevalence, the pathogenesis is still not fully understood. Unfortunately, there are few therapeutic interventions available and most treatment options are often palliative to either artificially replace the tears or increase retention on the ocular surface. TLRs may play a role in mediating some of the events in dry eye inflammation. In such a scenario it is envisaged that TLR activation would, most likely, be via various endogenous ligands and/or normal flora bacteria rather than pathogens. With this in mind, a sudden increase in the number of TLRs and/or endogenous ligands may lead to TLR over-activation. Alternatively, a breach of the superficial epithelial layers may provide access to additional TLRs normally hidden from the normal flora on the ocular surface. Therefore determining the potential role for TLRs in the pathogenesis of dry eye may lead to the development of much needed novel therapeutic options.

Dry eye inflammation is thought to be stimulated by tear film hyperosmolarity (Gilbard *et al.,* 1978; Farris 1994; Bron *et al.,* 2002). The range for normal tear film osmolality is 296-308 mOsm/L (Gilbart, 1978), while the peak value in dry eye patients has been documented to reach as high as 440 mOsm/L (Farris, 1994). Therefore, the tear film osmolality can increase by almost 150 mOsm/L *in vivo*. Considering this, ocular surface cells were cultured under HOS by increasing the osmolality of the culture media

by 70-170 mOsm/kg (to achieve 400-500 mOsm/kg) (Chapter 3). Under these conditions, TLR9 mRNA and protein expression were downregulated, TLR4 mRNA was upregulated, while TLR4 protein decreased. It unclear why TLR9 shows a corresponding change in both mRNA and protein expression whiles the same trend did not occur for TLR4. Perhaps these experiments captured a moment when the ocular surface is attempting to increase TLR4 protein expression by increasing TLR4 mRNA transcript levels. When compared to the other conditions examined here, this theory seems logical. For example, in response to desiccation, TLR4 mRNA and protein were significantly upregulated, and TLR4 mRNA was increased in dry eye patients with high OSDI (>65). In regards to TLR9, its expression was consistently downregulated in response to dry eye associated conditions. Specifically, TLR9 was significantly downregulated in response to HOS, desiccation and was significantly decreased in dry eye patients.

As HOS stimulates the production of cytokines, and modulates the expression of TLR, cytokines produced in response to HOS could be indirectly responsible for modulating TLR expression. To examine this, ocular surface cells were cultured with individual cytokines known to be found on the ocular surface in patients with dry eye. Despite trying several cytokines at different time points and concentrations, there was no change in TLR expression, suggesting another mechanism must exist in which HOS modulates TLR expression. However, the ocular surface is multifaceted and numerous cytokines are expressed in dry eye. Therefore other individual cytokines, not tested here, may be active *in vivo* or cytokines may act together synergistically to modulate TLR expression.

These results indicate that in ocular surface cells, the dry eye environment downregulated TLR9, upregulated TLR4, and TLR5 was upregulated or unchanged. This change in TLR expression may represent a response to try to minimize hyper-responsiveness in dry eye by balancing TLR expression.

To further recapitulate the environment that occurs in dry eye, a dry eye mouse model was used to examine TLR expression and also determine if TLR activation on the dry eye ocular surface can stimulate an inflammatory response (Chapter 4). In these studies, EDE upregulated TLR2-4 and 9 mRNA expression in the palpebral conjunctiva and, with the exception of TLR4, also in the corneal epithelium. In the lacrimal gland, TLR2 and 5 were upregulated while TLR9 and 4 were downregulated. When comparing these results to the *in vitro* studies done in human ocular surface cells (Chapter 3), a few discrepancies were apparent. In the EDE model, TLR9 expression was anticipated to be downregulated to correlate with the *in vitro* human data found in Chapter 3. However TLR9 mRNA expression was increased on the ocular surface but decreased in the lacrimal gland (Chapter 4). Furthermore, TLR4 mRNA expression was increased in the conjunctiva but there was no corresponding increase in the protein and there was no change in mRNA and protein expression on the corneal epithelium, suggesting that TLR4 expression does not change in EDE. In addition to this, there were also significant increases in TLR2, 3 and 5 expression on the ocular surface and lacrimal gland in mice with EDE, whereas in the human *in vitro* studies, TLR5 was only upregulated in response to desiccation in the organ culture model. Unfortunately, TLR2 and TLR3 were not examined in the experiments in Chapter 3, therefore additional studies are needed to expand upon their role in dry eye.

Some studies suggest that dry eye increases the risk for microbial infections (Derk and Vivino, 2004) while a recent study in mice with EDE suggests mice with EDE are resistant to microbial infections. Therefore the expression of AMP in humans and mice was then examined. In humans, there was an increase in hBD-2 but not LL-37. In the mouse model, there was an increase in mBD-3 and 4 (hBD-2 homolog) but this was not statistically significant and a significant decrease in CRAMP (LL-37 homolog). Suggesting, as found with TLR expression, a balance in expression often occurs to compensate for changes in expression.

To examine the functional consequence of an upregulation of several TLRs on the ocular surface in EDE, mice were subjected to a one time dose of TLR agonist application. Interesting, despite an increase in TLR protein expression, TLR agonist did not stimulate a robust recruitment of inflammatory cells to the cornea stroma as shown in the positive control, the corneal scratch model. These data suggest that the epithelial defects that occur in dry eye are not significant enough to stimulate TLR induced inflammation. However, in mice with EDE, TLR agonist treatment downregulated mBD-4 protein in the cornea and decreased the central corneal thickness presumably by large epithelial defects.

These data suggest that TLRs are expressed on the ocular surface and their activation triggers the production of LL-37 and hBD-2, with LL-37 being particularly important for protecting the ocular surface against PA infection. Furthemore, EDE, dry eye and dry eye associated conditions modulated the expression of TLRs which may alter the immune response on the ocular surface. However, when specific microbial TLR agonist were applied to the ocular surface, mice with EDE were resistant to TLR induced

inflammation, suggesting that mice with EDE may not be at an increased risk for microbial infection.

It is unknown if altered ocular surface TLR expression in inflammatory conditions is cause or effect. However, regardless of the etiology a change in expression pattern may have both beneficial and detrimental effects. Upregulated expression may confer an enhanced ability for pathogen recognition, whereas reduced expression may lead to an inadequate recognition response and therefore increased risk of infection. However, the latter may be compensated for by the fact that many pathogens are recognized in more than one way, by interactions with multiple TLRs and interaction with other pattern recognition receptors. Enhanced TLR expression may also lead to inappropriate and exacerbated inflammatory responses thus contributing to disease processes such as allergy and dry eye, whereas reduced expression would be expected to be anti-inflammatory. Furthermore, additional studies are needed to determine if endogenous TLR ligands are increased in patients with dry eye, which may further exacerbate the inflammatory response. In general, activation of the various TLRs leads to a similar response by ocular surface epithelial cells i.e. cytokine/chemokine and antimicrobial peptide production. Therefore it is possible that a downregulation of the expression of some TLRs, is a compensatory response for the upregulation of other TLRs to try to minimize hyper-responsiveness.

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