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THE TRANSCRIPTIONAL REGULATION OF FLAGELLIN-INDUCED INNATE PROTECTION OF THE CORNEA: ROLE OF IRF1 AND ATF3

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2013

MAJOR: ANATOMY AND CELL BIOLOGY

Approved by:

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Advisor   Date

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DEDICATION

I dedicate this to my family and those who suffer from ocular diseases.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor Dr. Fu-Shin X. Yu for his guidance, support and encouragement in science and in life. Dr. Yu set up high goals for me when I first came to the lab, displayed great professionalism and patience, provided me numerous opportunities and showed endless support throughout my entire graduate study. I would not be who I am today without him.

I also wish to thank my committee members: Drs. D. Randall Armant, Andrei Tkatchenko. Dr. Armant, a pioneer in reproductive biology, has provided me with his expertise in cell and developmental biology. Dr. Tkatchenko, an outstanding eye development scientist, has given me helpful technical skills and strong guidance in eye research.

I would like to extend my appreciation to Drs. Paul D. Walker, the Graduate Program Director of Anatomy and Cell Biology. He answered my numerous questions about life and provided valuable advice to my graduate study. It has been my honor to receive the Rumble Fellowship.

I would like to acknowledge the members in our laboratory: Drs. Nan Gao, Dong Chen, Jia Yin and Ashok Kumar. They taught me experimental skills, shared their scientific experiences, and have been a family to me. I also thank the technicians and research assistants in our laboratory for their hard work.

I thank Selina Hall and Terri Larrew at Anatomy and Cell Biology and Mark Allen at KEI for their administrative service.

I also would like to thank the staff of the Graduate Programs at the School of Medicine and the Graduate School of Wayne State University for their assistance to my study and dissertation.

I appreciate my friends both at Wayne State and elsewhere for their encouragement, camaraderie and friendship.

Lastly, I thank my family for their unconditional love and support; they gave me the strongest support and love anyone could ever ask through the years from more than six thousand miles away.

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LIST OF ABBREVIATIONS

AMP: antimicrobial peptide

ATF3: activating transcription factor 3

CRAMP: cathelicidin-related antimicrobial peptide

CXCL10: chemokine (C-X-C motif) ligand 2

CXCL10: chemokine (C-X-C motif) ligand 10

EC: epithelial cell

HCEC: human corneal epithelial cell

hBD-2: human β-defensin-2

IFN: interferon

IFNγR: interferon gamma receptor

IL-8: interleukin 8

IRF1: interferon regulatory factor 1

LPS: lipopolysaccharide

MIP-2: macrophage inflammatory protein 2

MPO: myeloperoxidase

NF-κB: nuclear factor –κB

NK cell: natural killer cell

PAMP: pattern-associated molecular patterns

PMN: polymorphonuclear

TF: transcription factor

TIR: Toll/IL-1R

TLR: Toll-like receptor

TNF: tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1 The Cornea

The cornea is the transparent, dome-shaped anterior portion of the eye. It measures around 0.9 mm thick in the center, and 1.1mm in the periphery. The cornea serves two specialized functions: i) It is the main refractive element of the visual system; and ii) it provides a protective barrier between the external environment and the inner eye (Kumar and Yu, 2006). The cornea is composed of five layers: corneal epithelium, Bowman's layer, stroma, Decemet's membrane, and endothelium (Forrester, 2008).

1.1.1 Corneal epithelium

The corneal epithelium is a stratified, squamous non-keratinized epithelium, ranging from 50 to 60 µm in thickness (Fig. 1A). The surface of corneal epithelium is characterized by abundant microvilli and ridges, which house a glycocalyx coat that interacts with and stabilizes the tear film. Below the tear film lays the epithelium, which forms a physical barrier via tight junctions that prevents the invasion of bacteria. Cell turnover starts from the mitotic activity in the limbal basal layer, which displaces existing cells both superficially and centripetally. The epithelium responds rapidly to rupture by cell migration on the wound margin to cover the wound, followed by cell proliferation to restore the lost cell population. The basal epithelial cells are anchored on a thin, but prominent basal lamina. Antigen presenting dendritic cells (the Langerhan's cells) are present in the limbus and peripheral cornea, but their population decreases sharply towards the center of the cornea (Forrester, 2008).

1.1.2 Bowman's layer and Stroma

Bowmans' layer is an acellular region of the stroma consisting of fine, randomly arranged collagen fibrils. The anterior part of the layer is separated from the epithelium by the

thin basal lamina, while the posterior section merges with the stroma. Bowman's layer terminates at the limbus (Forrester, 2008).

The corneal stroma makes up the majority of the cornea, consisting of dense connective tissue of incredible regularity and keratocytes. The stroma consists of mostly thick, flattened collagenous lamellae oriented parallel to the corneal surface, with flattened and modified fibroblasts, known as keratocytes, squeezed in between the lamellae (Fig. 1C, D). The stroma is normally free of blood or lymphatic vessels, but sensory nerve fibers that terminate at the epithelium pass through the stroma (Forrester, 2008). Studies have shown that there is a population of macrophages throughout the stroma (Chinnery et al., 2007). The stromal macrophages and epithelial dendritic cells are of monocytic lineage, and are the resident antigen presenting cells in the cornea.

1.1.3 Decemet's membrane and the endothelium

Decemet's membrane is a thin, modified basement membrane of the corneal endothelium that lies between the posterior stroma and the endothelium (Fig. 1B, E). The corneal endothelium is simple squamous epithelium at the posterior surface of the cornea. It anchors on the Decemet's membrane and has an essential role in maintaining the cornea (de)hydrated and transparent (Forrester, 2008).

1.2 Corneal Defensive Mechanisms

The cornea is constantly exposed to a wide array of microorganisms. The ability of the cornea to recognize pathogens and eliminate them on a timely fashion is critical to maintain corneal transparency and preserve sight. Innate immunity is the first line of defense against corneal infection, and there are many elements of the innate defense machinery, including tear film, epithelium, keratocytes, and polymorphonuclear (PMN) cells (Akpek and Gottsch, 2003).

The cornea is coated with a layer of tear film that flushes away foreign particles from the ocular surface, and transports antimicrobial proteins (AMPs) and immunoglobulin (especially IgA) to the ocular surface to limit the colonization of bacteria (Pepose et al., 1996). The major AMPs present in the ocular surface are defensins and LL-37 which directly kill microbes through electrostatic disruption of the microbial cell membrane (McDermott, 2009). While invading pathogens can directly infect the stroma (for example, in a penetrating wound), in most cases (such as contact lens wearing) the epithelial cells are the first cells to encounter the pathogens. Therefore, these cells act as sentinels that possess the ability to detect the presence of pathogens and coordinate the innate defense system. Upon injury or infection, the corneal epithelium releases chemotactic factors such as IL-1β, IL-6, IL-8, and TNFα (Hazlett, 2004a; Ruan et al., 2002; Xue et al., 2000), which recruit PMNs, and lymphocytes (Nassif, 1996). PMNs are critical effector cells in the cornea and play vital roles in phagocytosis and microbial killing (Burg and Pillinger, 2001). Keratocytes in the stroma also have a defensive role during microbial invasion, by synthesizing IL-6 and IL-8 (Akpek and Gottsch, 2003). The other components that participate in corneal innate immunity are the Langerhan's cells (dendritic cells), which orchestrate B and T lymphocyte activity in the cornea, and immunoglobulins (IgA and IgG) that are concentrated in the stromal layer (Kumar and Yu, 2006). Resident macrophages have also been discovered in the murine corneal stroma and may play a role in host immune responses (Brissette-Storkus et al., 2002).

Figure 1. Histology of cornea and its constituent layers. **(A)** Electron microscope of corneal epithelium. B, basal layer; W, wing cells; S, superficial layer. **(B)** Light micrograph of the five layers of the human cornea. Ep, epithelium; BL, Bowman's layer; S, stroma; DM, Descemet's membrane; E, endothelium. **(C)** Electron microphage illustrating keratocyte (K) among regularly spaced collagenous lamellae. Inset – higher magnification showing collagen fibers. **(D)** Schematic diagram showing arrangement of the collagenous lamella (CL) and the interposed keratocytes (K). **(E)** Schematic diagram of the human eye in horizontal section. AC, anterior chamber. Corneoscleral envelope (blue), uveal tract (orange), inner neural layer (purple) (Forrester, 2008).

A
BL

C

W

B

BL
S
DM
E

K
F

CL
K

D

Pupil
Iris
Conjunctiva
Posterior chamber
Lens zonules
Corneal epithelium
Cornea
Iridocorneal angle
AC
Limbus
Canal of Schlemm
Pars plana
Ora serrata
Lens
Ciliary body
Rectus muscle
tendon and belly
VITREOUS
Hyaloid (Cloquet's)
canal
Choroid
Retinal pigment epithelium
Retina
(neural layer)
Optic disk
Sclera
Fovea
Lamina cribrosa
Subarachnoid space
Dura mater
Optic nerve
E

1.3 Bacterial Keratitis

Bacterial infection of the cornea is considered as a relatively rare but serious medical condition that requires urgent medical attention due to potential vision reduction or loss in the affected eye. Factors that increase the chances of infection include extended wear of soft contact lenses; ocular surgical procedures; ocular disease, and ocular injury (Fig. 2). Infecting bacteria come from environmental sources, patients' skin and nasopharyngeal flora, contact lens care solution or lens cases, topical drugs, irrigation solutions or ocular instruments (Fleiszig and Evans, 2002). In the United States, microbial keratitis is most frequently associated with complications related to contact lens usage, with an incidence rate of 25,000 to 30,000 cases per year (Khatri et al., 2002).

The most common cause of bacterial infection in contact lens wearers is the Gram-negative pathogen, *Pseudomonas aeruginosa* (*P. aeruginosa*). It has an arsenal of cell-associated and extracellular virulence factors, including toxins and proteases that help it to initiate and maintain infection. *P. aeruginosa* can activate several pathways of the immune system during bacterial keratitis. Such activation often involves receptors on the corneal epithelial cells called Toll-like receptors (TLRs), especially TLR5 (Kumar and Yu, 2006; Zhang et al., 2003b). TLR5 recognizes the major component protein of flagella, flagellin, resulting in the epithelial production of cytokines/chemokines that recruit white blood cells and antimicrobial peptides that kill invading pathogens directly. The infiltrated white blood cells, mainly neutrophils (polymorphonuclear leukocytes), to the infection site can phagocytose and kill the bacteria. However, continuous presence and recruitment of white blood cells to the infection site leads to tissue destruction, which could eventually lead to scaring and vision loss. TLR5 also mediates the expression of proteins that are directly antimicrobial, such as defensins, from corneal epithelial cells (Willcox, 2007). Due to the increase in antibiotic resistant strains of bacteria and unsuccessful attempts to use antimicrobial peptides to control keratitis, there is an urgent need

to better understand the pathways involved in induction and suppression of inflammation by this bacterium so that improved therapeutic strategies can be developed (Hazlett, 2004a).
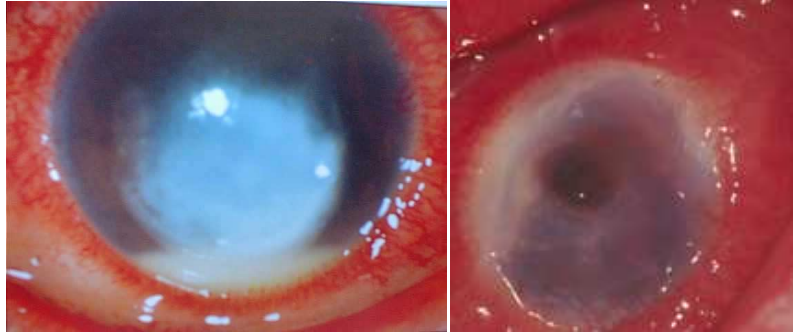
Figure 2. Bacterial keratitis. Left, bacterial keratitis following lasik surgery; Right, a perforated corneal ulcer caused by P. aeruginosa infection.
("http://img.medscape.com/pi/emed/ckb/ophthalmology/1189694-1221604-327.jpg," ; "http://www.revophth.com/publish/images/1_119_1.jpg,")[18, 19](18,19).

1.4   Flagellin and Toll-like Receptor 5 (TLR5)

Bacterial flagella are complex organelles composed of a basal body, hook, motor, and filament, which play a central role in motility and chemotaxis. The protein flagellin is the major protein of the flagellar filament with highly conserved sequences at the amino and carboxyl termini, which play critical roles in the structure and function of flagella. The region between the conserved termini is termed the *hypervariable* region due to its high variability in length and sequence. Analysis of the crystal structure of flagellin has shown that it is a "boomerang-shaped" protein with four major domains (Fig. 3, box). The D0 domain is comprised of the amino and carboxyl termini and is responsible for polymerization. The D1 domain is primarily α-helical in structure and contains highly conserved regions required for flagellin signaling. The D2 and D3 domains are the hypervariable domains consisting of mostly β-strands (Honko and Mizel, 2005).

In mucosa, the flagellar structure is required for bacterial motility, adhesion, invasion and secretion of virulence factors (Ramos et al., 2004). Flagella can serve in aiding the attachment of bacteria to the host cells, and assisting bacterial invasion (Honko and Mizel, 2005). Also flagellum can act as a "syringe", mediating the secretion of several extracellular toxins, including the phospholipase Yp1A (Young et al., 1999).
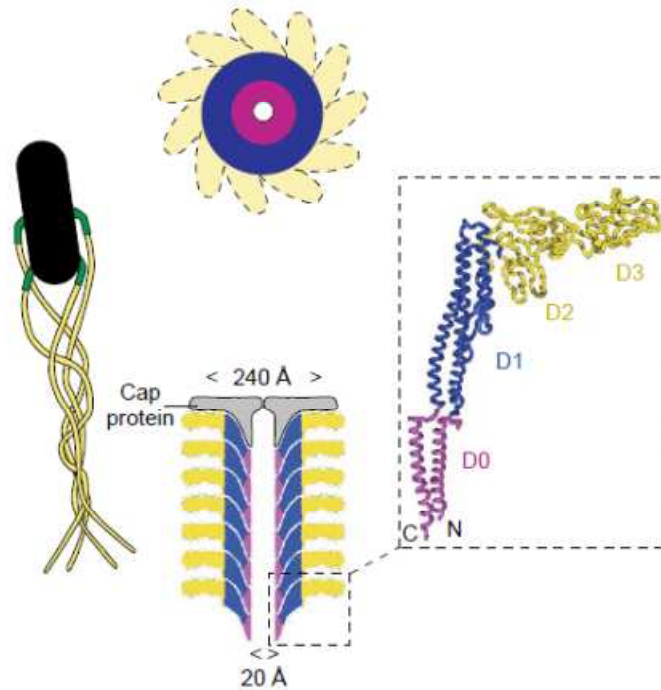
Figure 3. Structure and organization of flagellum and flagellin. Flagella structure is comprised of a hook (green) and a filament, referred to as flagellum. Schematic transversal and longitudinal views of the flagellum are also shown (center figures). On the right is the ribbon diagram of flagellin, which is color-coded to show the domains (Ramos et al., 2004).

1.5   TLRs of the Cornea and their PAMPs

Flagellin is recognized by the cell surface receptor TLR5. At present, 10 (in human) TLRs have been identified. TLRs 1-9 are common to both human and mouse, while TLR10 is unique to humans and TLR11-13 belong to the mouse (Kawai and Akira, 2007). TLR's extracellular domain contains leucine-rich repeats (LRRs), which are responsible for binding of the ligand (Honko and Mizel, 2005). The intracellular domain contains a region called the Toll/IL-

1R (TIR) domain, which exhibits high homology with that of Interleukin-1 receptor (IL-1R) (Fig. 4). TLRs recognize Pathogen-Associated Molecular Patterns (PAMPs) - conserved structural moieties of pathogens that are critical for their survival. This gives the host three big advantages. Firstly, PAMPs are produced only by microbes and not the host, which enables the host to distinguish between self and non-self. Secondly, as PAMPs are critical for the survival of the microbe, mutations or loss of patterns can be lethal. Therefore, PAMPs do not have a high mutation rate. Lastly, PAMP sequences are invariant between microorganisms of a given class. Hence, PAMPs are ideal targets for detection by the innate immune system (Kumar and Yu, 2006). Table 1 summarizes common PAMPs of *P. aeruginosa* that are involved in infectious keratitis and the TLRs that recognize them.
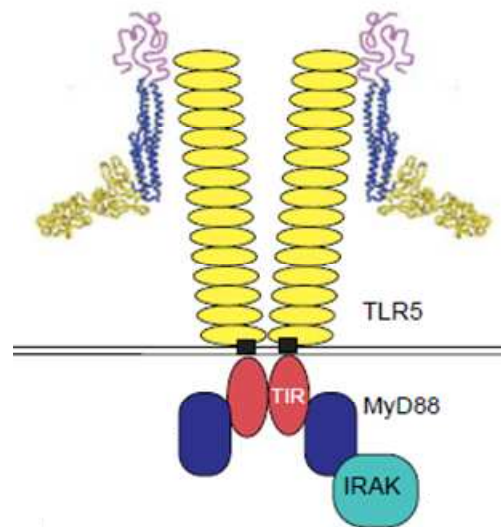
Figure 4. Toll-like receptor 5 with bound flagellin monomers. The leucine-rich repeats (yellow) detect distinct regions of flagellin monomers. TLR5 requires the TLR-specific adaptor molecule MyD88 that transfers the stimulus from TIR to downstream molecules of the signaling cascade, such as IRAK1. IRAK1, interleukin-1 receptor-associated kinase; MyD88, myeloid differentiation factor 88; TIR, Toll–interleukin 1 receptor domain; TLR5, Toll-like receptor 5 (Ramos et al., 2004).

Pathogen	PAMP	PRR
Pseudomonas aeruginosa	Flagellin	TLR5
	Lipoprotein	TLR2
	LPS	TLR4

Table 1. The PAMPs of *Pseudomonas aeruginosa* and their respective TLRs (Kumar and Yu, 2006).

TLR5's only ligand discovered thus far is flagellin (Eaves-Pyles et al., 2001). Studies by Andersen et al. demonstrated that TLR5 is only activated by flagellated bacteria, as compared to non-flagellated bacteria, suggesting that flagellin is a specific ligand for TLR5 (Andersen-Nissen et al., 2005; Hayashi et al., 2001). Several groups, including our lab, have reported that the major function of epithelial TLR5 is sensing of Gram negative bacteria, such as *P. aeruginosa*, and initiating the signaling pathways leading to NF-κB activation and inflammation in mucosal surfaces of our body including the cornea, intestine and airway/lung (Tallant et al., 2004; Zhang et al., 2003a; Zhang et al., 2005). According to these data, *P. aeruginosa* strains that lack the flagellin gene are almost nonfunctional in inducing NF-κB activation and proinflammatory cytokine production. It seems that TLR2 plays only a minor, if any, role in sensing of *P. aeruginosa* by corneal epithelial cells, while TLR4 is not responsive to LPS since it lacks the critical co-receptor, MD-2 (Zhang et al., 2009). TLR5 is expressed in the internal cell layers of the corneal epithelium and on the basolateral side of intestinal epithelial cells (Gewirtz et al., 2001a; Zhang et al., 2003a). This means that in normal conditions, TLR5 is separated from the bacteria or bacterial products that can be found on the lumen or mucosal surface. However, when the apical barrier is breached and TLR5 becomes exposed to pathogens, the epithelial cells initiate the innate immune response (Kumar and Yu, 2006). Studies by West et al. evaluated the localization of TLR5 during flagellin signaling by blocking flagellin/TLR5 internalization using monodansylcadverine, and concluded that TLR5 signaling occurs at the cell membrane and does not require internalization (West et al., 2005).

1.6   TLR5 signaling

To date, two major TLR signaling pathways have been identified: MyD88-dependent and MyD88-independent (TRIF-dependent) pathways. All TLR activation culminates in the activation

of nuclear factor (NF)-κB and activating protein-1 (AP-1) (Kawai and Akira, 2006). Flagellin signaling via TLR5 is MyD88-dependent. Therefore, we will discuss the MyD88-dependent pathway in more detail (Fig. 5).

Normally, upon ligand binding, TLRs dimerize and undergo conformational changes that favor the recruitment of the adaptor molecule IL-1R-associated kinase 1 (IRAK-1) (Burns et al., 1998; Wesche et al., 1997). However, in the case of TLR5, IRAK-1 seems to be constitutively associated with TLR5 (Mizel and Snipes, 2002), most probably through the adaptor molecule, MyD88, which interacts with the intracellular domain of TLR5 through homotypic interactions of the TIR domain (Burns et al., 1998). Flagellin binding causes the dissociation of phosphorylated and activated IRAK-1 from the TLR5/MyD88 complex (Mizel and Snipes, 2002) to associate with tumor necrosis factor receptor-associated factor 6 (TRAF6) (Kumar and Yu, 2006). TRAF6 forms a complex with Ubc13 and Uev1A, becoming the E3 ligase to promote synthesis of lysine 63-linked polyubiquitin chains (Chen, 2005). Lysine 63-linked ubiquitination is linked with various cellular responses such as signal transduction and cellular localization (Sun and Chen, 2004). TRAF6 then associates with and activates TGF-β-activated protein kinase (TAK-1), a member of the MAP kinase kinase kinase (MAP3K) family, in an ubiquitin-dependent manner (Chen, 2005). TAK-1 is constitutively associated with TAK-1 binding proteins, TAB-1, TAB-2 and TAB-3. Particularly, TAB-2 and TAB-3 bind lysine 63-linked polyubiquitination chains via zinc-finger domains, resulting in the activation of TAK-1(Chen, 2005). In turn, TAK-1 phosphorylates the inhibitor of NF-κB (IκB)-kinase (IKK) complex, which is formed by IKKa, IKKb, and (NEMO)/IKKg. Consequently, IκB becomes phosphorylated by the IKK complex and becomes the target for ubiquitination and subsequent degradation by the 26S proteosome. In unstimulated cells, NF-kB is kept inactive by interacting with inhibitor of NF-κB (IκB). NF-κB is then released into the nucleus to activate transcription of κB sites. NF-κB is a dimeric transcription factor that belongs to the Rel-homology domain-containing protein family, which

includes p65/RelA, p50/NF-κB1, p52/NF-κB2, RelB and c-Rel (Karin and Greten, 2005). The most widely known and prototypical NF-κB is thought to be the heterodimer consisting of subunits p65 and p50. Moreover, TAK-1 simultaneously phosphorylates two members of the MAP kinase kinase family, MKK3 and MKK6, which activate c-Jun N-terminal kinase (JNK) and p38, respectively (Chen, 2005). Furthermore, extracellular signal-related kinase (ERK) is activated in response to TLR ligands through the activation of MEK1 and MEK2. TAK-1 plays a key role in the cellular response to a variety of stimuli, as TAK-1 -deficient cells fail to activate NF-κB and MAP kinases (p38, JNK and ERK) in response to not only TLR stimuli, but also to TNFα and IL-1β (Sato et al., 2005). Activated p38, JNK and ERK mediate the activation of AP-1. AP-1 is a dimeric basic region leucine zipper (bZIP) protein composed of members of Jun, Fox, activating transcription factor (ATF), and the Maf subfamily, which binds to TPA-response elements or cAMP-response elements. Of the AP-1 family proteins, c-Jun is believed to play a central role in the inflammatory response (Shaulian and Karin, 2002).

Flagellin ligation of epithelial TLR5 also causes the rapid activation of phosphoinositide 3-kinase (PI3K) activation which serves to limit pro-inflammatory gene expression mainly via activation of phosphotases that downregulate p38 (Yu et al., 2006). We have shown that prolonged activation of TLR5 by flagellin dampens NF-κB activation but persistently activates PI3K-AKT pathways in two phases. The first phase of activation is associated with NF-κB and declines to the basal level in about 2 hrs, and the second arises around 8 h and persists in the presence of flagellin. The p85 subunit of PI3K physically interacts with MyD88 in response to flagellin, resulting in the activation of the catalytic subunit p110 (Rhee et al., 2006). This pathway may be responsible for the first phase activation. We propose different pathways leading to these two phases of PI3K-AKT activation. The work from our lab demonstrated that flagellin transactivates epidermal growth factor receptor (EGFR) leading to activation of PI3K-AKT and ERK pathways. This is likely responsible for the second phase of PI3K and AKT

activation (Zhang et al., 2004). STAT1 activation by TLR5 stimulation has been documented in osteoclasts. Ha et al. showed that TLR5 stimulation with flagellin caused STAT1 serine727 and tyrosine701 phosphorylation (Ha et al., 2008).
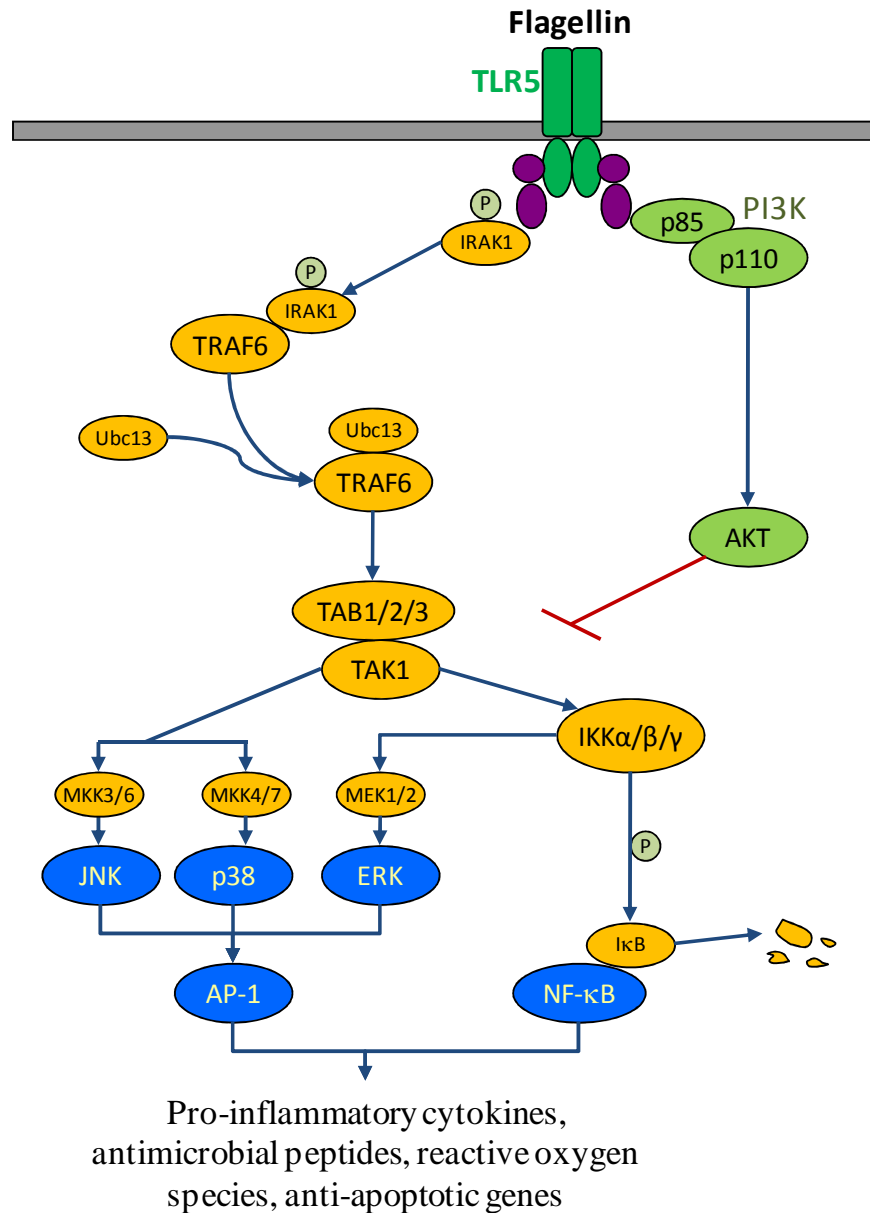
Figure 5. The TLR5 signaling pathway. Modified from (Kumar and Yu, 2006).

1.7 Consequences of TLR5 Activation

The overall downstream outcome of TLR5 signaling is the transcriptional activation of approximately 500 genes that protect cells against various challenges (Zeng et al., 2003). These activated genes include those with direct antibacterial function (e.g. defensins and LL-37), immune cell chemoattractants, and a number of general stress-induced genes such as heat-shock proteins (Vijay-Kumar et al., 2008a). Defensins are small cationic peptides containing sulfide bonds that can damage the bacterial cell membrane, but they also possess chemotactic properties (Ganz, 2003). Human corneal epithelial cells (HCECs) constitutively express human β-defensin-1, but can be stimulated to induce human β-defensin-2 (hBD-2) expression in response to *P. aeruginosa* infection (Huang et al., 2007; Kumar et al., 2007; McDermott et al., 2001). It seems that hBD-2 acts through the MAP kinase and NF-κB pathways when HCECs are stimulated with a proinflammatory cytokine, IL-1β (McDermott et al., 2003). LL-37 is another antimicrobial peptide that is induced in response to *P. aeruginosa* ocular infection (Kumar et al., 2007). LL-37 is derived from human cationic antimicrobial protein 18 (hCAP18), and possesses other biological activities, such as chemoattraction and wound healing (Lehrer and Ganz, 2002).

Flagellin induces the expression and secretion of IL-8 in HCECs, which is essential for recruitment of neutrophils and macrophages at sites of injury. Furthermore, epithelial cells also upregulate TNFα, IL-6, inducible nitric oxide synthase (iNOS) and nitric oxide, matrix metalloproteinase (MMP)-7, and macrophage inflammatory protein (MIP)-2α (Ramos et al., 2004; Zheng et al., 2010). These factors participate in anti-microbial activity, recruitment of professional killer and antigen-presenting phagocytes, and production of inflammatory mediators that activate phagocytes (Ramos et al., 2004).

Recent studies by the Gewirtz lab have shown that flagellin treatment protects against a variety challenges, such as chemicals, γ-irradiation, bacteria, and viruses (Vijay-Kumar et al., 2008a). Their group also demonstrated the non-pathogenic effects of systemically administered

flagellin compared to LPS. LPS, when administered systemically, induces the expression of a panel of proinflammatory cytokines, also known as a "cytokine storm" (Lin and Yeh, 2005). Their results showed that very little TNF-α, IL-1α, and RANTES, and only modest levels of IL-6 were induced by equal or 5-fold greater amounts of flagellin, compared to LPS. In general, their results indicate that, compared to LPS, flagellin has less potential to induce severe adverse systemic events (Vijay-Kumar et al., 2008a). Thus, these studies suggest that therapeutic administration of flagellin may provide temporary broad protection against a variety of adverse stimulants. In addition, potential uses of flagellin in vaccine formulations to promote mucosal immunity are being explored, due to its highly effective mucosal adjuvant activity (Ben-Yedidia and Arnon, 2007). Besides, flagellin has also been linked with some epithelium–based cancers (carcinomas), as studies have shown that flagellin promotes necrosis of tumors *in vivo*, possibly by promoting immune cell recruitment to the tumor site (Rhee et al., 2008).

In modulating adaptive immunity, flagellin promotes the development of T helper 2 (Th2)-biased and antibody response (Didierlaurent et al., 2004). Flagellin induces the maturation of TLR5-expressing DCs, but does not increase the production of IL-12, a T helper 1 (Th1)-driving cytokine, in DCs, which may be why a Th2 response is favored (Means et al., 2003). The direct activation of DCs within mucosa by flagellin might participate in Th2 differentiation, which favors antibody responses (Ramos et al., 2004). In epithelial cells, flagellin induces the transient production and secretion of a DC-specific cytokine, CCL20, in a NF-κB-dependent manner, which attracts immature DCs in a CCL20-dependent manner (Sierro et al., 2001). At the same time, IL-8 is also secreted to attract PMNs to the site, which provides the proinflammatory signals for DC maturation (Gewirtz et al., 2001b).

1.8   Flagellin–induced Cell Reprogramming

The ability to modulate the destructive consequences of uncontrolled inflammation caused by TLR signaling could be greatly beneficial for the host. Studies using several TLR

agonists have demonstrated that initial exposure to a TLR agonist results in a state of tolerance, more recently seen as cell reprogramming (Cavaillon and Adib-Conquy, 2006; Marsh et al., 2009; Nahid et al., 2011a). In the case of the TLR4 agonist, LPS, a number of mechanisms have been proposed, including the downregulation of TLR4 expression on the cell surface, degradation of IRAK-1, or TLR signaling suppression by IRAK-M (Buckley et al., 2006; Vartanian and Stenzel-Poore, 2010).

Flagellin also induces self-tolerance in a variety of cell types. For example, Mizel and Snipes (Mizel and Snipes, 2002) reported that flagellin-treated human peripheral blood monocytes or THP1 cells failed to produce TNF-α in a second exposure to flagellin. Flagellin tolerance is not due to reduced TLR5 surface expression or IRAK-1 degradation. Flagellin induced tolerance rapidly, within 2 hours after initial exposure to flagellin, and was protein-synthesis independent (Mizel and Snipes, 2002). Flagellin-induced tolerance or "reprogramming" is transient, with responsiveness returning to its original level after 12-96 hours (Honko and Mizel, 2005). In flagellin-reprogrammed cells, IRAK-1 activation is dramatically reduced in response to flagellin, indicating that reprogramming occurs at an early stage in TLR5 signaling (Mizel and Snipes, 2002). The interaction between flagellin and TLR5 is hypothesized to promote the release of phosphorylated IRAK-1 from TLR5 in exchange for unphosphorylated IRAK-1. However, in flagellin-reprogrammed cells, this exchange mechanism appears to be blocked, preventing the release of active IRAK-1 and the initiation of downstream signaling events (Honko and Mizel, 2005).

Studies from our lab using primary and immortalized HCECs, and B6 mice corneas have also shown that pretreatment with a low dose of flagellin can induce a reprogrammed state that is more resistant towards a subsequent higher dose of flagellin or live bacteria (*P. aeruginosa*). This was characterized *in vitro* by impaired activation of NF-κB, p38 and JNK pathways and reduced IL-8 and TNFα production. However, we also found that there was enhanced

expression of antimicrobial genes, such as LL-37 and hBD2 (Kumar et al., 2008; Kumar et al., 2007). This could be partially explained by more recent studies in our lab, which demonstrated that the production of antimicrobial peptides is uncoupled from the inflammatory response mediated by NF-κB. It seems that the EGFR pathway, which is indirectly activated in response to flagellin, is involved in AMP production (Gao et al., 2010). *In vivo* studies also showed that subconjunctival and intraperitoneal administration of flagellin 24 hours prior to *P. aeruginosa* inoculation resulted in suppression of the inflammatory response and enhancement of bacterial and fungal clearance (Gao et al., 2011a; Kumar et al., 2008; Kumar et al., 2007). Further analysis proved that flagellin pretreatment resulted in the inhibition of late-stage PMN infiltration (but not early-stage), decrease in proinflammatory cytokines, and enhancement of bacterial clearance (Kumar et al., 2008). We have also been able to reproduce the results by applying flagellin topically on to the cornea (Kumar et al., 2010b). These results suggest a beneficial effect of flagellin in modulating the host's innate immune system, and warrant further investigation into the underlying mechanism of flagellin-mediated cell reprogramming/tolerance.

1.9   Interferon (IFN) γ/Jak/STAT signaling

IFNs are widely expressed cytokines regarded as the first line of defense against viral infections. The IFN family includes two main classes of related cytokines: type I IFNs and type II IFNs. All type I IFNs, which includes IFNα, IFNβ, IFNε, IFNκ, and IFNω in humans, bind a common cell-surface receptor, type I IFN receptor. There is only one type II IFN, IFNγ, which binds to the type II IFN receptor. Type II IFN receptors have multichain structures, composed of at least two distinct subunits: IFNGR1 and IFNGR2 (for type II IFNs). Each of these subunits interacts with a member of the Janus activated kinase (JAK) family at the intracellular portion of the receptor: IFNGR1 with JAK1, and IFNGR2 with JAK2 (Fig. 6). Once the ligand binds, the receptor subunits undergo rearrangement and dimerization, followed by autophosphorylation and activation of JAKs. Interferon mediated signaling is initiated by the activated JAKs, which

activate not only the classical JAK-STAT signaling pathway, but also regulate several other downstream cascades (Platanias, 2005). IFN-γ plays a key role in inflammation, host defense against intracellular pathogens, Th1 cell responses, and tumor surveillance (Hu and Ivashkiv, 2009). Considering the multi-faceted role of IFNs on target cells and tissues, this diversity in signaling is not surprising. Expression of the IRF1 gene is strongly induced by IFNγ (Der et al., 1998).
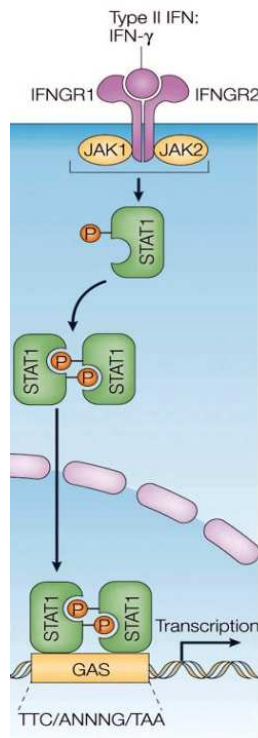
Figure 6. IFN receptors and activation of classical JAK-STAT pathways by type II IFNs (Platanias, 2005).

1.10   Interferon Regulatory Factors (IRFs)

The number of known mammalian IRF family members has grown to 9 members since the first report of IRF1 in 1988: IRF1-9 (Fujita et al., 1988; Honda and Taniguchi, 2006a). All IRF members have a well-conserved DNA binding domain (DBD) of approximately 120 amino acids located in the amino terminus. Forming a helix-turn-helix motif, this region recognizes a consensus DNA sequence termed the IRF-E [consensus sequence: A(G)NGAAANNGAAACT],

which is almost identical to the interferon-stimulated response element [ISRE; consensus sequence: $G(A)AAA^G/_C^T/_CGAAA^G/_C^T/_C$] (Honda and Taniguchi, 2006a; Taniguchi et al., 2001).

1.10.1 **IRF1**

IRF1 was the first IRF family member that was discovered (Miyamoto et al., 1988). IRF1 mRNA is expressed in a variety of cell types and it is dramatically upregulated upon viral infection or IFN stimulation (Harada et al., 1989; Miyamoto et al., 1988). Several cDNA transfection experiments have revealed that IRF1 can activate IFNα/β promoters, albeit at low efficiency (Taniguchi et al., 2001). It has a short half-life of approximately 30 minutes (Watanabe et al., 1991), and is expressed at low levels in unstimulated cells, but can be induced by many cytokines such as IFNs (-α, -β, -γ), TGFα, IL-1, IL-6 and leukocyte inhibitory factor (LIF), and by viral infection. IFNγ is the strongest known inducer of IRF1 expression, while certain combinations of cytokines, such as IFNγ and TNFα, induce even higher expression of IRF1 mRNA (Ohmori and Hamilton, 1997). On the other hand, cytokines such as IL-4 have been reported to inhibit IFNγ-induced IRF1 expression (Coccia et al., 2000). Key promoter elements in the IRF1 promoter include GAS and NF-κB binding sites, where activated STAT1 and NF-κB bind, respectively, and induce transcription (Kroger et al., 2002). Interestingly, IRF1 can activate the IRF2 promoter and upregulate IRF2, which inhibits the transcription of IRF1-activated genes. IRF2 is thought to act as a negative feedback mechanism to limit IRF1 activity.

1.10.2 **Activities of IRF1**

The most crucial activity of IRF1 is its ability to promote transcription of specific promoters. It is constitutively localized in the nucleus, as the transcription factor contains two nuclear localization signals (NLS) [120]RKERKSK and [132]KSKTKRK (Fig. 7). Two activator fragments function in an additive manner and are located between amino acids 185 and 256. The N-terminal 60 amino acids contain a repression domain that strongly inhibits its transcriptional activity (Kroger et al., 2002). IRF1 is regulated by different means, but primarily, it

is regulated at the transcriptional level. Due to its short half-life of 30 minutes, it is predicted that IRF1 mRNA levels correlate with IRF1 protein levels (Watanabe et al., 1991). A multifunctional domain 1 (Mf1) exists inside the enhancer domain, and it seems to be required for recruitment of coactivators (Dornan et al., 2004), maximal IRF1-mediated growth suppression (C-terminal repression domain) (Eckert et al., 2006), and plays a key role in determining the rate of IRF1 degradation (Pion et al., 2009).
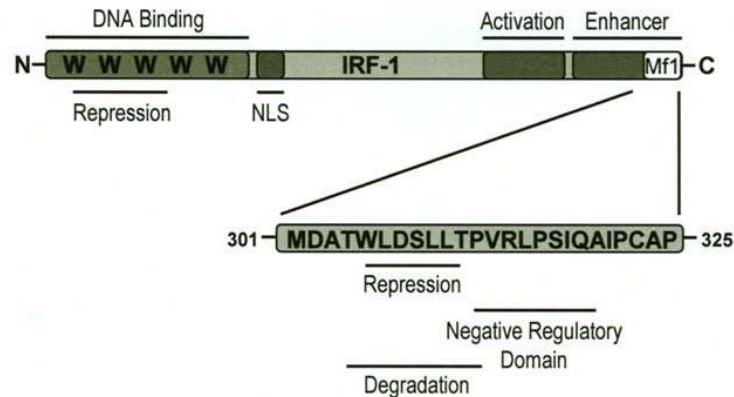
Figure 7. IRF1 domain organization. The five tryptophan (W) repeats in the DBD are common to all IRFs. N-terminal Repression domain functions to repress the transcriptional activity of IRF1, while the C-terminal Repression domain is required for target gene and growth repression. Mf1, multifunctional domain 1; NLS, nuclear localization signal (Narayan et al., 2009).

1.10.3 **IRF1-Induced genes**

Target gene	Role	Reference
IP-10 (CXCL10)	Antiviral response, angiostasis	(Buttmann et al., 2007)
Inducible nitric oxide synthase (iNOS)	Antibacterial response	(Kroger et al., 2002)
PIGR	IgA transport across mucous membranes	(Blanch et al., 1999)
Caspase 1	Apoptosis	(Kroger et al., 2002)

IL-12/p35	Th1 type immune response	(Kroger et al., 2002; Liu et al., 2003)
Secreted leukocyte peptidase inhibitor (SLPI)	Protects epithelial cells from serine proteases, broad antibiotic activity	(Nguyen et al., 1999)

Table 2. IRF1 target genes. Modified from (Kroger et al., 2002).

1.10.4 Regulation of Immune Responses by IRF1

IRF1 has been implicated in the development of immune cells, including dendritic, NK, and T cells (Tamura et al., 2008a). The generation of DC subsets is mainly regulated by IRF4 and IRF8, and studies have shown that DC function is regulated by IRF8 (Tamura et al., 2008a). However, IRF1 also contributes in DC subset development. IRF1-/- mice show a predominance of plasmacytoid DCs and decrease in conventional DC numbers, especially in CD8α+ DCs (Gabriele et al., 2006). This study also indentified the increased levels of IL-10, TGF-β, and the tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO), and the defect in IL-12p40 production in IRF1-/- DCs. As a result, IRF1-/- DCs fail to mature fully and stimulate proliferation of allogenic T cells, and induce IL-10-mediated suppression of CD4+CD25+ regulatory T cells. This suggests a novel role of IRF1 in regulating the tolerogenic features of DCs (Gabriele et al., 2006).

IRF1 plays a critical role in natural killer cell development. NK cells are dramatically reduced in IRF1-/- mice, resulting in the absence of NK cell activities such as cytotoxicity and IFNγ production. It seems that IRF1 expression is required in the stromal cells that constitute the microenvironment for NK cell development, as it is required for IL-15 induction, a cytokine essential for NK cell development (Ogasawara et al., 1998). Moreover, this IL-15 deficiency also causes impairment in the development of intestinal intraepithelial T cells (Ohteki et al., 1998).

The development of mature CD4- CD8+ T cells in the thymus and peripheral lymphoid organs is severely impaired in IRF1-/- mice (Matsuyama et al., 1993). This defect in thymocyte development is caused by intrinsic defects in T cell maturation, rather than environmental causes, as IRF1 controls both the negative and positive selection of CD8+ thymocytes (Penninger et al., 1997). In addition, IRF1 seems to mediate T cell receptor (TCR) signal transduction and may regulate the expression of genes required for lineage commitment and selection of CD8+ T cells (Penninger et al., 1997). One of those genes could be Bcl2, as introduction of a Bcl2 transgene into IRF1-/- restored CD8+ T cell development. This suggests that IRF1 may be required for survival signals to support CD8+ T cell development (Ohteki et al., 2001).

IRF1 deficiency leads to the induction of only Th2-type immune responses (Lohoff et al., 1997). This lack of Th1 differentiation of Cd4+ T cells is due to defects in multiple cell types. Firstly, IRF1-/- macrophages and dendritic cells are defective in IL-12, which is a cytokine essential for Th1 differentiation. Secondly, IRF1 activates the Il12rb1 (IL-12 receptor, β1) promoter, rendering IRF1-/- CD4+ cells hyporesponsive to IL-12. Thirdly, IRF1-/- mice lack NK cells, which produce IFNγ to stimulate macrophages into secreting IL-12 (Tamura et al., 2008a).

1.10.5 **IRF1 and TLR signaling**

IRF1 has been reported to directly interact with the adaptor molecule MyD88 (Negishi et al., 2006). Although IFNγ can strongly induce IRF1 expression, full activation of IRF1 is achieved when it associates with the MyD88-TLR complex, as it undergoes post-translational modifications and migrates to the nucleus more efficiently than non-MyD88-associated IRF1. This TLR-MyD88 "licensing" of IRF1 is further emphasized by the observation that a subset of genes activated by the TLR-MyD88 pathway, such as inducible nitric oxide synthase (iNOS),

IFNβ, and IL-12p35, are impaired in IRF1-/- dendritic cells and macrophages stimulated with IFNγ and CpG (Negishi et al., 2006).

This study is the first to investigate the details of the signaling mechanism of flagellin-stimulated IRF1 expression and its role in regulating the immune response in the cornea. Flagellin/TLR5 activation of STAT1 has only recently been discovered, and may have a crucial role in modulating IRF1 expression. Suppression of IRF1 expression by flagellin-induced reprogramming could explain the benefit of flagellin in alleviating corneal inflammation. Stimulation with flagellin or suppression of IRF1 seems to result in a similar Th2-biased response. Therefore, this study is to delineate a pathway that is stimulated by flagellin and mediated by IRF1 that can alleviate/exacerbate the pathogenesis of bacterial keratitis.

1.11 C-X-C Motif Chemokine 10 (CXCL10)

The C-X-C motif chemokine 10 (CXCL10) also known as interferon γ-induced protein 10 (IP-10), is a cytokine belonging to the CXC chemokine family. The CXCL10 protein is composed of 98 amino acids and has a molecular mass of 10,000 Daltons. CXCL10 exerts its biological effects by binding to CXCR3, a seven trans-membrane-spanning G protein-coupled receptor in a paracrine or autocrine fashion (Lo et al., 2010). CXCL10 is a pleiotropic molecule capable of exerting potent biological functions, including promoting the chemotactic activity of CXCR3+ cells, inducing apoptosis, regulating angiogenesis in infectious and inflammatory diseases and cancer (Liu et al., 2011a).

1.12 CXCL10 in bacterial infection

CXCL10 performs ''homing'' functions to chemoattract CXCR3-positive cells, including NK cells and activated T lymphocytes (CD4+ Th cells, CD8 (CD4+ Th cells, CD8+ Tc cells) toward inflamed and/or infected areas (Liu et al., 2011a). CXCL10 has been shown to play a role in *Helicobacter pylori* and *Mycoplasma* infections by recruiting inflammatory T cells into

the mucosal tissues (Kabashima et al., 2002). Elevation of CXCL10 levels appears to be an early host response to scrub typhus (*Orientia tsutsugamushi*) infection (DE FOST et al., 2005) and is associated with the severity of Legionnaire's disease and tuberculosis (TB). On the other hand, impaired CXCL10 production leads to increased susceptibility to *Leginella pneumophila* infection (Lettinga et al., 2003). In contrast, high levels of CXCL10 mediated protection against Leishmania amazonensis infection in mice, delayed lesion development and reduced parasite burden via IFNγ secretion (Vasquez and Soong, 2006).

1.13   Activating Transcription Factor 3 (ATF3)

ATF3 is a member of the larger AP-1 DNA binding protein group composed of the basic-region leucine zipper (bZIP) transcription factors Jun (c-Jun, JunB, JunD), Fos (C-Fos, FosB, Fra-1, Fra-2), and ATF (ATF2, ATF3, B-ATF) (Shaulian and Karin, 2001). ATF3, a 21 kDa protein, can bind DNA at ATF/cAMP responsive element (ATF/CRE) consensus sequences (TGACGTCA) (70, 71). In general, ATF3 is considered a repressor of target genes, but when partnered with other transcription factors it can both activate and repress transcription (Hai and Curran, 1991). For example, ATF3/c-Jun and ATF3/JunD activate promoters with ATF/CRE binding sites, whereas the ATF3/JunB heterodimer activates genes with CRE-containing promoters but represses genes with AP-1-containing promoters (Hsu et al., 1992). Thus, ATF3's role in transcription cannot be generalized. Evidence suggests that ATF3 may repress or activate the transcription of target genes depending on its dimerizing partner and the promoter context (Chen et al., 1994).

The ATF3 gene has four exons (A, B, C, E) spread over 15 kilobases and it is transcribed into two splice variants (Chen et al., 1994). The full-length transcript binds DNA, whereas the alternatively spliced variant, ATF3ΔZip (14 kDa), is truncated which prevents it from properly binding DNA at ATF/CRE consensus sites (Chen et al., 1994). Although the regulation of *ATF3*'s alternative splicing remains to be discovered, there are clues towards the

splice variants' distinct function. Several studies have observed that truncated ATF3 acts as a transcriptional activator, possibly by sequestering transcriptional co-repressors (Chen et al., 1994; Hashimoto et al., 2002; Pan et al., 2003). Another study found that the ATF3 splice variant represses NFκB-dependent transcription by displacing a positive cofactor (Hua et al., 2006). There are several transcription factor binding sites located within the *ATF3* gene promoter. ATF/CRE, AP-1 and NF-κB are inducible sites, which are known to be activated upon stress signals, and the Myc/Max, E2F, and p53 binding sites are involved in cell cycle regulation (Liang et al., 1996; Zhang et al., 2002). The expression of ATF3 mRNA is transient due to its ability to repress its own promoter (Wolfgang et al., 2000). There is a lack of evidence for posttranslational modifications of the ATF3 protein, although there are many potential modification sites that include several serine, threonine, tyrosine and lysine residues (Hai et al., 2011).

1.14   ATF3 in Immunity

ATF3 induction by genotoxic agents can lead to the activation and repression of proteins involved in cell cycle regulation and apoptotic pathways. However, there is an emerging regulatory role for ATF3 in immunity and the inflammatory response. Gilchrist *et al.* were the first group to link ATF3 with the innate immune system (Gilchrist et al., 2006). A DNA microarray coupled with systems biology analysis revealed that ATF3 is induced upon TLR4 activation (Gilchrist et al., 2006). TLR4 activates the IL-6 and IL-12b cytokines through NF-κB and ATF3 was found to repress the cytokine signal shortly after it was initiated (Gilchrist et al., 2006). The same study also found that ATF3 repressed 11 genes involved in macrophage signalling, thus highlighting ATF3's role in a negative feedback loop of the innate immune system. This new function was validated by another study that found ATF3 to be induced by a wide range of TLRs (TLR-2/6, -3, -5, -7, -9) located on the cell surface membrane and in intracellular endosomes (Whitmore et al., 2007). ATF3 is induced in an asthma mouse model and *atf3*-null mice had

more severe asthmatic symptoms than wild type mice (Gilchrist et al., 2008). ATF3 appeared to elicit its inflammatory suppression by directly antagonizing the transcription of pro-inflammatory cytokines (IL-4, IL-5, IL-13) (Gilchrist et al., 2008) and a chemokine (chemokine C-C motif ligand 4, CCL4) (Khuu et al., 2007). Interestingly, ATF3 appears to be repressed in patients with severe asthma compared to patients with mild disease (Roussel et al., 2011).

As a cell cycle regulator, ATF3 can directly bind the p53 protein to prevent its ubiquitination and degradation, and thus augment its function and causing cell cycle arrest and apoptosis (Yan et al., 2005). Cyclin D1 is another regulator of cell proliferation that can be under the control of ATF3, which can directly target the *cyclin D1* gene promoter, repressing its expression and subsequently leading to cell cycle arrest at the G1-S checkpoint (Lu et al., 2006). Another ATF3 target involved in the cell cycle is inhibitor of differentiation (Id1), an oncogene involved in cell growth and invasion (Zigler et al., 2011). ATF3 has consistently been shown to repress the Id1 gene promoter in several *in vitro* models, supporting ATF3's role as a transcriptional regulator of cell cycle control genes (Kang et al., 2003; Zigler et al., 2011). The ability of ATF3 to activate and repress transcription can help explain ATF3's apoptotic roles upon cellular stress.

1.15   Overview and significance

Bacterial keratitis is considered as a serious medical condition that requires urgent medical attention due to potential vision reduction or loss in the affected eye. Factors that increase the chances of infection include extended wear of soft contact lenses; ocular surgical procedures; ocular disease and ocular injury. The infecting bacteria come from environmental sources, patients' skin and nasopharyngeal flora, contact lens care solution or lens cases, topical drugs, irrigation solutions or ocular instruments (Fleiszig and Evans, 2002). In the United States, microbial keratitis is most frequently associated with complications related to contact lens usage, with an incidence rate of 25,000 to 30,000 cases per year (Khatri et al., 2002).

The integrated human immune response to infection has traditionally been divided into 2 branches: innate and adaptive immunity. The protective ability of innate immunity of the corneal epithelium is largely dependent on germ-line encoded pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2011). TLR-PAMP binding initiates the innate immune response, which includes the release of inflammatory mediators, antimicrobial effectors, and signals inducing adaptive immune responses (Nish and Medzhitov, 2011).   However, the corneal epithelial innate and inflammatory response to pathogens, if not properly controlled, can result in the development of bacterial keratitis. But multiple regulatory mechanisms exist in corneal epithelial cells to control the inflammatory response including the expression of negative regulators and the induction of hyporesponsiveness.

Previous work from our lab has shown that application of purified flagellin, the ligand of TLR5, prior to microbial inoculation induces profound protection in the cornea against infectious pathogens (Gao et al., 2011a; Kumar et al., 2010a). Pre-exposure of corneal epithelial cells to flagellin dampens the expression of inflammatory cytokines and augments the induced expression of antimicrobial, anti-oxidative and/or cytoprotective genes in response to pathogens and other adverse challenges, a phenomenon now being renamed "TLR-mediated genomic reprogramming" (Biswas and Lopez-Collazo, 2009; Vartanian and Stenzel-Poore, 2010). TLR5 reprogramming has also been shown to induce protection against a variety of adverse challenges or diseases conditions such as stroke (Vartanian and Stenzel-Poore, 2010), infection (Kumar et al., 2008; Vijay-Kumar et al., 2008b), radiation (Burdelya et al., 2008; Jones et al., 2011), and chemicals (Vijay-Kumar et al., 2008b). In addition, recent reports have also highlighted the increase in antibiotic resistant strains of bacteria, which fortifies the need to better understand the targeted genes and pathways involved in TLR5-induced cell reprogramming so that improved therapeutic strategies can be developed (Hazlett, 2004a).

As TLR-mediated expression of proinflammatory and cytoprotective genes are mostly controlled at the transcription level, it is of much interest to identify the transcription factors (TF) involved in TLR-induced reprogramming and mucosal surface protection. Hence, it is reasonable to postulate that in addition to NF-κB, other transcription factors (TFs), the effectors controlling gene expression, may also be involved in cell reprogramming. In this regard, the goal of this dissertation is to test the following three hypotheses:

1. TLR5-mediated human corneal epithelial cell reprogramming results in suppressed IRF1 and CXCL10 expression, but enhanced ATF3 expression.

2. TLR5-mediated mouse corneal protection requires the enhancement of IRF1 and CXCL10 expression, which is caused by accelerated infiltration of IFNγ-secreting NK cells.

3. ATF3 is essential for reducing pathogen-induced corneal inflammation, enhancing bacterial clearance and maintaining TLR5-mediated mouse corneal protection

CHAPTER 2

CHARACTERIZATION OF EXPRESSION AND FUNCTION OF IRF1 IN HUMAN CORNEAL EPITHELIAL CELLS

2.1   ABSTRACT

We previously showed that pre-exposure of the cornea to TLR5 ligand flagellin induces profound mucosal innate protection against infections by modifying gene expression. To understand the regulation at the transcriptional level, we used Superarray and identified Interferon Regulatory Factor (IRF) 1 and Activating Transcription Factor (ATF) 3 as the most drastically affected genes by flagellin pretreatment in *P. aeruginosa* challenged human corneal epithelial cells (CEC). However, flagellin pretreatment had opposite effects on IRF1 (inhibition) and ATF3 (enhancement) gene expression in response to *P. aeruginosa*, and other IRFs were not affected. To find the functional target gene of IRF1, we knocked-down IRF1 using siRNA and identified the pleiotropic chemokine CXCL10, but not IL12-p35 or iNOS, as a specific target. The IRF1-CXCL10 axis is also strongly expressed in response to IFNγ stimulation, but flagellin pretreatment could not reprogram the IRF1-CXCL10 axis in response to IFNγ, indicating the different signaling mechanisms used by flagellin and IFNγ to induce IRF1-CXCL10 in hCECs. Together, our results indicate that flagellin profoundly reprograms the gene expression of IRF1 and ATF3, and CXCL10 plays a key role in corneal innate immunity against microbial infection.

2.2 INTRODUCTION

The ocular surface, like other mucosal surfaces including the respiratory (Bals and Hiemstra, 2004), gastrointestinal (Santaolalla et al., 2011), and urogenital tracts (Song and Abraham, 2008), is covered by epithelial cells (ECs) that form a physiological barrier. These ECs also possess the ability to sense and initiate the host immune response, which is largely achieved through germ-line encoded pattern-recognition receptors (PRRs) (Kumar and Yu, 2006; Ueta and Kinoshita, 2010; Yu and Hazlett, 2006). Toll-like receptors (TLRs) are well-known PRRs that recognize pathogen-associated molecular patterns (PAMPs) to initiate the innate immune response, which includes production of inflammatory mediators, antimicrobial effectors, and signals promoting the adaptive immune response (Nish and Medzhitov, 2011). However, the epithelial innate and inflammatory response to pathogens caused by TLRs, if not properly controlled, can also cause tissue damage, resulting in the development of human disease such as corneal scarring, airway asthma, or allergic rhinitis. Multiple regulatory mechanisms exist in epithelial cells to control the inflammatory response including the expression of negative regulators and the induction of hyporesponsiveness, a phenomenon similar to endotoxin (TLR4) tolerance (West and Heagy, 2002). Pre-exposure of mucosal surfaces or cultured cells to TLR ligands has resulted in suppression of inflammatory cytokine release yet enhanced tissue resistance to infection and other adverse environmental challenges – a phenomenon called "reprogramming" (Fan and Cook, 2004; Nahid et al., 2011b). Cell reprogramming induced by TLR5/flagellin interaction has been shown to induce protection against a variety of adverse challenges or diseases conditions such as stroke (Vartanian and Stenzel-Poore, 2010), infection (Gao et al., 2011a; Kumar et al., 2008; Vijay-Kumar et al., 2008b), radiation (Burdelya et al., 2008; Jones et al., 2011), and chemicals (Vijay-Kumar et al., 2008b).

Previous studies from our lab have shown that TLR5 activation by flagellin in primary human corneal epithelial cells (HCECs) induces "cell reprogramming" that has greatly reduced proinflammatory cytokine production. This is mainly due to impaired activation of NF-κB and AP-1 in flagellin-reprogrammed cells (Kumar et al., 2008; Kumar et al., 2007). However, the underlying mechanisms for the expression of antimicrobial effectors, such as hBD2 and LL-37, and other protective genes remain elusive.

Hence, it is reasonable to postulate that in addition to NF-κB, other transcription factors (TFs), the effectors controlling gene expression, may also be involved in TLR ligand-mediated cell reprogramming. Identification of these factors is of great interest as they may mediate the expression of a subgroup of genes, and serve as a more specific target for controlling TLR-triggered inflammation and/or accelerating the resolution of inflammation. Moreover, it may lead to the development of more advanced therapeutic molecules to treat bacterial keratitis and other infectious diseases. As TLR-mediated expression of proinflammatory and cytoprotective genes are mostly controlled at the transcription level, it is of interest to identify the TFs involved in TLR-induced reprogramming and mucosal surface protection.

In this study, we used a real time PCR array to identify TFs with differential expression profiles in HCECs challenged with bacteria with or without flagellin pretreatment. We identified IRF1 as one such TF and demonstrated that CXCL10 expression is regulated by in part by IRF1 in response to flagellin and IFNγ stimulation, albeit by different pathways in HCECs.

2.3   MATERIALS AND METHODS

Human corneal epithelial cell culture

Primary HCECs were isolated from human donor corneas obtained from the Midwest Eye Bank. The epithelial sheet was separated from the underlying stroma after overnight Dispase treatment. The dissected epithelial sheet was trypsinized, and the epithelial cells were

collected by centrifugation (500g, 5 minutes). HCECs were cultured in keratinocyte growth medium (KBM supplemented with growth factors; BioWhittaker) in T25 flasks coated with fibronectin-collagen (FNC) and use between passage 3 and 5.

Reagents and antibodies

Anti-ATF3, anti-IRF1 and anti-hBD2 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-β-actin and anti-CXCL10 antibodies were purchased from Sigma (St. Louis, MO) and Abcam (Cambridge, MA), respectively. Anti-LL-37 antibody was purchased from Panatechs (Tubingen, Germany). All other primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA). Human IRF1 siRNA and negative control siRNA was purchased from Dharmacon (Chicago, IL). Defined keratinocyte serum-free medium (DK-SFM), reduced serum media (Opti-MEM), transfection reagent (Lipofectamine 2000), and reagent (TRIzol) were purchased from Invitrogen (Carlsbad, CA). Keratinocyte basal medium (KBM) was purchased from BioWhittaker (Walkersville, MD).

Bacterial strains and flagellin

Flagellin was prepared from PAO1 using a previously described method (Zhang et al., 2003b). For direct bacterial challenge of HCECs, *P. aeruginosa* were grown in tryptic soy broth (Sigma-Aldrich) at 37℃ until absorbance at 600 nm reached O.D. 0.5. The bacterial culture was centrifuged at 6000 × g for 10 min. Bacteria were washed in PBS and resuspended in KBM (PAO1) or PBS (PAO1 or ATCC 19660) and then used to challenge HCECs at a ratio of 1:50 (cell to bacteria) or to infect mouse corneas at 10^5 for PAO1 and 10^4 cfu for ATCC18660, respectively.

RNA isolation and RT-PCR

Total RNA was isolated from HCECs using the TRIzol solution (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 1 µg of total RNA was reverse-transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). cDNA was amplified by PCR using primers for human IRF1, CXCL10, BD2, IL-8, and GAPDH (Table 3);. The PCR products and internal control GAPDH were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide. Stained gels were captured using a digital camera and band intensity was quantified using 1D Image Analysis Software (EDAS 290 system; Eastman Kodak, Rochester, NY).

Transcription factor PCR array

Total RNA was isolated using the protocol below. First strand DNA was created using RT^2 First Strand kit along with the protocol and cycling times as recommended by the manufacturer (SABiosciences, Frederick, MD). Two-stage real-time reverse transcriptase PCR of Toll-like Receptor-related transcription factors was performed on the RT^2 Profiler PCR Array (SABiosciences, Catalogue no. PAHS-018). The PCR was performed on an ABI 7000 (Applied Biosystems). The cycle threshold (C_T) was determined for each sample and normalized to the average C_T of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Comparative C_T method was used to calculate relative gene expression. Data are represented as fold change relative to control. All solutions, including the SYBR Green reverse transcriptase PCR mix, were purchased from SABiosciences Corporation. The data was analyzed using online analysis software provided by the manufacturer. Briefly, the PCR Array Pathway Number is entered, followed by uploading the MS Excel file containing the PCR data. Housekeeping genes on the PCR array were used for data normalization, whereas HCECs without any treatment were used as the reference for infected with PAO1 and for flagellin pretreated and infected with the same strain. The "Fold Change" (increase as positive and decrease as negative) and "p-value" (only <0.05 were chosen) were used by the software for subsequent graphical presentation.

Western blot analysis

HCECs challenged with either flagellin or bacteria were lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mm NaCl, 100 mm Tris-HCl [pH 7.5], 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 50 mm NaF, 100 mm sodium pyrophosphate, and 3.5 mm sodium orthovanadate). A protease inhibitor cocktail (aprotinin, pepstatin A, leupeptin, and antipain, 1 mg/mL each) and 0.1 M phenylmethylsulfonyl fluoride were added to the RIPA buffer (1:1000 dilution) before use. The protein concentration in cell lysates was determined with the bicinchoninic acid detection assay (MicroBCA; Pierce). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) and electroblotted onto nitrocellulose membranes (0.45-μm pores; Bio-Rad, Hercules, CA). After blocking for 1 hour in Tris-buffered saline/Tween (TBST; 20 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween) containing 5% nonfat milk, the blots were probed with primary antibodies overnight at 4℃. The membranes were washed with 0.05% (vol/vol) Tween 20 in TBS (pH 7.6) and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 60 minutes at room temperature. Protein bands were visualized by chemiluminescence (Supersignal reagents; Pierce) using the Kodak Image Station 4000R Pro.

ELISA measurement of cytokines

Secretion IL-8 and TNFα from HCECs was determined by ELISA. HCECs were plated at 1 x 10^6 cells/well in six-well plates. After growth factor starvation, the cells were either left untreated or pretreated with flagellin followed by challenge with a higher dose of flagellin or live bacteria (~MOI 50). At the end of the incubation period, the media were harvested for measurement of cytokines and ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The amount of cytokines in cultured media was expressed as nanogram per milligram of cell lysate. All values are expressed as the mean ±

SEM. Statistical analysis was performed with an unpaired, two-tailed Student t test, and $P <$ 0.05 was considered statistically significant.

Slot blot determination of CXCL10 and LL-37

Accumulation of human CXCL10 in the culture media was detected by slot blot (Kumar et al., 2006). Briefly, 150 µl supernatant was applied to a nitrocellulose membrane (0.2 µm; Bio-Rad) by vacuum using a slot-blot apparatus (Bio-Rad). The membrane was fixed by incubating with 10% formalin for 1 hour at room temperature, followed by blocking in Tris-buffered saline (TBS) containing 5% nonfat powdered milk for 1 h at room temperature. The membrane was then incubated overnight at 4℃ with rabbit anti-human C XCL10 antibody diluted 1:500 in TBS containing 5% nonfat powdered milk, and 0.05% Tween-20. After washing, the membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to HRP diluted 1:2000 with 5% nonfat powdered milk. Immunoreactivity was visualized by chemiluminescence (Supersignal reagents; Pierce) using the Kodak Image Station 4000R Pro.

Statistical analysis

An unpaired, two-tailed Student t test was used to determine statistical significance for data from bacterial count, cytokine ELISA and MPO assay. Mean differences were considered significant at a P value of <0.05. Experiments were repeated at least twice to ensure reproducibility.

2.4   RESULTS

Flagellin Pretreatment Suppresses PAO1-induced IRF1, but Enhances ATF3 Expression in HCECs.

The activation of NF-κB in response to bacterial and TLR challenges occurs at both transcriptional and post-transcriptional levels, whereas other transcription factors (TFs) are regulated primarily at the transcriptional level.   To determine the TFs involved in flagellin-

induced cell reprogramming and protection against microbial infection, we used the transcription factor RT^2 Profiler PCR Array to compare the expression profiles of TFs in HCECs in response to PAO1 (multiplicity of infection, MOI, 50 bacteria per cell) with or without flagellin pretreatment, 50 ng/ml for 24 h (Fig. 8A). Compared to the control, PAO1 challenge of HCECs resulted in greatly elevated expression of IRF1 (29.1 fold) and ATF3 (42 fold). Strikingly, flagellin pretreatment further enhanced the expression of ATF3 (61.5 fold) while suppressing IRF1 expression by -2.93 fold compared to control, giving an 85.2 fold decrease for IRF1 mRNA in PAO1 challenged HCECs.

To further confirm the expression pattern of IRF1 at the protein level, Western blotting of HCEC lysates, ELISA for IL-8, and dot blot for LL-37 detection in culture media were performed (Fig. 8B-D). As we have demonstrated previously (Kumar et al., 2007), flagellin-pretreatment (50 ng/ml for 24 h) by itself had minimal effects on HCECs, including the expression of IRF1 and its target gene CXCL10 (data not shown). PAO1 (50 MOI)-stimulated production of IL-8 (Fig. 8B) was blocked while LL-37 levels increased (Fig. 8C) in HCECs pretreated with flagellin, indicating flagellin-induced cell reprogramming. Consistent with the PCR array data, PAO1 challenge induced an abundant expression of IRF1, and flagellin pretreatment dampened PAO1-induced IRF1 expression (Fig. 8D). Thus, IRF1 is differentially expressed in HCECs in response to bacterial challenge in the presence or absence of flagellin pretreatment, and this disparity of expression suggests that IRF1 is a good candidate to study flagellin-induced cell reprogramming. Given its distinction as an important regulator of inflammation and immunity (Koetzler et al., 2009; Stirnweiss et al., 2010; Zaheer and Proud, 2010), we focused on IRF1 in subsequent experiments in this chapter and the next.

IRF1 is the only IRF upregulated by TLR5 activation in HCECs

The 9 IRFs identified have been reported to play significant roles in innate immunity and PRR-mediated induction of cytokines (Fujita et al., 1988; Honda and Taniguchi, 2006a). It has

been shown that IRF5, which associates with MyD88, an adaptor of most TLR, is a critical regulator of the induction of proinflammatory cytokine genes (Takaoka et al., 2005). It was also reported that IRF4 and IRF8 participate in TLR-mediated signaling in dendritic cells (DC) (Negishi et al., 2005). In addition, recent studies have reported the up-regulation of IRF3 and 7 induced by TLR3 and 4 through the TRIF-mediated pathway, and of IRF5, 7 and 1 by intracellular TLRs (7, 8, and 9), the latter only in myeloid dendritic cells (Battistini, 2009; Savitsky et al., 2010a). Thus, many of the IRF family members are essential regulators in TLR-mediated signaling. Therefore, we analyzed the mRNA expression levels of other IRF family members (IRF1-9) in HCECs under the same treatments using RT-PCR (Fig. 9) to verify whether other IRFs are subjected to reprogramming. All IRFs, except IRF4 and IRF8, were detectable, but did not display any alterations in RNA expression after stimulation with flagellin or infection with live PAO1 bacteria for 4h. IRF2 is known as a natural antagonist of IRF1 expression (Choo et al., 2006) but was not altered at 4h post-stimulation. Hence, all observed effects related to IFNs are likely the results of IRF1 upregulation in HCECS.

IRF1 targets the expression of CXCL10 in HCECs

CXCL10 is a major target gene of IRF1 (O'Neill and Bowie, 2007; Shultz et al., 2009). We have shown that the expression of CXCL10 in CECs is very sensitive to the environmental challenges including wounding (Gao et al., 2011b) and infection (Fig. 8). To determine if TLR5-induced CXCL10 expression is regulated by IRF1, siRNA knockdown of IRF1 was performed (Fig. 10). Here, we used a higher dose of flagellin (250ng/ml), to mimic the inflammatory response induced by bacterial challenge (Kumar et al., 2007) to stimulate CXCL10 expression. At the mRNA level, IRF1 siRNA treatment reduced flagellin-induced IRF1 expression (Fig. 10A). Consistent with IRF1 downregulation, CXCL10 expression was affected, while, as expected, IL-8 and hBD2 were not. At the protein level (Fig. 10B), IRF1 was not detected in the control but induced by 250 ng/ml flagellin as stimulus for 4 h. This induced elevation of IRF1 was

dampened by IRF1-specific, but not by non-specific siRNA. Equal quantity of culture media, normalized with protein concentration, from the same samples shown in Panel B were subjected to Slot Blot for CXCL10 secretion with hBD2 as the positive control (Fig. 10C). The accumulation of CXCL10, but not hBD2, in culture media was affected by IRF1 siRNA. Taken together, figure 10 shows a strong correlation between IRF1 levels and CXCL10 expression and production in cultured HCECs.

Flagellin-induced HCEC reprogramming cannot suppress IFNγ-induced IRF1 and CXCL10 expression

Flagellin caused reprogramming of HCECs to suppress PAO1-induced IRF1 expression. However, we wanted to find if flagellin can reprogram the expression of IFNγ-stimulated IRF1 and CXCL10 in HCECs, as IFNγ is a powerful stimulator of IRF1 and CXCL10 expression (Ohmori and Hamilton, 1997). We pretreated primary HCECs with or without flagellin for 24h and then stimulated with IFNγ (10ng/ml) for 4h, after which the cells and culture media were collected and subjected to Western blot for IRF1 detection and Slot Blot for CXCL10 detection (Fig.11A & B). IFNγ 10 ng/ml produced a robust expression of IRF1 and CXCL10 (lane 3), and flagellin pretreatment did not cause noticeable reduction in IFNγ-induced IRF1 and CXCL10 expression (lane 3 vs lane 4). A high dose of flagellin (250ng/ml) was used as control for IRF1 and CXCL10 expression (lane 2). These results suggest that TLR5 and IFNγR utilize different signaling pathways to regulate IRF1 expression, and that suppression of IRF1 expression by TLR5/flagellin-induced reprogramming does not extend to IFNγ-induced IRF1 expression.

2.5 DISCUSSION

Previous studies from our lab have shown that flagellin pretreatment has profound effects on mucosal innate immunity in mouse cornea and lung (Gao et al., 2011a; Kumar et al., 2010a; Yu et al., 2010). To understand the underlying mechanisms of flagellin-induced

reprogramming we started our study in cultured primary HCECs by screening for transcription factors (TF) differentially expressed in PAO1-infected primary HCECs with or without flagellin pretreatment. This revealed that IRF1 expression was greatly increased in response to bacterial challenge but pre-exposure of cells to a low dosage of flagellin for 24h prior to PAO1 challenge caused a dramatic decrease in IRF1 expression. IRF1 is one of the members of the IRF family of TFs consisting of nine members: IRF1-9 (Colonna, 2007; Honda and Taniguchi, 2006b; Savitsky et al., 2010b). IRFs were first characterized as IFN-inducible TFs and recent studies have revealed their involvement in the regulation of gene expression in responses triggered by TLRs. Our study is the first to show elevated expression of IRF1 in response to TLR5 stimulation by flagellin or Gram-negative bacterial challenge in non-professional immune cells. Other IRFs have been reported to be expressed in response to other TLRs, such as IRF3 and 7 by TLR3 and 4 (Battistini, 2009; Savitsky et al., 2010a) and IRF5 and 7 by intracellular TLRs (7, 8, and 9) (Battistini 2009). However, we did not detect alterations in gene expression of other IRFs, indicating the specificity of the TLR5-induced IRF1 expression in CECs. In accordance with reports that IRF4 and IRF8 are only expressed in immune cells (Tamura et al., 2008a), we could not detect them in HCECs.

TFs function as gene expression regulators, and in order to elucidate the function of IRF1 in HCECs, we screened for effector target genes of IRF1. Down regulation of IRF1 by siRNA in HCECs reduced the expression and secretion of CXCL10, a major targeted gene of IRF1. However, other reported target genes such as iNOS or IL-12p35 did not display reduction at the RNA level, indicating the specificity of CXCL10 as a target gene of IRF1 in corneal epithelial cells. CXCL10 is a chemokine for innate immune cells such as NK cells. In addition to being a chemokine, CXCL10 has also been shown to possess antimicrobial activities (Cole et al., 2001a; Egesten et al., 2007; Gotsch et al., 2007; Muller et al., 2010; Strieter et al., 1995; Yang et al., 2003). Indeed, we recently observed CXCL10 possessing bactericidal and

fungicidal activities *in vitro* and *in vivo* (Liu, Gao and Yu, Submitted). In the cornea, CXCL10 is among the earliest and most highly expressed chemokines during HSV-1 infection and plays a pivotal role in the coordinated immune response to HSV-1 (Carr et al., 2003; Wuest and Carr, 2008).

In summary, our results suggest that TLR5/flagellin prestimulation reprograms the expression of IRF1 in HCECs, by suppressing its expression in response to P. aeruginosa challenge, and exerts its function by regulating the expression CXCL10, a pleiotropic molecule with anti-microbial and angiostatic function. Future studies to delineate the function of the IRF1-CXCL10 axis in the animal model of flagellin-induced corneal protection against bacterial keratitis is warranted.
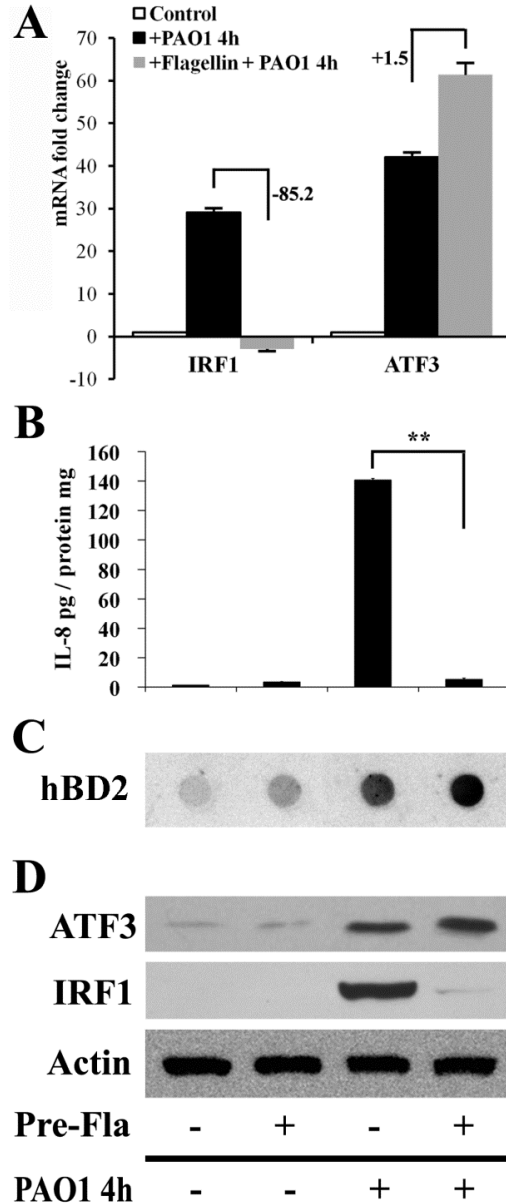
Figure 8. Reprogramming of transcription factor expression caused by flagellin pretreatment in primary HCECs. Normal or flagellin-pretreated (50 ng/ml, 24 h) cells were challenged with live PAO1 (1:50 MOI) for 4 h and transcription factor RNA expression alterations were quantified by comparing RNA fold changes of PAO1-infected normal cells vs PAO1-infected flagellin-pretreated cells using the real-time RT^2 Profiler PCR Array (A). The cycle threshold (C_T) was determined for each sample and normalized to the average C_T of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are represented as fold change relative to control. For confirmation of PCR array results, cells were cultured and challenged under the same conditions and lysed and subjected to Western blotting analyses with ATF3, IRF1 and β-actin antibodies (D). Cell culture media was collected 4 h post bacterial challenge for the analyses of IL-8 by ELISA (B) and LL-37 using slot-blot (C). P values were generated using unpaired Student's *t* test (** P<0.01). The figure is a representative of four independent experiments.
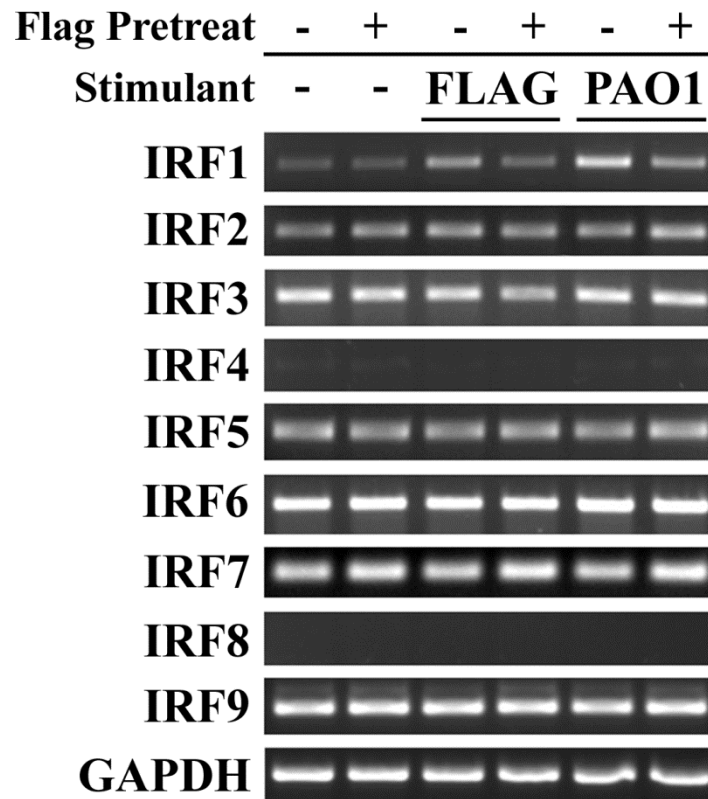
Figure 9. Expression of IRFs in HCECs. RT-PCR analysis of the IRF family gene expression. Normal or flagellin-pretreated (50 ng/ml, 24 h) cells were challenged with live PAO1 (1:50 MOI) or a high dose of flagellin (FLAG: 250 ng/ml) for 4 h and transcription factor RNA expression alterations were quantified using RT-PCR. The figure is a representative of two independent experiments.
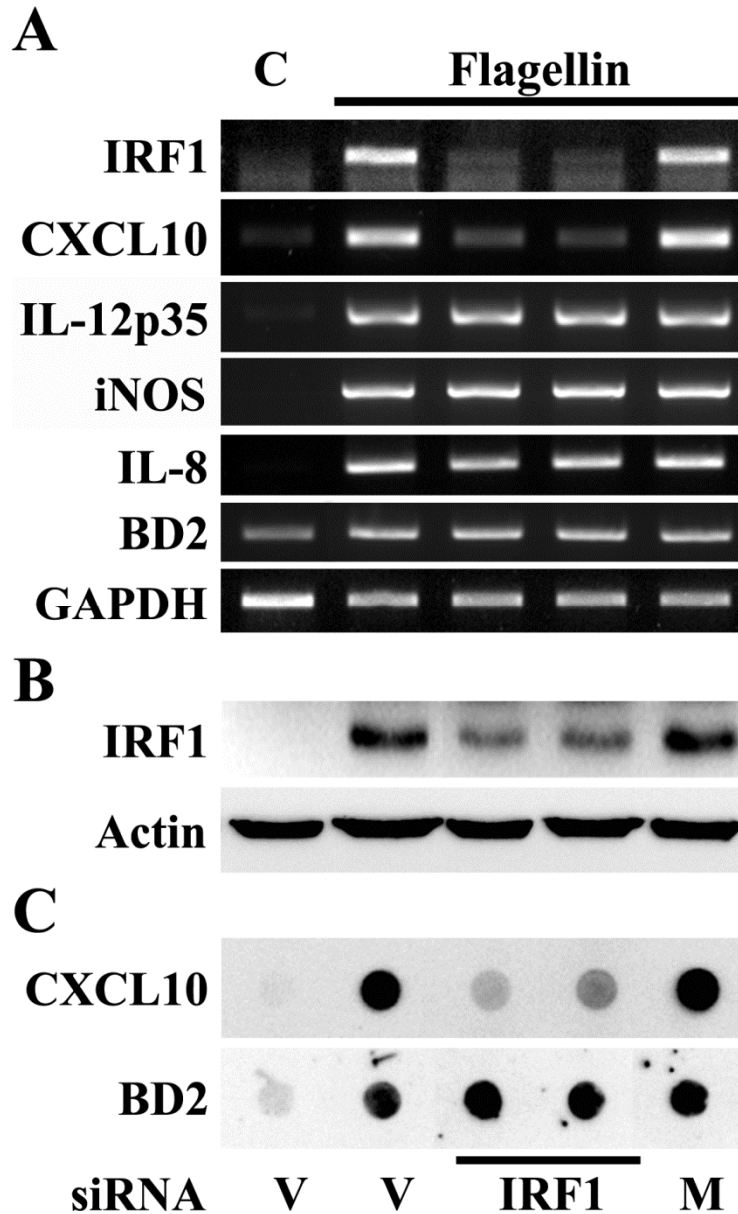
Figure 10. Requirement of IRF1 for flagellin-induced CXCL10 expression and production. HCECs were transfected with IRF1 siRNA with transfection vehicle alone (V) and scrambled siRNA (M) as the controls. At 48 h post-transfection, cells were challenged with 250 ng/ml flagellin and then lysed at 4 h for semi-quantitaive RT-PCR analysis (A) or Western blotting of IRF1 and actin (B). The culture media collected was used for dot blot analysis of CXCL10 and hBD2 production (B).   The figure is a representative of three independent experiments.
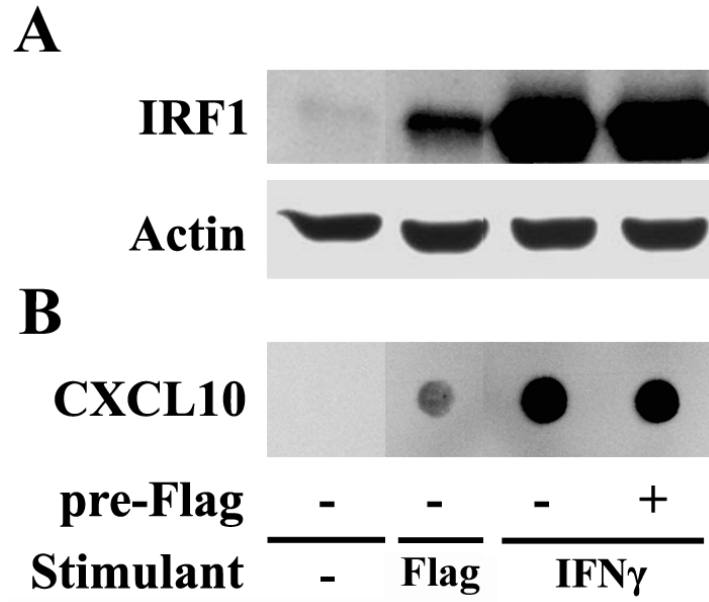
Figure 11. Flagellin pretreatment cannot suppress IFNγ-induced IRF1 and CXCL10 expression. Western blot of IRF1 and actin (A) and slot blot of CXCL10 (B). Pre-Flag, 50ng/ml flagellin-pretreatment for 24 hrs; FLAG, 250ng/ml flagellin rechallenged; IFNγ, 10ng/ml. Figure is a representation of three independent experiments.

Table 3. Human primer sequences used for PCR

Gene	Primer	Sequence (5'→3')	Product size (bp)
IRF1	Forward	TTCCCTCTTCCACTCGGAGT	379
	Reverse	GATATCTGGCAGGGAGTTCA	
IRF2	Forward	GATGCATGCGGCTAGACATGGGTG	548
	Reverse	GGTAGAGCTCGCTCATGCTGACCGG	
IRF3	Forward	AATCCCACTCCCTTCCCAAACC	430
	Reverse	-CAAACACGCCTCCTTCCTTGTC	
IRF4	Forward	TCCCCACAGAGCCAAGCATAAG	586
	Reverse	TAGCCCCTCAGGAAATGTCCACTG	
IRF5	Forward	AGACCAAGCTTTTCAGCCTGG	407
	Reverse	GCCTTGTTATTGCATGCCAGC	
IRF6	Forward	CAAAACTGAACCCCTGGAGATG	560
	Reverse	GCCATTCTTCCCCAAAGCATAAG	
IRF7	Forward	TCGTGATGCTGCGGGATAAC	175
	Reverse	TGTGTGTGTGCCAAGAATGG	
IRF8	Forward	CAGTGGCTGATCGAGCAGATTGA	360
	Reverse	ATTCACGCAGCCAGCAGTTGCCA	
IRF9	Forward	CTGCTCACCTTCATCTACAACGG	390
	Reverse	ATTCAGTGTTACCTGGAACTTCGG	
CXCL10	Forward	GTACCTCTCTCTAGAACCGTACG	229

	Reverse	GAGATCTTTTAGACATTTCC	
IL-12p35	Forward	TTCACCACTCCCAAAACCTGC	226
	Reverse	GAGGCCAGGCAACTCCCATTAG	
iNOS	Forward	CAACTCTATATTGCTGGAACATGGA	125
	Reverse	TGGAAGCCTGTGATACTTTCTGTACT	
IL-8	Forward	GCTTCTGCATTTGAGTTTGCTAGCT	294
	Reverse	TACAGACATTCACAGCCACC	
BD2	Forward	GAGAAACAGGATGGCTGAAGG	291
	Reverse	TGAGGATGGTGTAAGCGATGG	

CHAPTER 3

INTERFERON REGULATORY FACTOR-1 IN FLAGELLIN-INDUCED REPROGRAMMING: POTENTIAL PROTECTIVE ROLE OF ITS TARGET GENE CXCL10 IN CORNEA INNATE DEFENSE AGAINST *PSEUDOMONAS AERUGINOSA* INFECTION

3.1   ABSTRACT

Having shown Interferon Regulatory Factor (IRF) 1 as the most drastically affected gene by flagellin pretreatment in *P. aeruginosa* challenged human corneal epithelial cells (CEC) and CXCL10 as the target gene of IRF1, next we sought understand the role of IRF1 and CXCL10 in mucosal innate protection against infections *in vivo.* Flagellin augmented the *P. aeruginosa-*induced expression of CXCL10 in CECs in WT, but not in IRF1-/- mice at 6 hpi. IRF1 deficiency markedly increased the severity of *P. aeruginosa* keratitis and significantly attenuated flagellin-elicited protection compared to WT controls at 3 dpi. CXCL10 neutralization in the cornea of WT mice displayed similar pathogenesis to that of IRF1-/- mice. To understand the regulation of CXCL10 expression *in vivo,* we used neutralizing antibodies to target IFNγ and its producer NK cells. The neutralization of IFNγ receptor or NK cells prevented flagellin-augmented IRF1 and CXCL10 expression and increased the susceptibility to *P. aeruginosa* infection in mouse corneas. Together, our results indicate that IRF1 plays a role in the corneal innate immune response by regulating CXCL10 expression and that IFNγ-producing NK cells induces epithelial expression of IRF1, thus significantly contributing to the protection of the cornea from *P. aeruginosa* infection.

3.2   INTRODUCTION

Among the 10 identified human TLRs, TLR5 is unique as it recognizes a bacterial protein called flagellin and requires only MyD88 for signal transduction Using flagellin as a stimulus, we found that pre-stimulation of the cornea provided robust protection against microbial keratitis, including those caused by flagellin-bearing *Pseudomonas* (P.) *aeruginosa* or unrelated pathogens such as fungi (Gao et al., 2011a; Gao et al., 2010; Kumar et al., 2010a; Kumar et al., 2008; Kumar et al., 2007). Flagellin has also been reported to induce protection against lethal radiation and chemicals in mice and monkeys (Burdelya et al., 2008; Vijay-Kumar et al., 2008b), to restore antibiotic-impaired innate immune defenses (Kinnebrew et al., 2010), and to protect mice from acute *Clostridium difficile* colitis (Jarchum et al., 2011). Moreover, the flagellin-TLR5 axis, but not the Ipaf pathway (Miao et al., 2006; Miao et al., 2007), exhibits several distinctive properties: 1) more potent stimulation of mucosal ECs compared to immune cells such as dendritic cells and macrophages (Uematsu and Akira, 2009), 2) unique expression of anti-inflammatory genes (such as IL-1Ra, but not IL-1β) (Carvalho et al., 2010; Vijay-Kumar et al., 2008b), and 3) preservation of epithelial barrier function (Jarchum et al., 2011; Kinnebrew et al., 2010). While flagellin or its derivatives have great potential as a prophylactic and/or therapeutic reagent, its clinical application faces many obstacles due to its potential ability to alter the cell's response to other adverse challenges, its untested usability in repeated applications on the airway and cornea or through systematic injections, and the generation of antibodies in the host that neutralize its effects *in vivo* (Campodonico et al., 2010; Prince, 2006). Hence, harnessing the flagellin-induced protective power requires much more insight into the underlying mechanisms of cell reprogramming.

In this chapter, we utilized animal model of *P. aeruginosa* keratitis to verify the expression of IRF1 and its target gene CXCL10, and elucidate its role in protective effects of flagellin-induced protection of the cornea. Our results suggest that the IRF1-CXCL10 axis plays

a role in flagellin-induced corneal protection by accelerating the recruitment or proliferation of IFNγ-secreting NK cells into the cornea.

3.3   MATERIALS AND METHODS

Reagents and antibodies

Anti-human IRF1, and anti-BD2 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-β-actin and anti-mouse CXCL10 antibodies were purchased from Sigma (St. Louis, MO) and Peprotech (Rocky Hill, NJ), respectively. Anti-NK1.1 was purchased from eBiosciences (San Diego, CA). Anti-IFNγR2 was purchased from R&D Systems (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA). FITC-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Defined keratinocyte serum-free medium (DK-SFM), were purchased from Invitrogen (Carlsbad, CA). Keratinocyte basal medium (KBM) was purchased from BioWhittaker (Walkersville, MD).

Animals

Wild-type (WT) C57BL6 (B6) mice (8 weeks of age; 20–24 g weight) and IRF1-/-mouse breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). IRF1-/-mouse were bred in-house, and their pups were subjected to genotyping before use. All investigations conformed to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health, and the guidelines of the Animal Investigation Committee of Wayne State University.

Bacterial strains and flagellin

Two strains of *P. aeruginosa* PAO1 and ATCC 19660, were used in the study. ATCC 19660, a toxic strain, can infect the B6 mouse corneas at 10^4 cfu per cornea and cause severe keratitis and corneal perforation at about day 5, while 10^8 cfu of PAO1 are required for mild

keratitis. To illustrate the profound protection induced by flagellin we have been using ATCC 19660. However, our interest is in the early innate immune response in the cornea, particularly the epithelium, which has been shown to play a key role in innate immunity and in shaping adaptive immunity as well. ATCC 19660 usually causes epithelial cell death *in vitro* and epithelial sheet loss *in vivo*. To that end, we had to use PAO1 with 10 fold increase in cfu (10^5) to challenge B6 mouse corneas and assess host responses at 6 h by isolate epithelial cells for PCR analysis or immunohistochemistry. Flagellin was prepared from PAO1 using a previously described method (Zhang et al., 2003b). *P. aeruginosa* were grown in tryptic soy broth (Sigma-Aldrich) at 37℃ until absorbance at 600 nm reached O.D. 0.5. The bacterial culture was centrifuged at 6000 × *g* for 10 min. Bacteria were washed in PBS and resuspended in PBS and then used to infect mouse corneas at 10^5 for PAO1 and 10^4 cfu for ATCC19660, respectively.

ELISA measurement of cytokines

To measure mouse CXCL2 and CXCL10, corneal extracts were prepared by homogenization in 200µl phosphate-buffered saline (PBS) with a glass micro tissue grinder, followed by centrifugation at 14,000 × g for 10 min. Protein concentration of the corneal lysate was determined by Micro BCA™ protein assay kit (Pierce) and equal amounts of protein was used to perform ELISA according to the manufacturer's instructions (R & D Systems).

RNA isolation, RT-PCR and Real-time PCR

Total RNA was isolated from mice corneal epithelia or whole cornea using RNeasy Mini Kit (Qiagen, Valencia, CA) according to each manufacturer's instructions. 1 µg of total RNA was reverse-transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). cDNA was amplified by PCR using primers for mouse IFNγ, IRF1, CXCL10, CXCL2, BD3, and GAPDH (Table 1). The PCR products and internal control GAPDH were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide. Stained gels were captured using a digital camera and band intensity was quantified using 1D Image Analysis Software

(EDAS 290 system; Eastman Kodak, Rochester, NY). Real-time PCR was conducted using the Power SYBR Green PCR Master Mix (AB Applied Biosystems, Carlsbad, CA), based on expression of GAPDH, on the StepOnePlus Real-Time PCR System (AB Applied Biosystems, Carlsbad, CA).

PMN infiltration assay

Measurement of myeloperoxidase (MPO) activity was employed to determine PMN infiltration in the cornea as previously described.(Kumar et al., 2008) The corneas were excised from enucleated eyes at 5 dpi and homogenized in 1 ml of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). The samples were then subjected to four freeze-thaw cycles, followed by centrifugation at 16,000 × g for 20 min. Each supernatant was mixed with 50 mM phosphate buffer (pH 6.0) containing 16.7 mg/ml O,O-dianisidine hydrochloride and 0.0005% hydrogen peroxide at a 1:30 ratio to obtain a total volume of 3 ml. The change in absorbance at 460 nm was monitored continuously for 5 min. The results were expressed in units of MPO activity/cornea.

Bacterial CFU determination in the cornea

Corneas (n=5 to 7/group) from PA -infected mice were collected, and the numbers of viable bacteria were determined by plate count method. Individual corneas were homogenized in sterile PBS, and aliquots (100 µl) of serial dilutions were plated onto *Pseudomonas* isolation agar (BD biosciences) plates in triplicate. The plates were incubated overnight at 37℃ and bacterial colonies were counted. The results are expressed as the mean number of cfu/cornea.

Immunohistochemistry

Mouse eyes were enucleated and embedded in Tissue-Tek (Miles Inc., Elkhart, IN) optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Sections (10-µm thick) were cut and mounted to poly-lysine-coated glass slides. After a 10-minute fixation in 4% paraformaldehyde, slides were blocked with 10 mmol/L sodium phosphate buffer containing 2%

BSA for 1 hour at room temperature. Sections were then incubated with primary antibody for CXCL10, 5 µg/mL. For fluorescence microscopy, the slides were incubated with anti-rabbit IgG conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:100), and then mounted with Vectorshield mounting medium containing DAPI mounting media. Controls were similarly treated, but the primary antibody was replaced with rabbit IgG.

Statistical analysis

An unpaired, two-tailed Student t test was used to determine statistical significance for data from bacterial count, cytokine ELISA, MPO assay, real-time PCR. Mean differences were considered significant at a P value of <0.05. Experiments were repeated at least twice to ensure reproducibility.

3.4   RESULTS

IRF1 Deficiency Dramatically Reduced Pseudomonas-induced CXCL10 Expression in B6 Mouse Corneal Epithelium

Having shown the dramatic effects of flagellin pretreatment on *P. aeruginosa* PAO1-induced IRF1 expression in human CECs, we next investigated if flagellin pretreatment can also dampen IRF1 expression *in vivo* in mouse CECs in response to PAO1 at 6 hpi (Fig. 12). Previously we established an animal model demonstrating that pre-exposure of the mouse cornea to flagellin suppressed infection-induced inflammation and enhanced pathogen clearance, rendering it resistant to bacterial and fungal infection (Gao et al., 2011a; Kumar et al., 2010a). Surprisingly, unlike *in vitro* cultured human CECs, we observed augmented expression of IRF1 by flagellin pretreatment in PAO1 infected corneas (Fig. 12A). To assess the consequences of altered expression of IRF1, we evaluated the expression of the IRF1 target gene CXCL10 in comparison with two known flagellin reprogrammed genes, CXCL2 (MIP-2, a

homolog of human IL-8) and mBD3 (a human homolog of inducible hBD2) in WT and IRF1 knockout mice. The expression patterns of CXCL10 in these mice were consistent with IRF1 expression except a faint band that can be detected in IRF1-/- mice by RT-PCR, suggesting additional pathways to induce CXCL10 expression in response to PAO1. Flagellin-augmented CXCL10 expression in PAO1-exposed WT, but not IRF1-/- mice CECs was verified with real time PCR showing 32 and 47 fold increases in CXCL10 mRNA levels in infected WT CECs without or with flagellin pretreatment, respectively. There was no significant increase of CXCL10 in PAO1-infected corneas of IRF1-/- mice. As a control, the expression pattern of mBD3 was the same in WT and IRF1-/- mice (Fig. 12B).

We next investigated CXCL10 expression and distribution in the corneas in response to flagellin pretreatment and PAO1 infection using immunohistochemistry with epifluorescence microscopy to show whole cornea and confocal microscopy to focus on epithelia (Fig. 13). Consistent with mRNA levels, no expression of CXCL10 was detected in uninfected corneas of WT and IRF1-/- mice (Fig. 13: A-C and J-L). PAO1 infection at 6 h induced CXCL10 expression in WT **(**Fig. 13: D-F) but not in IRF1-/- (Fig. 13: M-O) mice. Flagellin pretreatment greatly increased CXCL10 immunoreactivity in corneal epithelia of WT (Fig. 13: G-I with the insert i as the isotype control). No CXCL10 staining was observed in epithelium of IRF1-/- mice (Fig. 13: P-R). Hence, IRF1 is required for PAO1-induced and flagellin-augmented CXCL10 expression in mouse CECs.

Flagellin-induced Corneal Protection against Pseudomonas is Reduced in IRF1-/- Mice

To examine the role of IRF1 on *P. aeruginosa* keratitis, we used a well-accepted *P. aeruginosa* keratitis model using a toxic strain (ATCC 19660) which causes severe corneal keratitis and perforation, allowing better assessment of the protective effects of flagellin. As we showed previously (Kumar et al., 2008), 10^4 cfu ATCC 19660/cornea caused severe inflammation in WT B6 mice with high bacterial burden and pretreatment with 500ng

flagellin/eye for 24 hrs resulted in total protection with no sign of inflammation and no viable bacteria at 3 dpi (Fig. 14). The corneas of IRF1-/- mice at 3 dpi exhibited signs of perforation which usually occurs in WT B6 mice at 5 dpi or later, albeit the bacterial burden was comparable to B6 mice (Fig. 14A). Flagellin pretreatment was able to protect the cornea of IRF1-/- mice from perforation with much reduced bacterial burden compared to the controls injected with PBS at 3 dpi (Fig. 14B). Compared to the WT, the protection was much reduced and incomplete. The severe keratitis observed in IRF1-/- mice appeared to be correlated to PMN infiltration while expression of CXCL2 was correlated to bacterial burden in these mice (Fig. 14C and 14D). Thus, IRF1-/- mice are susceptible to *P. aeruginosa* with dimished flagellin-induced protection.

Neutralization of CXCL10 Activity Impedes the Protective Properties of Flagellin Pretreatment against PA Infection

How might IRF1 influence corneal innate immunity and flagellin induced protection? As CXCL10 was a target gene of IRF1 and upregulated more apparently in protected corneas, we reasoned that it could play a role as an effector of IRF1 in bacterial keratitis. To test this hypothesis, we injected into the mouse subconjunctival space an anti-CXCL10 neutralizing antibody or isotype rabbit IgG 4 h prior to flagellin/PBS pretreatment, followed by *P. aeruginosa* infection. At 3 dpi, we assayed viable bacterial count, MPO activity and CXCL2 expression in infected corneas (Fig. 15). CXCL10 neutralization did not significantly increase bacterial burden, PMN infiltration and CXCL2 expression in infected corneas at 3 dpi in PBS pretreated WT B6 mice. However, in flagellin-pretreated corneas CXCL10 neutralization resulted in visible infection and inflammation (Fig. 15A), significant increases in bacterial count (Fig. 15B), MPO activity (Fig. 15C) and CXCL2 expression (Fig. 15D) compared to anti-rabbit IgG-injected controls. However, compared to the control, PBS-pretreated corneas, flagellin-induced protection remained apparent. These results support the role of CXCL10 in mediating flagellin-induced corneal protection in the mouse model of PA keratitis.

Flagellin-augmented CXCL10 Expression and protection involve IFNγ and NK cells in the corneas

There was a major disparity between *in vitro* and *in vivo* results regarding IRF1 expression in flagellin-pretreated CECs in response to *P. aeruginosa:* human CECs *in vitro* flagellin pretreatment dampened IRF1 expression, whereas *in vivo* infection-induced IRF1 expression was further augmented. This disparity could be due to human versus mouse CECs and/or to the pathogen used, PAO1 versus ATCC 19660. We would argue that the disparity is primarily due to the response of other cells such as infiltrated innate immune cells that are lacking in the cell culture setting. We hypothesized that IFNγ, the strongest inducer of IRF1 and CXCL10, secreted from infiltrating NK cells (Souza-Fonseca-Guimaraes et al., 2012), is the missing link in cultured human CECs. To test this hypothesis, we first blocked IFNγ function by injecting anti-IFNγR2 neutralizing antibodies (2.5μg per cornea) subconjunctivally 4h prior to flagellin pretreatment, followed by ATCC 19660 inoculation. As shown in Figure 16, IFNγR2 neutralization increased the severity of keratitis and reduced flagellin-induced protection (Fig. 16A). Immunohistological staining of IFNγR2-neutralized and infected corneas revealed that while isotype IgG had no effects on CXCL10 expression, IFNγR2 neutralization attenuated infection-induced and flagellin-augmented expression of CXCL10 (Fig. 16B) in epithelia; there appeared a large influx of immune cells that expressed CXCL10 in PA-infected, IFNγR2-neutralized corneas. Moreover, IFNγR2 neutralization resulted in significantly reduced bacterial clearance (Fig 16C), increased neutrophil infiltration (Fig. 16D), and elevated CXCL2 (Fig. 16E) expression compared to isotype IgG injected controls.

Using the same approach, we depleted NK cells with antibody against NK1.1 (Fig. 17). Similarly, the isotype matched antibody had no detectable effects on PA keratitis and on flagellin-induced protection. Depletion of NK cells resulted in an increase in the severity of PA keratitis and impaired flagellin-induced protection, including increased opacity (Fig. 17A),

bacterial burden (Fig. 17B), PMN infiltration (Fig. 7C), and CXCL2 expression (Fig. 17D). As expected, the infection-induced and flagellin-augmented expressions of IFNγ, IRF1, and CXCL10 was greatly suppressed by NK cell depletion as detected by realtime PCR (Fig. 17E) and by ELISA for CXCL10 (Fig. 17F). These results support the importance of IFNγ and NK cells in the enhanced expression of CXCL10 and corneal innate immunity against *P. aeruginosa in vivo*.

3.5   DISCUSSION

Our study of screening for TFs differentially expressed in PAO1-infected primary HCECs revealed that IRF1 expression was increased in response to bacterial challenge but pre-exposure of cells to a low dosage of flagellin for 24h prior to PAO1 challenge caused a dramatic decrease in IRF1 expression.   While our *in vitro* study of cultured human primary CECs revealed suppressive effects of flagellin on IRF1 expression, our *in vivo* study using B6 mice indicates an enhanced expression of IRF1 as well as its target gene CXCL10 in flagellin-pretreated CECs exposed to PAO1 for 6 h. The expression of CXCL10 was primarily in epithelia. To understand the role of IRF1 in infectious keratitis, we used the mouse model of *P. aeruginosa* keratitis using ATCC 19660 as pathogen. Our results indicated that IRF1 deficiency increased cornea susceptibility to *P. aeruginosa* infection and abolished flagellin-induced protection against this common keratitis-causing pathogen in B6 mice. To assess if CXCL10 is an effector gene of IRF1, we neutralized CXCL10 and found indeed that CXCL10 is required for optimal flagellin-induced protection. To understand how the IRF1-CXCL10 axis is regulated, we blocked IFNγ signaling by neutralizing its receptor and found that the infection-induced and flagellin-augmented epithelial expression of CXCL10 was dampened in anti-IFNγR2-, but not the control antibody-treated corneas. IFNγR2 neutralization also increased the severity of *P. aeruginosa* keratitis and significantly reduced flagellin-induced protection. Moreover, as NK cells are known to be the major source of IFNγ in the cornea (Souza-Fonseca-Guimaraes et al.,

2012), depletion of NK cells had similar effects as IFNγR2 neutralization on the severity of *P. aeruginosa* keratitis and on flagellin-induced protection. The upregulated expressions of IFNγ, IRF1, and CXCL10, in NK depleted corneas were significantly dampened. Taken together, our data showed that infection-induced and flagellin-augmented expression of CXCL10 in CECs is IRF1-dependent and plays a major role in the elimination of inoculated *P. aeruginosa* and that NK cells and their production of IFNγ are critical for flagellin-induced protection and innate defense against *P. aeruginosa* in the cornea.

IRF1 is one of the members of the IRF family of TFs consisting of nine members: IRF1-9 (Colonna, 2007; Honda and Taniguchi, 2006b; Savitsky et al., 2010b). IRFs were first characterized as IFN-inducible TFs, but recent studies have revealed their involvement in the regulation of gene expression in responses triggered by TLRs (Battistini, 2009; Savitsky et al., 2010a). In the mouse model of *P. aeruginosa* infection, we showed that *P. aeruginosa* infection resulted in the upregulation of IRF1 and this upregulation, in contrast to *in vitro* data, was further augmented by flagellin pretreatment. This upregulation is important for controlling *P. aeruginosa* keratitis. While it is mostly studied in viral infection, IRF1 has been shown as a master regulator of mycobacteria-induced immunopathology, involved in the development of centrally necrotizing granulomatous lesions in the lung (Aly et al., 2009; Yamada et al., 2002). Interestingly, our results showed that IRF1 mediated gene expression plays a protective role in *P. aeruginosa* keratitis. IRF1 deficiency resulted in much severe keratitis and the corneas of IRF1-/- mice were perforated within 3 days while in WT B6 mice the pathology will need 5 or more days to be developed. Interestingly, while PMN infiltration is significantly higher than the WT mice, the bacterial burden and the levels of CXCL2 are similar between IRF1-/- and WT B6 mice, suggesting excessive infiltration of PMNs are a major pathogenic factor for cornea tissue destruction. Importantly, although it is significantly compromised in IRF1-/- mice, the flagellin induced protection remains strong in IRF1-/- mice as the severity of keratitis, evidenced by

increased corneal opacity, bacterial burden, PMN infiltration and the expression of CXCL10 in IRF1-/- mice compared to WT B6 mice. Our study further revealed that the epithelial expressed CXCL10 is a major effector of IRF1-mediated protection in the corneas.

CXCL10 is a member of the interferon-inducible tripeptide motif Glu-Leu-Arg-negative (ELR⁻) CXC chemokines (Cole et al., 2001a). It, along with CXCL9 and CXCL11, signals through a G-protein-coupled receptor, CXCR3, and functions primarily in the recruitment of activated CD4$^+$ and CD8$^+$ T cells, NK cells, and plasmacytoid dendritic cells to sites of infection and inflammation (Cao and Liu, 2007; Mohan et al., 2005). In addition to their roles in leukocyte recruitment, CXCL9, CXCL10, and CXCL11 exert direct antimicrobial effects that are comparable to the effects mediated by cationic antimicrobial peptides, including defensins (Cole et al., 2001b). Our study showed that flagellin pretreatment greatly augmented infection-induced upregulation of CXCL10 in CECs in an IRF1-dependent manner.

The epithelium-expressed CXCL10 may play a key role in bacterial clearance due to its antimicrobial properties at the early stage of infection. In our epithelial scratch model, pathogens were inoculated on epithelial injury sites with intact or minimally injured basement membrane. It was reported that the basement membrane possesses physical barrier effects, resulting in the trapping of pathogens in epithelium for several hours (Alarcon et al., 2009; Alarcon et al., 2011), allowing the elevated CXCL10, along with other antimicrobial peptides such as β-defensins (Augustin et al., 2011) and LL-37 (or cathelicidin-related antimicrobial peptide (CRAMP)) (Huang et al., 2006; Kumar et al., 2010a), to kill these invading pathogens before they reach the stromal layer. Indeed, neutralizing CXCL10 significantly diminished but not totally abolished flagellin induced protection, including much reduced bacterial burden, PMN infiltration and CXCL2 expression. Hence, we conclude that flagellin-induced expression of CXCL10 contributes to innate defense activated by topical flagellin and epithelium-expressed CXCL10 is involved in pathogen clearance in the corneas.

Surprisingly, in contrast to *in vitro* results, flagellin pretreatment in B6 mice further enhanced the expression of IRF1. We suspected that this disparity is due to the molecular and/or cellular component(s) that are present in the mouse cornea and/or tears *in vivo* but not in cultured human CECs. One such factor is IFNγ which is known not to be expressed in epithelial cells. Our recent study of genome-wide cDNA array revealed a large number of interferon-induced genes in the epithelial cells of flagellin-pretreated and PAO1-infected B6 mouse corneas (Gao et al., 2013).   Using IFNγ receptor neutralizing antibody, we showed that inactivation of IFNγ signaling increased the severity of *P. aeruginosa* keratitis in the control corneas and significantly reduced flagellin-induced protection to a level similar to non-treated corneas. As expected, infection-induced and flagellin-augmented expressions of IRF1 and CXCL10 were dampened in IFNγ receptor-neutralized corneas.

What are the cellular sources of IFNγ in infected corneas? NK cells are known to produce large amount of IFNγ (Goodridge et al., 2009; Quaranta et al., 2007). In a parallel study, we demonstrated that epithelial expressed CXCL10 may target CXCR3 expressing NK cells (Liu et al, under review in Mucosal Immunology). We then used neutralizing antibody to deplete NK cells by subconjectival injection and assess its effects on *P. aeruginosa* and on the expression of IFNγ, IRF1 and CXCL10, an axis of IFNγ signaling pathway. While depletion of NK cells had similar effects on the severity of *P. aeruginosa* keratitis as IFNγ receptor neutralization, NK depletion indeed significantly affected the expression of all 3 genes. Intriguingly, the most affected is CXCL10, the effector of the axis. The suppression of CXCL10 expression in flagellin-pretreated and *P. aeruginosa*-infected corneas by IFNγ neutralization and NK cell depletion may provide an explanation for diminished protection from microbial keratitis in these corneas. Nevertheless, our results indicate that the involvement of infiltrated NK cells, through production of IFNγ, in mediating IRF1 expression in the cornea. In cultured cells, IRF1

is induced by NF-κB, which was blunted by prolonged exposure to flagellin. Lack of IFNγ in cell culture resulting in IRF1 being reprogrammed *in vitro*, but not *in vivo*.

Taken together, our data suggests that IRF1 is an important responsive TF in corneal innate immunity and in flagellin-induced protection mediated by CXCL10 expression. The much elevated epithelial production of CXCL10 may contribute to flagellin-elicited corneal protection by recruiting NK cells to the site of infection and by acting as an AMP that directly kills invading pathogens. Moreover, NK cells that infiltrated in the cornea in the early phase of infection are required for sustaining CXCL10 expression through production of IFNγ that induces IRF1 expression through an NFκB independent mechanism, and thus play an important role in corneal innate defense and more apparently in flagellin induced mucosal protection against *P. aeruginosa* infection.
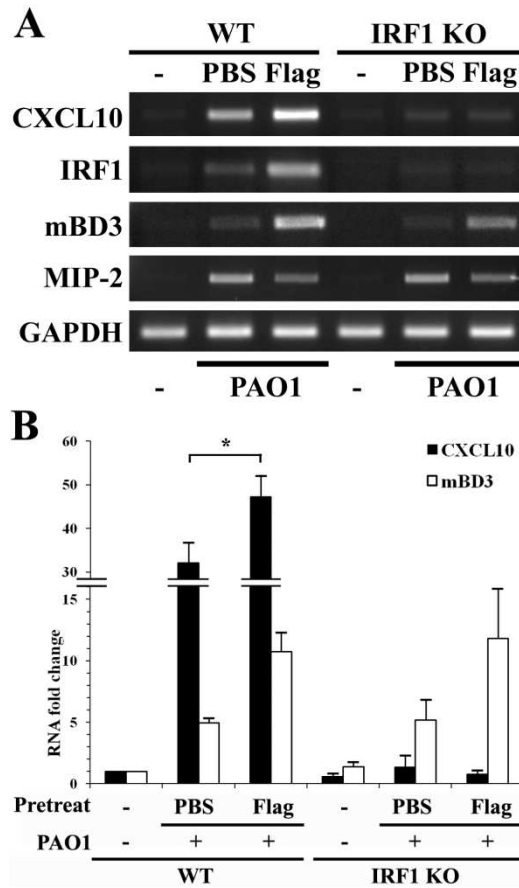
Figure 12. Detection of CXCL10 expression in corneal epithelia of B6 WT and IRF1^{-/-} mice.
WT and IRF1^{-/-} B6 mice were topically pretreated with 500 ng flagellin in 5 μl PBS, a dosage we showed to induce protection in B6 mice, or 5 μl PBS for 24h, and then infected with PAO1 at 10^5 cfu for 6h. The epithelium was collected and RNA isolated for semi-quantitative RT-PCR (A) and quantitative real-time PCR (B) analysis of CXCL10, mBD3 and/or MIP-2. GAPDH was used as the internal control. P values were generated using unpaired Student's *t* test (∗ P<0.05). The figure is a representative of six corneas (***n*** = 3 mice) per condition.
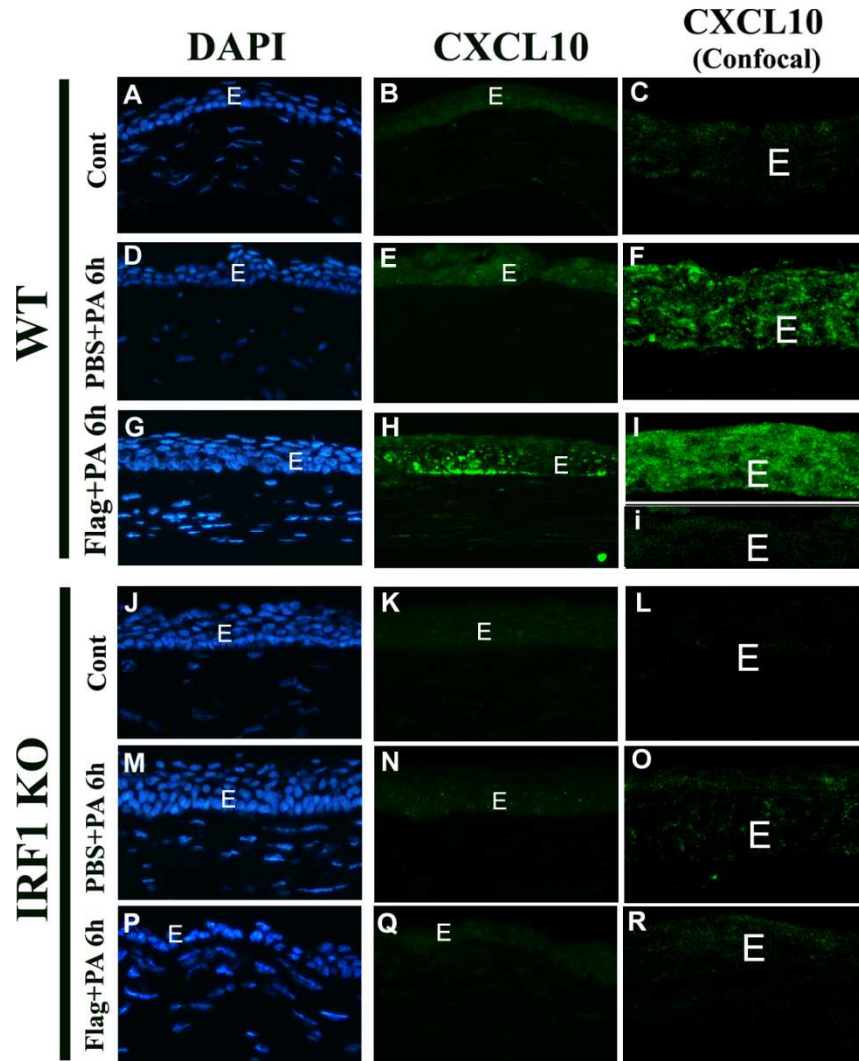
**Figure 13. Distribution of CXCL10 expression in corneal epithelia of B6 WT and IRF1-/-
mice.** WT and IRF1-/- B6 mice were topically pretreated with 500 ng flagellin in 5 µl PBS, a
dosage we showed to induce protection in B6 mice, or 5 µl PBS for 24h, and then infected with
PAO1 at 10^5 cfu for 6h. The whole cornea was snap-frozen in OCT, sectioned, stained with
mouse anti-CXCL10 antibody and examined with fluorescence microscopy (magnification at
40x, DAPI used to stained the nuclei) or confocal microscopy (maginification at 80x). The figure
is a representative of six corneas (**n** = 3 mice) per condition.
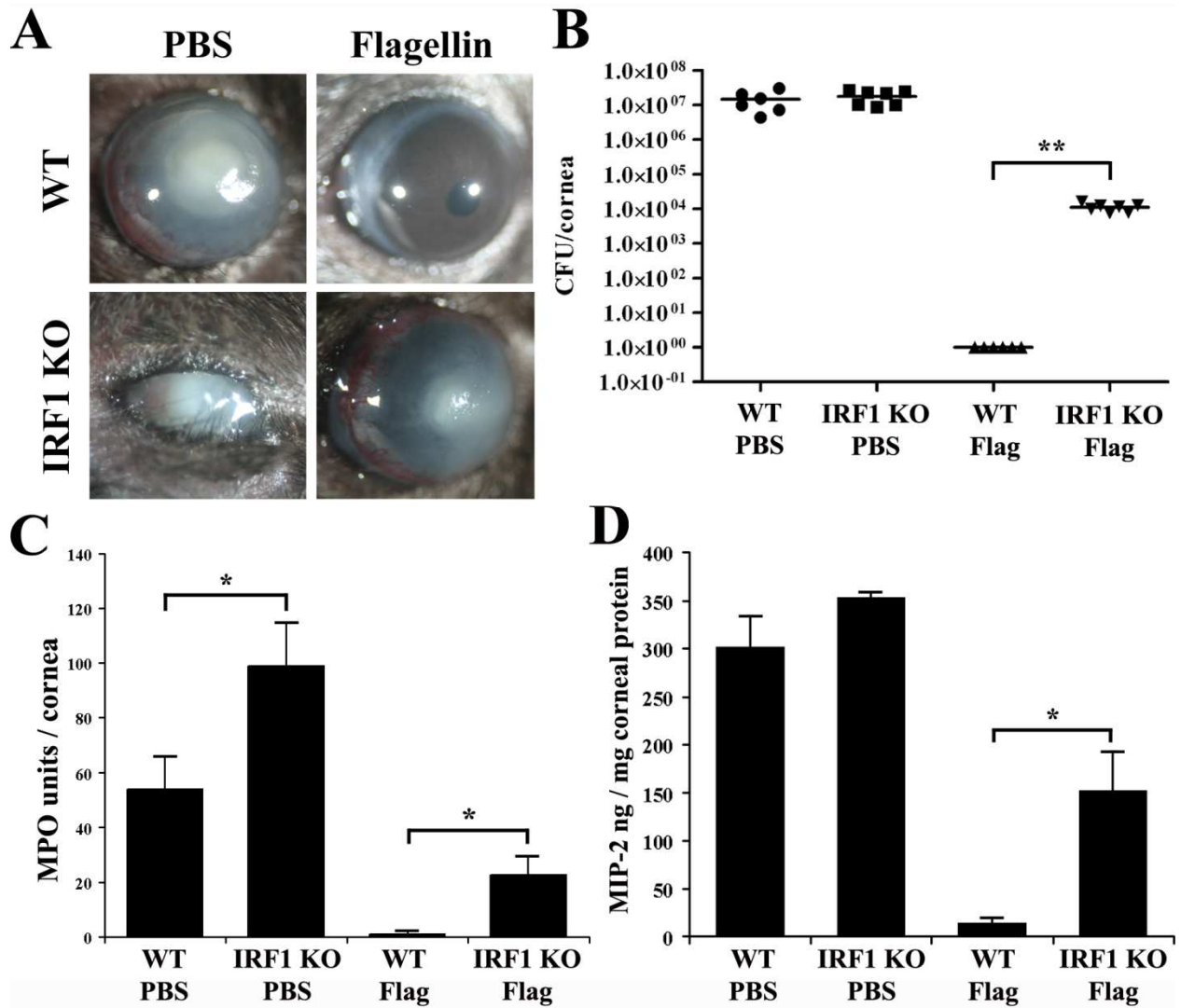
Figure 14. IRF1 deficiency increased the severity of *Pseudomonas* keratitis and attenuated flagellin-induced protection in B6 mice. WT or IRF1$^{-/-}$ B6 mice were topically pretreated with 500 ng flagellin or PBS for 24h, and then infected with ATCC 19660 at 10^4 cfu for 3 days (A). The whole cornea was collected for viable bacterial count (B), MPO activity assay and MIP-2 ELISA (C). P values were generated using unpaired Student's *t* test (* P<0.05; ** P<0.01). The figure is a representative of three independent experiments.
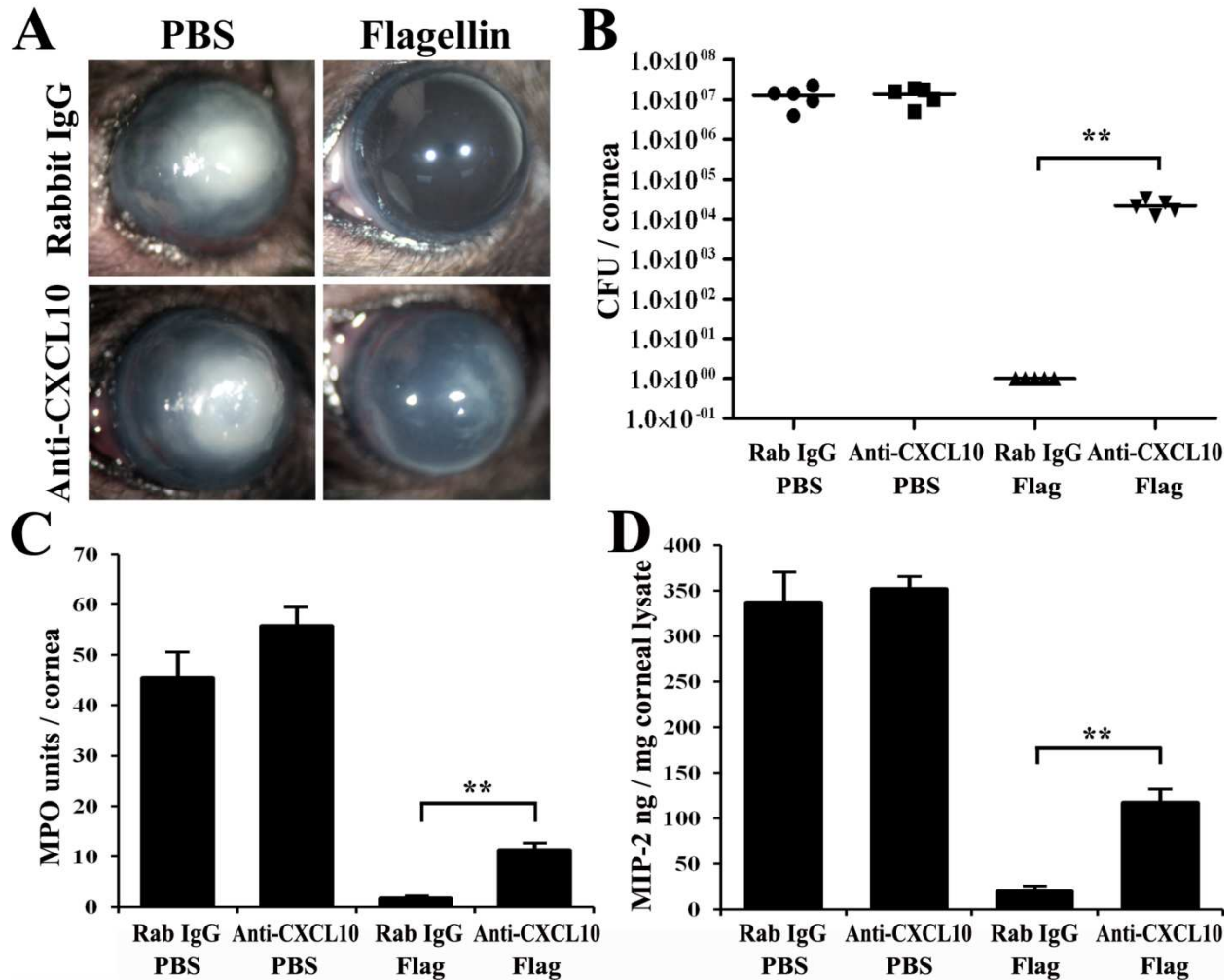
Figure 15. Neutralization of CXCL10 activity in the cornea attenuated flagellin-induced protection in B6 mice. WT mice were injected with neutralizing anti-CXCL10 antibody (3 ug in 5 µl) or isotype rabbit IgG 24h prior to topical PBS or flagellin pretreatment, and then infected with ATCC19660 at 10^4 cfu. At 3 dpi the corneas were photographed (A) and then excised and homogenized for viable bacterial count (B), MPO activity assay and MIP-2 ELISA (C). P values were generated using unpaired Student's *t* test (** P<0.01). The figure is a representative of two independent experiments.
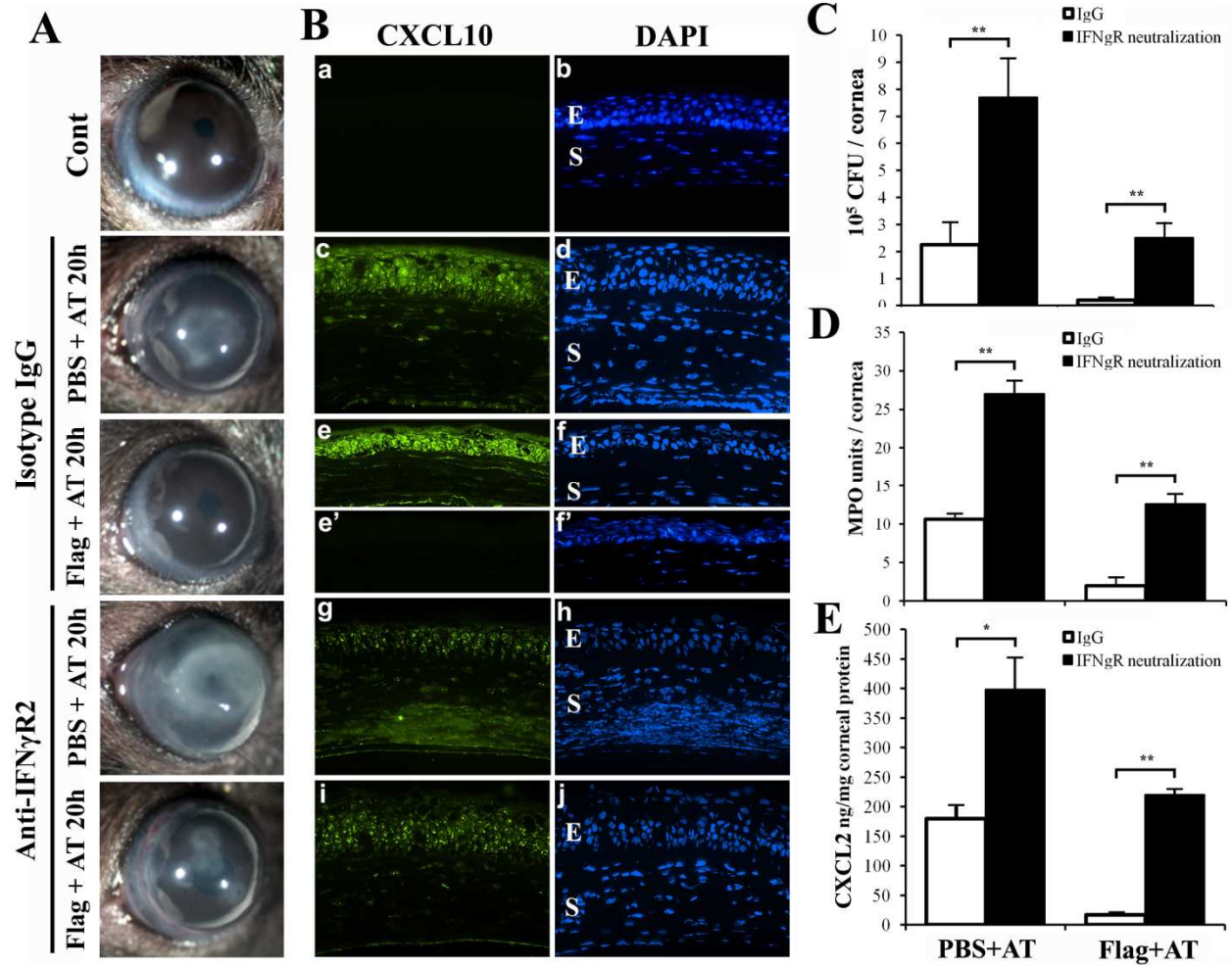
Figure 16. Neutralization of IFNγR2 activity in the cornea attenuated epithelial CXCL10 production and flagellin-induced protection in B6 mice. WT mice were injected with neutralizing anti-IFNγR2 antibody (3 ug in 5 µl) or isotype rabbit IgG 24h prior to topical PBS or flagellin pretreatment, and then infected with ATCC19660 at 10^4 cfu. At 20h post-infection the corneas were photographed (A) and then harvested for immunohistochemistry analysis of CXCL10 expression (B) or bacterial count (C), MPO activity assay (D) and MIP-2 ELISA (E). P values were generated using unpaired Student's *t* test (∗ P<0.05; ∗∗ P<0.01). The figure is a representative of 6 corneas (***n*** = 3 mice) per condition.
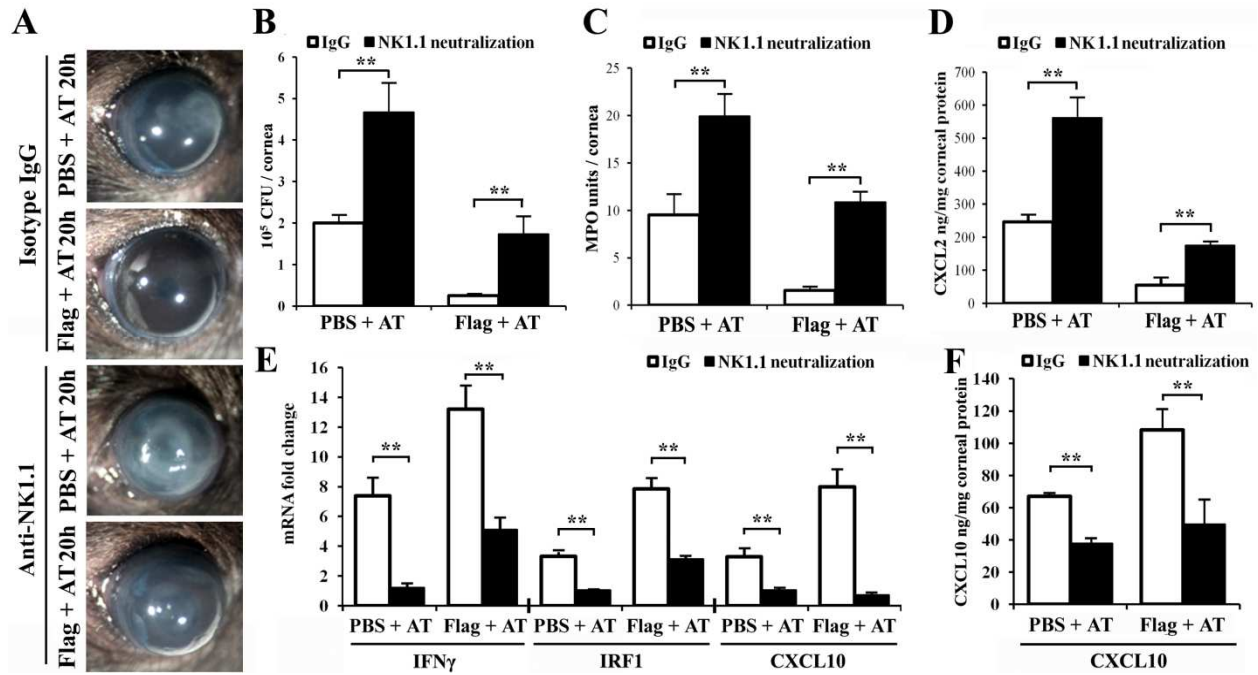
Figure 17. Neutralization of NK cells in the cornea blocked IFNγ, IRF1, and CXCL10 expression and compromised flagellin-induced corneal protection in B6 mice. WT mice were injected with NK cell neutralizing anti-NK1.1 antibody (3 ug in 5 μl) or isotype rabbit IgG 24h prior to topical PBS or flagellin pretreatment, and then infected with ATCC19660 at 10^4 cfu. At 20h post-infection the corneas were photographed (A) and then harvested for bacterial count (B), MPO activity assay (C), MIP-2 (D) and CXCL10 (F) ELISA, real-time PCR analysis of IFNγ, IRF1, and CXCL10 expression (E). P values were generated using unpaired Student's *t* test (** P<0.01). The figure is a representative of 6 corneas (***n*** = 3 mice) per condition.

Table 4. Mouse primer sequences used for PCR

Gene	Primer	Sequence (5'→3')	Product size (bp)
IFNγ	Forward	ACGGCACAGTCATTGAAAGC	198
	Reverse	TGCTGATGGCCTGATTGTCTT	
IRF1	Forward	CAGAGGAAAGAGAGAAAGTCC	208
	Reverse	CACACGGTGACAGTGCTGG	
CXCL10	Forward	ATGAACCCAAGTGCTGCCGTC	297
	Reverse	TTAAGGAGCCCTTTTAGACCTTT	
CXCL2	Forward	AGTTTGCCTTGACCCTGAAGCC	466
	Reverse	TGGGTGGGATGTAGCTAGTTCC	
BD3	Forward	GCTTCAGTCATGAGGATCCATTACCTTC	318
	Reverse	GCTAGGGAGCACTTGTTTGCATTTAATC	
GAPDH	Forward	ACCACAGTCCATGCCATCAC	452
	Reverse	TCCACCACCCTGTTGCTGTA	

CHAPTER 4

ACTIVATING TRANSCRIPTION FACTOR 3 IN FLAGELLIN-INDUCED REPROGRAMMING: POTENTIAL ROLE IN THE REGULATION OF INFLAMMATION AND BACTERIAL CLEARANCE IN CORNEA AGAINST *PSEUDOMONAS AERUGINOSA* INFECTION

4.1   ABSTRACT

We previously identified Activating Transcription Factor (ATF) 3 as one of the most drastically affected genes by flagellin pretreatment in *P. aeruginosa* challenged human corneal epithelial cells (CEC). Next we sought to understand the functional role of ATF3 in mucosal innate protection against infections *in vivo.* Consistent with *in vitro* results, flagellin augmented the *P. aeruginosa*-induced epithelial expression of ATF3 in CECs in WT at 6 hpi. ATF3 deficiency markedly increased the severity of *P. aeruginosa* keratitis, indicated by significantly increased bacterial count, neutrophil infiltration and CXCL2 expression. In addition, ATF3-null mice displayed compromised flagellin-induced corneal protection compared to PBS-pretreated ATF3-/- littermates at 3 dpi. Together, our results indicate that ATF3 plays a role in the corneal innate immune response by regulating inflammation and bacterial clearance, and ATF3 is indispensable to maintain the protective properties of flagellin against bacterial keratitis.

4.2   INTRODUCTION

Epithelial innate immune defense against microbial pathogens rely on membrane-associated pattern recognition receptors. Toll-like receptors (TLRs) recognize pathogens extracellularly by binding to specific microbial patterns occurring at the cell surface (Tamura et al., 2008b). TLRs recognize microbial lipids (TLR1, -2, -4, -6, and -10), viral or bacterial DNA (TLR9), or viral single- or double-stranded RNA (TLR3 and TLR7/8), and bacterial protein (flagellin/TLR5). The transcriptional program that is activated by pathogen exposure involves a plethora of molecular components, including cytokines, chemokines, and cell cycle/apoptosis regulatory proteins (Huang et al., 2001). In these transcriptional events, the biological roles and activation of transcription factors belonging to the interferon (IFN) regulatory factors (IRFs) and nuclear factor of κB (NF-κB) families have been previously studied.

Activating transcription factor 3 (ATF3), a 21-kDa (181 amino acid residues) nuclear protein belongs to the basic-region leucine zipper (bZIP) transcription factor family of proteins. ATF3 binds the ATF/cAMP response element (CRE) of many viral and cellular promoters to regulate its downstream target genes. Several genes have been identified as ATF3 target genes, including CCNE2 (Cyclin E2), CD82 (KAI1), DDIT (GADD153), LDLR (LDL receptor), SNAI1, and TP53 genes (Hagiya et al., 2011; Lim et al., 2011; Liu et al., 2011b; Yan et al., 2011). In quiescent cells, ATF3 expression is maintained at low levels (Lu et al., 2006), but it is dramatically induced upon exposure of cells to stress signals, which include cytokines, genotoxic agents, infections, nerve injury, tissue damage or physiological stress (Hai et al., 1999). Emerging evidence has implicated the importance of ATF3 in the host's innate defense against invading pathogens and in ameliorating inflammation. ATF3 induction occurs in response to a wide range of TLRs, as treatment of bone marrow macrophages with lipopolysaccharide (LPS; TLR4), pIC (TLR3), CpG-ODN (TLR9), pIC/CpG-ODN (TLR3 and TLR9), and zymosan (TLR2/6 heterodimer) was shown to significantly elevate ATF3 protein

expression (Whitmore et al., 2007). Accordingly, *atf3*-deficient primary macrophages produced elevated amounts of interleukin (IL)-6, IL-12p40, and tumour necrosis factor (TNF)-α cytokines in response to a range of activated TLRs (Gilchrist et al., 2006; Whitmore et al., 2007). This identified ATF3 as a transcriptional regulator of TLR signaling, possibly through a negative feedback loop response to suppress TLR-mediated cytokine expression in macrophages (Thompson et al., 2009). However, the role of ATF3 in TLR5-mediated inflammatory response has not been previously documented and warrants investigation.

In light of these reports, we predicted the involvement of ATF3 in regulating TLR5-mediated epithelial immune response, and also in flagellin/TLR5-induced cell reprogramming. Our results show that ATF3 is critical to regulate TLR5-mediated inflammatory response and bacterial clearance and is required to maintain the protective properties of flagellin against *P. aeruginosa* keratitis.

4.3   MATERIALS AND METHODS

Reagents and antibodies

Anti-human and anti-mouse ATF3 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-β-actin antibody were purchased from Sigma (St. Louis, MO) and Peprotech (Rocky Hill, NJ), respectively. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA). FITC-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Animals

Wild-type (WT) C57BL6 (B6) mice (8 weeks of age; 20–24 g weight) were purchased from The Jackson Laboratory (Bar Harbor, ME). ATF3-/- mouse were a gift from Dr. Tsonwin Hai of Ohio State University (Allen-Jennings et al., 2002; Zmuda et al., 2010) and bred in-house. All investigations conformed to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health, and the guidelines of the Animal Investigation Committee of Wayne State University.

Bacterial strains and flagellin

Two strains of *P. aeruginosa* PAO1 and ATCC 19660, were used in the study. ATCC 19660, a toxic strain, can infect the B6 mouse corneas at 10^4 cfu per cornea and cause severe keratitis and corneal perforation at about day 5, while 10^8 cfu of PAO1 are required for mild keratitis. To illustrate the profound protection induced by flagellin we have been using ATCC 19660. However, our interest is in the early innate immune response in the cornea, particularly the epithelium, which has been shown to play a key role in innate immunity and in shaping adaptive immunity as well. ATCC 19660 usually causes epithelial cell death *in vitro* and epithelial sheet loss *in vivo*. To that end, we had to use PAO1 with 10 fold increase in cfu (10^5) to challenge B6 mouse corneas and assess host responses at 6 h by isolate epithelial cells for

PCR analysis or immunohistochemistry. Flagellin was prepared from PAO1 using a previously described method (Zhang et al., 2003b). P. aeruginosa were grown in tryptic soy broth (Sigma-Aldrich) at 37℃ until absorbance at 600 nm reached O.D. 0.5. The bacterial culture was centrifuged at 6000 × g for 10 min. Bacteria were washed in PBS and resuspended in PBS and then used to infect mouse corneas at 10^5 for PAO1 and 10^4 cfu for ATCC19660, respectively.

ELISA measurement of cytokines

To measure mouse CXCL2, corneal extracts were prepared by homogenization in 200µl phosphate-buffered saline (PBS) with a glass micro tissue grinder, followed by centrifugation at 14,000 × g for 10 min. Protein concentration of the corneal lysate was determined by Micro BCA^{TM} protein assay kit (Pierce) and equal amounts of protein was used to perform ELISA according to the manufacturer's instructions (R & D Systems).

RNA isolation and RT-PCR

Total RNA was isolated from mice corneal epithelia or whole cornea using RNeasy Mini Kit (Qiagen, Valencia, CA) according to each manufacturer's instructions. 1 µg of total RNA was reverse-transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). cDNA was amplified by PCR using primers for mouse ATF3 and beta-actin (Table 5). The PCR products and internal control GAPDH were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide. Stained gels were captured using a digital camera and band intensity was quantified using 1D Image Analysis Software (EDAS 290 system; Eastman Kodak, Rochester, NY).

PMN infiltration assay

Measurement of myeloperoxidase (MPO) activity was employed to determine PMN infiltration in the cornea as previously described.(Kumar et al., 2008) The corneas were excised from enucleated eyes at 3 dpi and homogenized in 1 ml of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). The samples were

then subjected to four freeze-thaw cycles, followed by centrifugation at 16,000 × g for 20 min. Each supernatant was mixed with 50 mM phosphate buffer (pH 6.0) containing 16.7 mg/ml O,O-dianisidine hydrochloride and 0.0005% hydrogen peroxide at a 1:30 ratio to obtain a total volume of 3 ml. The change in absorbance at 460 nm was monitored continuously for 5 min. The results were expressed in units of MPO activity/cornea.

Bacterial CFU determination in the cornea

Corneas (n=5 to 7/group) from PA -infected mice were collected, and the numbers of viable bacteria were determined by plate count method. Individual corneas were homogenized in sterile PBS, and aliquots (100 μl) of serial dilutions were plated onto *Pseudomonas* isolation agar (BD biosciences) plates in triplicate. The plates were incubated overnight at 37℃ and bacterial colonies were counted. The results are expressed as the mean number of cfu/cornea.

Immunohistochemistry

Mouse eyes were enucleated and embedded in Tissue-Tek (Miles Inc., Elkhart, IN) optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Sections (10-μm thick) were cut and mounted to poly-lysine-coated glass slides. After a 10-minute fixation in 4% paraformaldehyde, slides were blocked with 10 mmol/L sodium phosphate buffer containing 2% BSA for 1 hour at room temperature. Sections were then incubated with primary antibody for mouse ATF3 5 μg/mL. For fluorescence microscopy, the slides were incubated with anti-rabbit IgG conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:100), and then mounted with Vectorshield mounting medium containing DAPI mounting media.

Statistical analysis

An unpaired, two-tailed Student t test was used to determine statistical significance for data from bacterial count, cytokine ELISA, MPO assay, real-time PCR. Mean differences were

considered significant at a P value of <0.05. Experiments were repeated at least twice to ensure reproducibility.

4.4   RESULTS

Flagellin pretreatment enhanced the expression of ATF3 in mouse corneal epithelium

In Chapter 2 we used the transcription factor RT^2 Profiler PCR Array (SAbiosciences) to compare the expression of TFs in human CECs (hCECs) with or without flagellin pretreatment (50ng/ml 24h) in response to *P. aeruginosa* challenge. ATF3 mRNA was upregulated the highest (42 fold) in response to bacterial challenge compared to unstimulated controls. Flagellin pretreatment further elevated ATF3 mRNA expression in response to *P. aeruginosa* to 61.5 fold.

To investigate whether flagellin-pretreatment can also enhance ATF3 mRNA and protein expression in the mouse corneal epithelium *in vivo*, we conducted PT-PCR, Western blot and immunohistochemsitry of mouse corneal epithelium with PBS or flagellin pretreatment for 24 hrs and subsequently rechallenged with *P. aeruginosa* (10^4 cfu, strain ATCC 19660) for 6hrs (Fig. 18). PBS or flagellin pretreatment alone for 24h did not result in altered ATF3 mRNA (Fig. 18A) or protein expression (Fig. 18B). Immunohistochemical analysis showed ATF3 to be expressed in the epithelium at this time point (Fig. 18C). In accordance with the human CEC PCR array results, flagellin-pretreatment enhanced ATF3 expression in response to *P. aeruginosa* challenge.at both mRNA and protein levels by 1.58 fold and 1.51 fold in measured densitometry, respectively (n=3).

ATF3-/- mice exhibit severe inflammation and enhanced expression of CXCL2 and MPO activity

To examine the effects of ATF3 on *P. aeruginosa* keratitis and flagellin-induced corneal protection, ATF3-/- mice were infected with *P. aeruginosa* (ATCC 19660 at 10^4 cfu/cornea) after pretreatment with PBS or 500ng flagellin for 24 hrs (Fig. 19). We previously showed in B6 mice

that 500 ng/eye flagellin was the minimal dosage to induce maximal protection in the cornea (Kumar et al., 2008). Disease progression was monitored daily and all experiments were terminated at 3 dpi as the corneas of ATF3-/- mice exhibited signs of perforation at 3 days post-infection (dpi) (Fig. 19A). ATF3^{-/-} mice displayed more severe keratitis at 3 dpi compared to WT B6 mice and increased PMN infiltration assessed by MPO assay. Bacterial burden was 10 fold higher in PBS pretreated ATF3-/- mice than their WT counterparts (Fig. 19). As shown previously, topical flagellin protected the corneas of WT B6 mice from PA infection. Specifically, no bacteria were recovered and reduced PMN infiltration and CXCL2 expression was markedly diminished in flagellin-pretreated B6 corneas at 3dpi (Fig. 19C). However, flagellin pretreatment failed to protect ATF3-/- corneas from infection, as bacterial count was 10^7 fold higher than flagellin-pretreated WT corneas. In addition, the corneas from these mice were heavily opaque and contained large amounts of PMN infiltration, and elevated expression of CXCL2. However, compared to PBS-pretreated ATF3-/- mice, flagellin-pretreated littermates displayed reduced bacterial burden, PMN infiltration, and CXCL2 concentration, suggesting flagellin-induced protection was only partially attenuated in ATF3-deficient mice. Intriguingly, while flagellin induces similar protection against P. aeruginosa infection in the cornea and in the lung and this protection requires the expression of CRAMP (mouse homolog of LL37), ATF3 knockout was protective in P. aeruginosa lung infection. Comparative analyses of the cornea and the lung infection in ATF3 knockout mice may reveal tissue specific defense mechanisms which currently are lacking. This however is out the scope of this dissertation.

4.5   DISCUSSION

Flagellin pretreatment of the mouse corneal epithelium has a profound effect on the innate immune response of the cornea in against a variety of pathogens (Gao et al., 2011a; Kumar et al., 2010a). We predicted that this profound effect of flagellin is mediated by reprogramming which involves many transcription factors (TF) in corneal epithelial cells, and in

Chapter 3 and 4 we were able to decipher the IRF1-CXCL10 axis as playing a critical role in bacterial killing and clearance in the cornea. However, transcriptional reprogramming in cells does not occur with only a single TF, but rather with multiple TFs. In chapter 2, we used the real-time PCR Superarray to indentify transcription factors IRF1 and ATF3 being reprogrammed by flagellin pretreatment in hCECs. In this Chapter, we sought to understand the role of ATF3 in regulating corneal innate immunity and flagellin-induced CEC reprogramming by flagellin stimulation as ATF3 is an important transcription factor that shapes the host immune response to pathogens (Hai et al., 2010). We found that flagellin pretreatment of the mouse cornea differentially regulated the expression of ATF3 by augmenting its expression, and enhancing the immunity of the corneal epithelium against bacterial infection. Unlike IRF1, the enhanced expression of ATF3 in the mouse corneal epithelium was without the influence of cyto/chemokines from infiltrating cells as the same enhancement was observed in a pure population of hCECs *in vitro*. However, we cannot rule out the influence of autocrine/paracrine factors, such as IL-6 (Hai et al., 1999), that may contribute to enhanced ATF3 expression.

The lack of ATF3 expression *in vivo* proved to be detrimental to corneal innate immunity, evidenced by more severe opacity of the cornea, neutrophil infiltration (5 fold) and CXCL2 (2 fold) expression compared to WT mice. This observation is in accord with previous findings that ATF3 functions as a negative regulator of inflammation and hyperresponsiveness (Gilchrist et al., 2008; Whitmore et al., 2007). In addition, the 10-fold increase in viable bacterial count of ATF3-deficient mice compared WT mice implies that the bacterial killing and clearing function of epithelial and/or infiltrating immune cells in the cornea have been compromised. By 3 dpi neutrophils and macrophages have infiltrated the cornea (Hazlett, 2004b) and previous reports describing the role of ATF3 in immune cell function may explain the uncontrolled inflammatory response in ATF3 knockout mice (Gilchrist et al., 2008; Rosenberger et al., 2008; Whitmore et al., 2007). Maruyama *et al.* demonstrated ATF3 to negatively regulate neutrophil differentiation (Maruyama et al., 2012), and lack of ATF3 may result in neutrophil overproduction in the host in

response to infection. In addition, ATF3 is involved in the negative regulation of TLR signaling pathways by suppressing proinflammatory cytokines in macrophages (Whitmore et al., 2007), which may explain the increase in CXCL2 expression in ATF3-null mice. In epithelial cells bacterial challenge induced the ATF3-dependent stress response and reprogramming of transcriptional response to the pathogen (Tattoli et al., 2012). These findings help us to understand the uncontrolled inflammatory response in the cornea resulting from the lack of ATF3 expression.

Aside from its role as a transcription factor that regulates gene expression, emerging evidence indicates that ATF3 can also regulate cellular functions through mechanisms beyond transcriptional regulation (Yan et al., 2005). ATF3 blocks the ubiquitination and degradation of p53, leading to up-regulation of p53 stability and activity, independent of ATF3 transcriptional activity (Mo et al., 2010; Yan et al., 2005). Furthermore, p53 is known to negatively regulate the activity of NF-κB (Ak and Levine, 2010), and lack of ATF3 may promote p53 inactivity leading to NF-κB hyperactivation, a combination that would inhibit epithelial and immune cell apoptosis, which is an essential mechanism to eliminate pathogens at the early stage of infection without emitting alarm signals (Ashida et al., 2011). These reports may explain the heavy influx and lack of clearance of immune cells and increased bacterial load witnessed in ATF3-null mouse.

In light of the important role of ATF3 in regulating inflammation and bacterial killing, we expected ATF3 to have also an essential role in flagellin-induced corneal protection, as flagellin-induced cell reprogramming results in accelerated bacterial clearance and resolution of inflammation. Indeed, ATF3 expression was enhanced after flagellin pretreatment and subsequent infection compared to PBS-pretreated counterparts, *in vitro* and *in vivo*. ATF3 has been characterized as an "adaptive response" gene because it participates in various cellular processes to adapt to extra- and/or intracellular changes (Lu et al., 2007), and the lack of ATF3 may compromise the ability of flagellin to reprogram epithelial cells and adapt to forthcoming pathogen attack. However, flagellin pretreatment of ATF3-null mice was still able to reduce

bacterial count, MPO activity and CXCL2 expression compared to PBS-pretreated littermates, which implies that flagellin-induced full protection requires the reprogramming of not only ATF3, but also other genes.

In summary, ATF3 is an essential transcription factor that contributes to corneal innate defense by the suppression of inflammation and the enhancement of bacterial and immune cell clearance from the site of infection. ATF3 is a critical component of flagellin-induced cell reprogramming and corneal protection and may serve as a marker to measure the efficacy of therapeutic drugs in reducing inflammation and enhancing bacterial clearance.
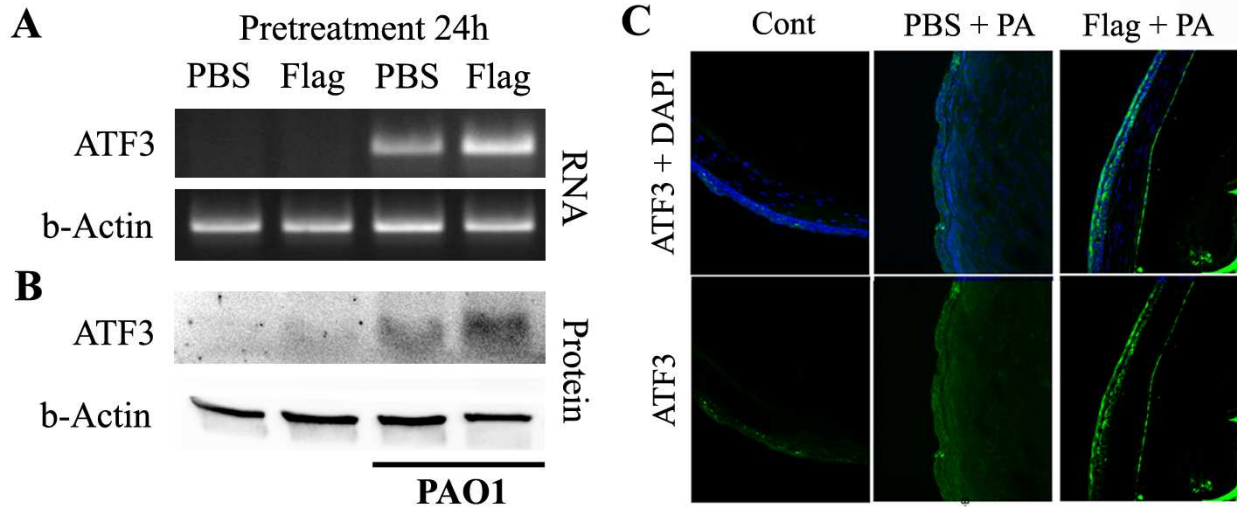
Figure 18. Detection of ATF3 expression in corneal epithelia of B6 WT mice. WT B6 mice were topically pretreated with 500 ng flagellin (in 5 µl PBS), or 5 µl PBS for 24h, and then infected with PAO1 at 10^5 cfu for 6h. The epithelium was collected and RNA isolated for semi-quantitative RT-PCR (A) and Western blot (B) analysis of ATF3. Beta-actin was used as the internal control. For immunohistochemical detection of ATF3, the whole cornea was snap-frozen in OCT, sectioned, stained with mouse anti-ATF3 antibody and examined with fluorescence microscopy (magnification at 20x, DAPI used to stained the nuclei). The figure is a representative of six corneas (**n** = 3 mice) per condition.
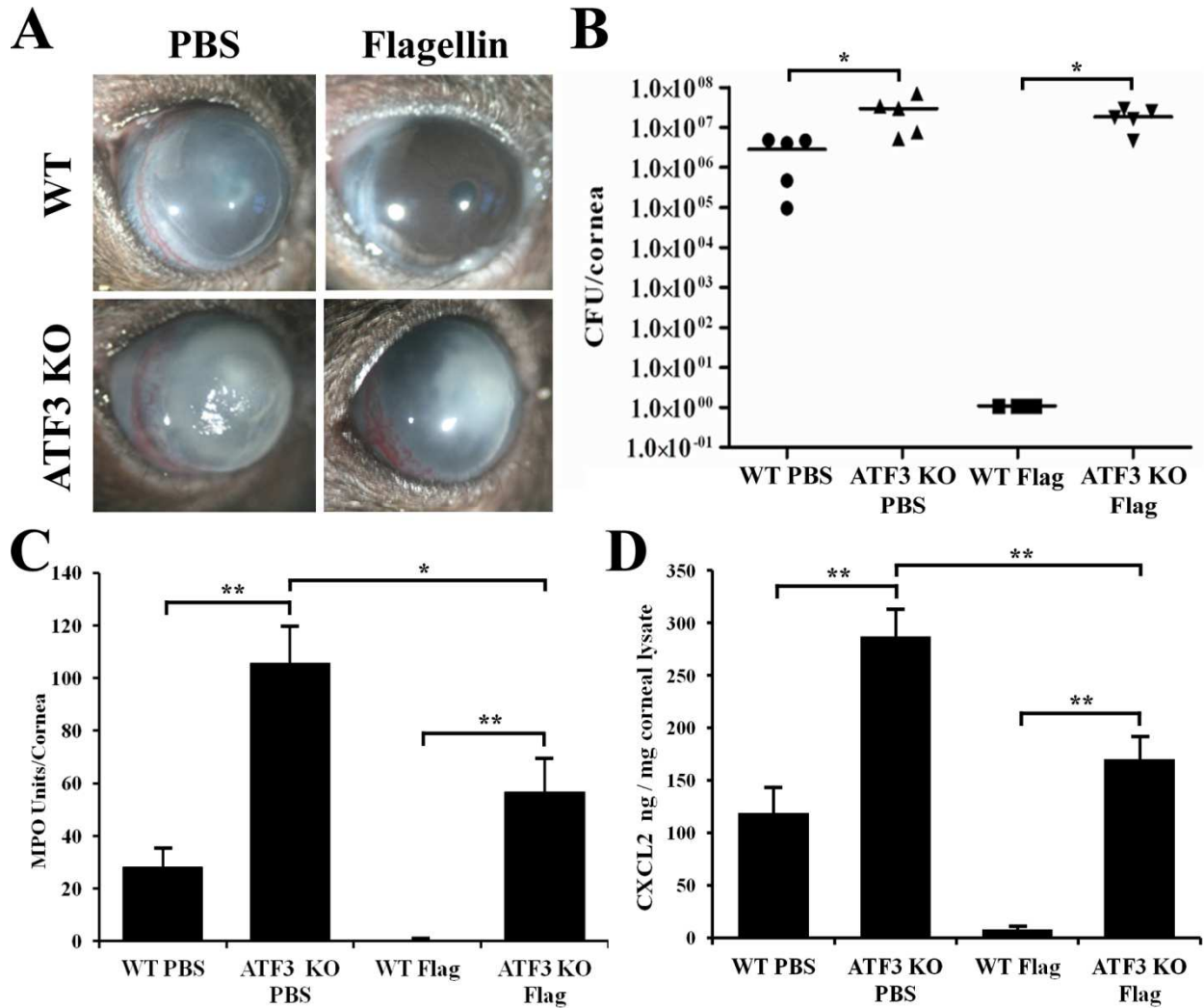
Figure 19. ATF3 deficiency increased the severity of *Pseudomonas* keratitis and abolished flagellin-induced protection in B6 mice. WT or ATF3-/- B6 mice were topically pretreated with 500 ng flagellin or PBS for 24h, and then infected with ATCC 19660 at 10^4 cfu for 3 days (A). The whole cornea was collected for viable bacterial count (B), MPO activity assay and MIP-2 ELISA (C). P values were generated using unpaired Student's *t* test (* P<0.05; ** P<0.01). The figure is a representative of three independent experiments.

Table 5. Mouse primer sequences used for PCR

Gene	Primer	Sequence (5'→3')	Product size (bp)
ATF3	Forward	ATGATGCTTCAACATCCAGGC	546
	Reverse	TTAGCTCTGCAATGTTCCTTC	
β-actin	Forward	CGTGGGCCGCCCTAGGCACC	443
	Reverse	TTGGCCTTAGGGTTCAGGGGGG	

CHAPTER 5

CONCLUSIONS

The innate immune response of the cornea is vital to maintaining corneal homeostasis and preserving vision against pathogens. The corneal epithelium plays a barrier function that separates the outside environment and contributes to host defense of the cornea. The ability of the corneal epithelium to recognize and respond to pathogens is attributed largely to the family of Toll-like receptors (TLRs), of which TLR5 plays a central role in detecting Gram-negative bacteria. Flagellin, the constituting protein of bacterial flagella, is the natural ligand of TLR5 which can elicit a cascade of signal transduction pathways, resulting in the production of proinflammatory cytokines/chemokines and antimicrobial molecules. Although the production of proinflammatory cytokines is important for mediating the initial host defense against invading pathogens, an excessive host inflammatory response can be detrimental. Thus, TLR-mediated corneal inflammation is a double-edged sword that must be precisely regulated.

Many studies have revealed that pre-exposure of TLR5 to flagellin causes cell reprogramming, which results in suppressed NF-κB activation and proinflammatory cytokine production in response to subsequent bacterial and fungal challenge in a variety of cells including epithelial cells. The purpose of this dissertation is to identify reprogramming of transcription factors caused by TLR5/flagellin pretreatment, and their target genes to better understand the mechanisms underlying flagellin-mediated corneal protection.

In Chapter 2, we investigated flagellin-induced cell reprogramming of transcription factors *in vitro* using primary human corneal epithelial cells (hCECs) and identified IRF1 and ATF3 as the most heavily altered transcription factors. *P. aeruginosa* challenge of HCECs resulted in profound increase of IRF1 expression, but flagellin-pretreatment of HCECs almost eliminated its expression in response to the same pathogen. In addition, of the 9 IRF family members only IRF1 was upregulated and reprogrammed, indicating its specificity in response to

TLR5 activation. We searched the literature and found several target genes of IRF1, including iNOS, IL-12p35 and CXCL10. By knocking down IRF1 in HCECs using siRNA, we were able to identify CXCL10, but not iNOS or IL-12p35, as a target gene of IRF1, implying the specificity of the TLR5-IRF1-CXCL10 axis in HCECs. In contrast to IRF1, ATF3 expression was enhanced by flagellin pretreatment, indicative of transcriptional reprogramming of its expression as well as its potential role as a regulator of reprogrammed gene expression.

In Chapter 3 we sought to assess how IRF1 and CXCL10 expression are reprogrammed in mouse cornea, especially in the epithelium. To our supprise, we observed that flagellin pretreatment caused enhanced IRF1 and CXCL10 expression in the mouse corneal epithelium. This contrast between *in vitro* and *in vivo* results concerning IRF1 and CXCL10 expression led us to explore further the involvement of infiltrated cells *in vivo*. We discovered that topical application of flagellin results in the accelerated infiltration of innate immune cells such as NK cells that produces IFNγ to sustain IRF1 and CXCL10 upregulation *in vivo* in response to infection. We further demonstrated that NK cells are required for corneal innate defense and flagellin induced protection. These findings suggest that enhanced epithelial expression of IRF1-CXCL10 witnessed *in vivo* by flagellin-induced reprogramming is caused by IFNγ secreted from accelerated infiltration of NK cells in the stroma (Fig. 20).

In Chapter 4, we investigated the reprogramming of ATF3 *in vivo*, comparing its expression pattern to hCECs. In contrast to IRF1, the enhanced expression of ATF3 in hCECs by flagellin pretreatment was also observed in our animal model of Pseudomonas infection at both the mRNA and protein levels. With the use of ATF3 knockout mice we observed a hyperresponsive inflammatory response towards Pseudomonas infection, culminating in accelerated corneal perforation, decreased bacterial clearance, increased CXCL2 expression and neutrophil infiltration. Moreover, flagellin-pretreatment was not able to protect the corneas of ATF3-null mice. Although the inflammatory response was somewhat dampened compared to

PBS-pretreated ATF3-null mice, flagellin pretreated ATF3-null corneas displayed more severe inflammation than that of control WT mice. These results coincide with numerous previous reports citing the essential role of ATF3 as a negative regulator of inflammation.

In conclusion, flagellin pretreatment of corneal epithelial cells reprograms the expression of epithelial IRF1 and ATF3, via different mechanisms, resulting in the enhancement of the innate immune response of the cornea. Both transcription factors play an indispensable role in protecting the cornea from bacterial infection and regulating infection associated inflammation, and may serve as important markers for the development of therapeutic drugs to treat and/or prevent bacterial keratitis.
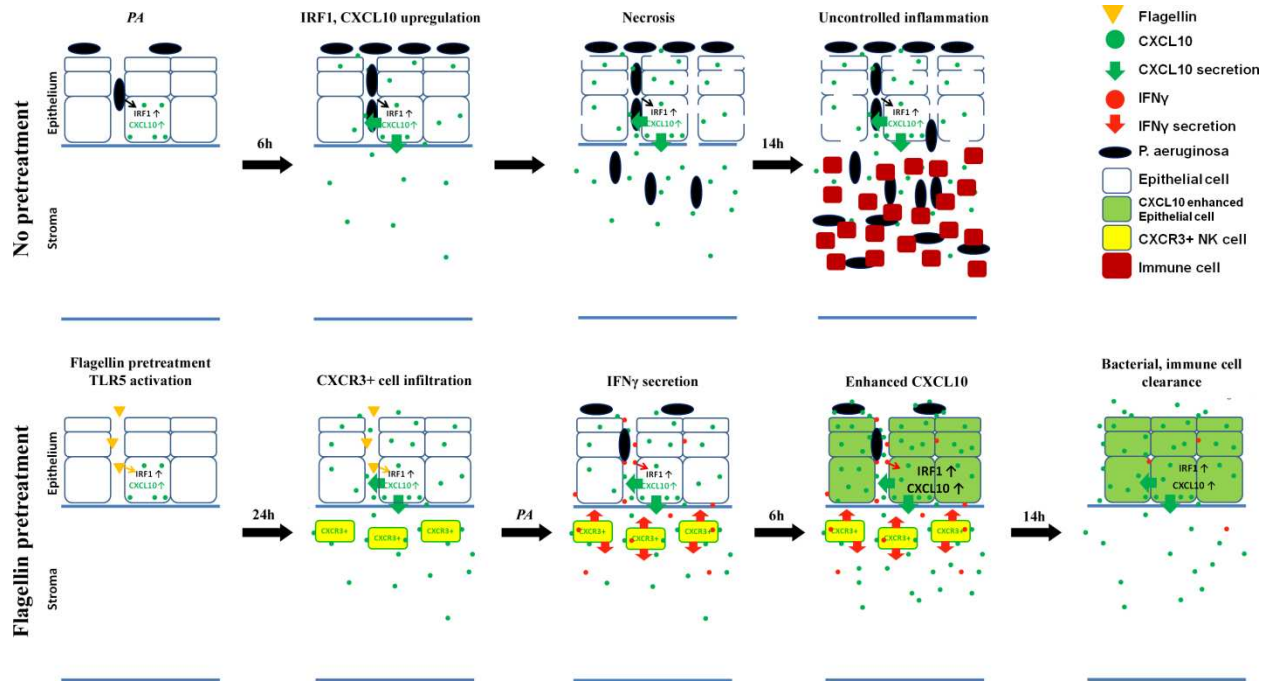
Figure 20. Schematic representation of the mechanisms of flagellin-induced protection of the cornea caused by the reprogramming of corneal epithelial cells.

REFERENCES

http://img.medscape.com/pi/emed/ckb/ophthalmology/1189694-1221604-327.jpg.

http://www.revophth.com/publish/images/1_119_1.jpg.

Ak, P., and A.J. Levine. 2010. p53 and NF-κB: different strategies for responding to stress lead to a functional antagonism. *The FASEB Journal*. 24:3643-3652.

Akpek, E.K., and J.D. Gottsch. 2003. Immune defense at the ocular surface. *Eye*. 17:949-956.

Alarcon, I., L. Kwan, C. Yu, D.J. Evans, and S.M. Fleiszig. 2009. Role of the corneal epithelial basement membrane in ocular defense against Pseudomonas aeruginosa. *Infect Immun*. 77:3264-3271.

Alarcon, I., C. Tam, J.J. Mun, J. LeDue, D.J. Evans, and S.M. Fleiszig. 2011. Factors impacting corneal epithelial barrier function against Pseudomonas aeruginosa traversal. *Invest Ophthalmol Vis Sci*. 52:1368-1377.

Allen-Jennings, A.E., M.G. Hartman, G.J. Kociba, and T. Hai. 2002. The roles of ATF3 in liver dysfunction and the regulation of phosphoenolpyruvate carboxykinase gene expression. *The Journal of biological chemistry*. 277:20020-20025.

Aly, S., J. Mages, N. Reiling, U. Kalinke, T. Decker, R. Lang, and S. Ehlers. 2009. Mycobacteria-induced granuloma necrosis depends on IRF-1. *J Cell Mol Med*. 13:2069-2082.

Andersen-Nissen, E., K.D. Smith, K.L. Strobe, S.L. Barrett, B.T. Cookson, S.M. Logan, and A. Aderem. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A*. 102:9247-9252.

Ashida, H., H. Mimuro, M. Ogawa, T. Kobayashi, T. Sanada, M. Kim, and C. Sasakawa. 2011. Cell death and infection: A double-edged sword for host and pathogen survival. *The Journal of Cell Biology*. 195:931-942.

Augustin, D.K., S.R. Heimer, C. Tam, W.Y. Li, J.M. Le Due, D.J. Evans, and S.M. Fleiszig. 2011. Role of defensins in corneal epithelial barrier function against Pseudomonas aeruginosa traversal. *Infect Immun*. 79:595-605.

Bals, R., and P.S. Hiemstra. 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J.* 23:327-333.

Battistini, A. 2009. Interferon regulatory factors in hematopoietic cell differentiation and immune regulation. *J Interferon Cytokine Res.* 29:765-780.

Ben-Yedidia, T., and R. Arnon. 2007. Epitope-based vaccine against influenza. *Expert Rev Vaccines*. 6:939-948.

Biswas, S.K., and E. Lopez-Collazo. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in immunology*. 30:475-487.

Blanch, V.J., J.F. Piskurich, and C.S. Kaetzel. 1999. Cutting edge: coordinate regulation of IFN regulatory factor-1 and the polymeric Ig receptor by proinflammatory cytokines. *J Immunol*. 162:1232-1235.

Brissette-Storkus, C.S., S.M. Reynolds, A.J. Lepisto, and R.L. Hendricks. 2002. Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci*. 43:2264-2271.

Buckley, J.M., J.H. Wang, and H.P. Redmond. 2006. Cellular reprogramming by gram-positive bacterial components: a review. *Journal of Leukocyte Biology*. 80:731-741.

Burdelya, L.G., V.I. Krivokrysenko, T.C. Tallant, E. Strom, A.S. Gleiberman, D. Gupta, O.V. Kurnasov, F.L. Fort, A.L. Osterman, J.A. Didonato, E. Feinstein, and A.V. Gudkov. 2008. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science*. 320:226-230.

Burg, N.D., and M.H. Pillinger. 2001. The neutrophil: function and regulation in innate and humoral immunity. *Clin Immunol*. 99:7-17.

Burns, K., F. Martinon, C. Esslinger, H. Pahl, P. Schneider, J.L. Bodmer, F. Di Marco, L. French, and J. Tschopp. 1998. MyD88, an adapter protein involved in interleukin-1 signaling. *J Biol Chem*. 273:12203-12209.

Buttmann, M., F. Berberich-Siebelt, E. Serfling, and P. Rieckmann. 2007. Interferon-b Is a Potent Inducer of Interferon Regulatory Factor-1/2-Dependent IP-10/CXCL10 Expression in Primary Human Endothelial Cells. *Journal of Vascular Research*. 44:51-60.

Campodonico, V.L., N.J. Llosa, M. Grout, G. Doring, T. Maira-Litran, and G.B. Pier. 2010. Evaluation of flagella and flagellin of Pseudomonas aeruginosa as vaccines. *Infect Immun*. 78:746-755.

Cao, W., and Y.J. Liu. 2007. Innate immune functions of plasmacytoid dendritic cells. *Curr Opin Immunol*. 19:24-30.

Carr, D.J., J. Chodosh, J. Ash, and T.E. Lane. 2003. Effect of anti-CXCL10 monoclonal antibody on herpes simplex virus type 1 keratitis and retinal infection. *J Virol*. 77:10037-10046.

Carvalho, F.A., J.D. Aitken, A.T. Gewirtz, and M. Vijay-Kumar. 2010. TLR5 activation induces secretory interleukin-1 receptor antagonist (sIL-1Ra) and reduces inflammasome-associated tissue damage. *Mucosal Immunol*.

Cavaillon, J.-M., and M. Adib-Conquy. 2006. Bench-to-bedside review: Endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Crit Care*. 10:233.

Chen, B.P., G. Liang, J. Whelan, and T. Hai. 1994. ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *The Journal of biological chemistry*. 269:15819-15826.

Chen, Z.J. 2005. Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol*. 7:758-765.

Chinnery, H.R., M.J. Ruitenberg, G.W. Plant, E. Pearlman, S. Jung, and P.G. McMenamin. 2007. The chemokine receptor CX3CR1 mediates homing of MHC class II-positive cells to the normal mouse corneal epithelium. *Invest Ophthalmol Vis Sci*. 48:1568-1574.

Choo, A., P. Palladinetti, T. Passioura, S. Shen, R. Lock, G. Symonds, and A. Dolnikov. 2006. The role of IRF1 and IRF2 transcription factors in leukaemogenesis. *Curr Gene Ther*. 6:543-550.

Coccia, E.M., E. Stellacci, G. Marziali, G. Weiss, and A. Battistini. 2000. IFN-gamma and IL-4 differently regulate inducible NO synthase gene expression through IRF-1 modulation. *Int Immunol*. 12:977-985.

Cole, A.M., T. Ganz, A.M. Liese, M.D. Burdick, L. Liu, and R.M. Strieter. 2001a. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol*. 167:623-627.

Cole, A.M., T. Ganz, A.M. Liese, M.D. Burdick, L. Liu, and R.M. Strieter. 2001b. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *Journal of immunology*. 167:623-627.

Colonna, M. 2007. TLR pathways and IFN-regulatory factors: to each its own. *European journal of immunology*. 37:306-309.

DE FOST, M., W. CHIERAKUL, K. PIMDA, A.M. DONDORP, N.J. WHITE, and T. VAN DER POLL. 2005. ACTIVATION OF CYTOTOXIC LYMPHOCYTES IN PATIENTS WITH SCRUB TYPHUS. *The American Journal of Tropical Medicine and Hygiene*. 72:465-467.

Der, S.D., A. Zhou, B.R. Williams, and R.H. Silverman. 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A*. 95:15623-15628.

Didierlaurent, A., I. Ferrero, L.A. Otten, B. Dubois, M. Reinhardt, H. Carlsen, R. Blomhoff, S. Akira, J.P. Kraehenbuhl, and J.C. Sirard. 2004. Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *J Immunol*. 172:6922-6930.

Dornan, D., M. Eckert, M. Wallace, H. Shimizu, E. Ramsay, T.R. Hupp, and K.L. Ball. 2004. Interferon Regulatory Factor 1 Binding to p300 Stimulates DNA-Dependent Acetylation of p53. *Mol. Cell. Biol*. 24:10083-10098.

Eaves-Pyles, T., K. Murthy, L. Liaudet, L. Virag, G. Ross, F.G. Soriano, C. Szabo, and A.L. Salzman. 2001. Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol.* 166:1248-1260.

Eckert, M., S.E.M. Meek, and K.L. Ball. 2006. A Novel Repressor Domain Is Required for Maximal Growth Inhibition by the IRF-1 Tumor Suppressor. *Journal of Biological Chemistry.* 281:23092-23102.

Egesten, A., M. Eliasson, A.I. Olin, J.S. Erjefalt, A. Bjartell, P. Sangfelt, and M. Carlson. 2007. The proinflammatory CXC-chemokines GRO-alpha/CXCL1 and MIG/CXCL9 are concomitantly expressed in ulcerative colitis and decrease during treatment with topical corticosteroids. *Int J Colorectal Dis.* 22:1421-1427.

Fan, H., and J.A. Cook. 2004. Molecular mechanisms of endotoxin tolerance. *J Endotoxin Res.* 10:71-84.

Fleiszig, S.M., and D.J. Evans. 2002. The pathogenesis of bacterial keratitis: studies with Pseudomonas aeruginosa. *Clin Exp Optom.* 85:271-278.

Forrester, J.V. 2008. The eye: basic sciences in practice. Saunders.

Fujita, T., J. Sakakibara, Y. Sudo, M. Miyamoto, Y. Kimura, and T. Taniguchi. 1988. Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-beta gene regulatory elements. *EMBO J.* 7:3397-3405.

Gabriele, L., A. Fragale, P. Borghi, P. Sestili, E. Stellacci, M. Venditti, G. Schiavoni, M. Sanchez, F. Belardelli, and A. Battistini. 2006. IRF-1 deficiency skews the differentiation of dendritic cells toward plasmacytoid and tolerogenic features. *J Leukoc Biol.* 80:1500-1511.

Ganz, T. 2003. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol.* 3:710-720.

Gao, N., A. Kumar, H. Guo, X. Wu, M. Wheater, and F.S. Yu. 2011a. Topical flagellin-mediated innate defense against Candida albicans keratitis. *Invest Ophthalmol Vis Sci*. 52:3074-3082.

Gao, N., A. Kumar, J. Jyot, and F.S. Yu. 2010. Flagellin-induced corneal antimicrobial peptide production and wound repair involve a novel NF-kappaB-independent and EGFR-dependent pathway. *PLoS One*. 5:e9351.

Gao, N., G. Sang Yoon, X. Liu, X. Mi, W. Chen, T.J. Standiford, and F.S. Yu. 2013. Genome-wide transcriptional analysis of differentially expressed genes in flagellin-pretreated mouse corneal epithelial cells in response to Pseudomonas aeruginosa: involvement of S100A8/A9. *Mucosal immunology*.

Gao, N., J. Yin, G.S. Yoon, Q.-S. Mi, and F.-S.X. Yu. 2011b. Dendritic Cell–Epithelium Interplay Is a Determinant Factor for Corneal Epithelial Wound Repair. *Am J Pathol*. 179:2243-2253.

Gewirtz, A.T., T.A. Navas, S. Lyons, P.J. Godowski, and J.L. Madara. 2001a. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol*. 167:1882-1885.

Gewirtz, A.T., P.O. Simon, Jr., C.K. Schmitt, L.J. Taylor, C.H. Hagedorn, A.D. O'Brien, A.S. Neish, and J.L. Madara. 2001b. Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest*. 107:99-109.

Gilchrist, M., W.R. Henderson, A.E. Clark, R.M. Simmons, X. Ye, K.D. Smith, and A. Aderem. 2008. Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation. *The Journal of Experimental Medicine*. 205:2349-2357.

Gilchrist, M., V. Thorsson, B. Li, A.G. Rust, M. Korb, J.C. Roach, K. Kennedy, T. Hai, H. Bolouri, and A. Aderem. 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature*. 441:173-178.

Goodridge, J.P., L.J. Lathbury, E. John, A.K. Charles, F.T. Christiansen, and C.S. Witt. 2009. The genotype of the NK cell receptor, KIR2DL4, influences INFgamma secretion by decidual natural killer cells. *Mol Hum Reprod*. 15:489-497.

Gotsch, F., R. Romero, L. Friel, J.P. Kusanovic, J. Espinoza, O. Erez, N.G. Than, P. Mittal, S. Edwin, B.H. Yoon, C.J. Kim, S. Mazaki-Tovi, T. Chaiworapongsa, and S.S. Hassan. 2007. CXCL10/IP-10: a missing link between inflammation and anti-angiogenesis in preeclampsia? *J Matern Fetal Neona*. 20:777-792.

Ha, H., J.-H. Lee, H.-N. Kim, H.B. Kwak, H.-M. Kim, S.E. Lee, J.H. Rhee, H.-H. Kim, and Z.H. Lee. 2008. Stimulation by TLR5 Modulates Osteoclast Differentiation through STAT1/IFN-{beta}. *J Immunol*. 180:1382-1389.

Hagiya, K., J. Yasunaga, Y. Satou, K. Ohshima, and M. Matsuoka. 2011. ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells. *Retrovirology*. 8:19.

Hai, T., and T. Curran. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proceedings of the National Academy of Sciences of the United States of America*. 88:3720-3724.

Hai, T., S. Jalgaonkar, C.C. Wolford, and X. Yin. 2011. Immunohistochemical detection of activating transcription factor 3, a hub of the cellular adaptive-response network. *Methods Enzymol*. 490:175-194.

Hai, T., C.D. Wolfgang, D.K. Marsee, A.E. Allen, and U. Sivaprasad. 1999. ATF3 and stress responses. *Gene expression*. 7:321-335.

Hai, T., C.C. Wolford, and Y.S. Chang. 2010. ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? *Gene Expr*. 15:1-11.

Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi. 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell*. 58:729-739.

Hashimoto, Y., C. Zhang, J. Kawauchi, I. Imoto, M.T. Adachi, J. Inazawa, T. Amagasa, T. Hai, and S. Kitajima. 2002. An alternatively spliced isoform of transcriptional repressor ATF3 and its induction by stress stimuli. *Nucleic acids research*. 30:2398-2406.

Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 410:1099-1103.

Hazlett, L.D. 2004a. Corneal response to Pseudomonas aeruginosa infection. *Prog Retin Eye Res*. 23:1-30.

Hazlett, L.D. 2004b. Corneal response to Pseudomonas aeruginosa infection. *Progress in Retinal and Eye Research*. 23:1-30.

Honda, K., and T. Taniguchi. 2006a. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol*. 6:644-658.

Honda, K., and T. Taniguchi. 2006b. Toll-like receptor signaling and IRF transcription factors. *IUBMB Life*. 58:290-295.

Honko, A.N., and S.B. Mizel. 2005. Effects of flagellin on innate and adaptive immunity. *Immunol Res*. 33:83-101.

Hsu, J.C., R. Bravo, and R. Taub. 1992. Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G1 phase of liver regeneration. *Molecular and Cellular Biology*. 12:4654-4665.

Hu, X., and L.B. Ivashkiv. 2009. Cross-regulation of Signaling Pathways by Interferon-[gamma]: Implications for Immune Responses and Autoimmune Diseases. *Immunity*. 31:539-550.

Hua, B., M. Tamamori-Adachi, Y. Luo, K. Tamura, M. Morioka, M. Fukuda, Y. Tanaka, and S. Kitajima. 2006. A Splice Variant of Stress Response Gene ATF3 Counteracts NF-κB-

dependent Anti-apoptosis through Inhibiting Recruitment of CREB-binding Protein/p300 Coactivator. *Journal of Biological Chemistry*. 281:1620-1629.

Huang, L.C., T.D. Petkova, R.Y. Reins, R.J. Proske, and A.M. McDermott. 2006. Multifunctional roles of human cathelicidin (LL-37) at the ocular surface. *Invest Ophthalmol Vis Sci*. 47:2369-2380.

Huang, L.C., R.L. Redfern, S. Narayanan, R.Y. Reins, and A.M. McDermott. 2007. In Vitro Activity of Human β-Defensin 2 against Pseudomonas aeruginosa in the Presence of Tear Fluid. *Antimicrob Agents Chemother*. 51:3853-3860.

Huang, Q., D. Liu, P. Majewski, L.C. Schulte, J.M. Korn, R.A. Young, E.S. Lander, and N. Hacohen. 2001. The plasticity of dendritic cell responses to pathogens and their components. *Science*. 294:870-875.

Jarchum, I., M. Liu, L. Lipuma, and E.G. Pamer. 2011. Toll-like receptor-5 stimulation protects mice from acute Clostridium difficile colitis. *Infect Immun*.

Jones, R.M., V.M. Sloane, H. Wu, L. Luo, A. Kumar, M.V. Kumar, A.T. Gewirtz, and A.S. Neish. 2011. Flagellin administration protects gut mucosal tissue from irradiation-induced apoptosis via MKP-7 activity. *Gut*. 60:648-657.

Kabashima, H., M. Yoneda, K. Nagata, T. Hirofuji, and K. Maeda. 2002. THE PRESENCE OF CHEMOKINE (MCP-1, MIP-1α, MIP-1β, IP-10, RANTES)-POSITIVE CELLS AND CHEMOKINE RECEPTOR (CCR5, CXCR3)-POSITIVE CELLS IN INFLAMED HUMAN GINGIVAL TISSUES. *Cytokine*. 20:70-77.

Kang, Y., C.R. Chen, and J. Massague. 2003. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Molecular Cell*. 11:915-926.

Karin, M., and F.R. Greten. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*. 5:749-759.

Kawai, T., and S. Akira. 2006. TLR signaling. *Cell Death Differ*. 13:816-825.

Kawai, T., and S. Akira. 2007. TLR signaling. *Semin Immunol*. 19:24-32.

Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 34:637-650.

Khatri, S., J.H. Lass, F.P. Heinzel, W.M. Petroll, J. Gomez, E. Diaconu, C.M. Kalsow, and E. Pearlman. 2002. Regulation of endotoxin-induced keratitis by PECAM-1, MIP-2, and toll-like receptor 4. *Invest Ophthalmol Vis Sci*. 43:2278-2284.

Khuu, C.H., R.M. Barrozo, T. Hai, and S.L. Weinstein. 2007. Activating transcription factor 3 (ATF3) represses the expression of CCL4 in murine macrophages. *Molecular immunology*. 44:1598-1605.

Kinnebrew, M.A., C. Ubeda, L.A. Zenewicz, N. Smith, R.A. Flavell, and E.G. Pamer. 2010. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. *J Infect Dis*. 201:534-543.

Koetzler, R., R.S. Zaheer, R. Newton, and D. Proud. 2009. Nitric oxide inhibits IFN regulatory factor 1 and nuclear factor-kappaB pathways in rhinovirus-infected epithelial cells. *J Allergy Clin Immunol*. 124:551-557.

Kroger, A., M. Koster, K. Schroeder, H. Hauser, and P.P. Mueller. 2002. Activities of IRF-1. *J Interferon Cytokine Res*. 22:5-14.

Kumar, A., N. Gao, T.J. Standiford, R.L. Gallo, and F.S. Yu. 2010a. Topical flagellin protects the injured corneas from Pseudomonas aeruginosa infection. *Microbes Infect*. 12:978-989.

Kumar, A., N. Gao, T.J. Standiford, R.L. Gallo, and F.S. Yu. 2010b. Topical flagellin protects the injured corneas from Pseudomonas aeruginosa infection. *Microbes and infection / Institut Pasteur*. 12:978-989.

Kumar, A., L.D. Hazlett, and F.S. Yu. 2008. Flagellin suppresses the inflammatory response and enhances bacterial clearance in a murine model of Pseudomonas aeruginosa keratitis. *Infect Immun*. 76:89-96.

Kumar, A., J. Yin, J. Zhang, and F.S. Yu. 2007. Modulation of corneal epithelial innate immune response to pseudomonas infection by flagellin pretreatment. *Invest Ophthalmol Vis Sci*. 48:4664-4670.

Kumar, A., and F.S. Yu. 2006. Toll-like receptors and corneal innate immunity. *Curr Mol Med*. 6:327-337.

Kumar, A., J. Zhang, and F.S. Yu. 2006. Toll-like receptor 2-mediated expression of beta-defensin-2 in human corneal epithelial cells. *Microbes Infect*. 8:380-389.

Lehrer, R.I., and T. Ganz. 2002. Cathelicidins: a family of endogenous antimicrobial peptides. *Current Opinion in Hematology*. 9:18-22.

Lettinga, K.D., S. Weijer, P. Speelman, J.M. Prins, T. van der Poll, and A. Verbon. 2003. Reduced interferon-γ release in patients recovered from Legionnaires' disease. *Thorax*. 58:63-67.

Liang, G., C.D. Wolfgang, B.P. Chen, T.H. Chen, and T. Hai. 1996. ATF3 gene. Genomic organization, promoter, and regulation. *The Journal of biological chemistry*. 271:1695-1701.

Lim, J.H., H.J. Lee, Y.K. Pak, W.H. Kim, and J. Song. 2011. Organelle stress-induced activating transcription factor-3 downregulates low-density lipoprotein receptor expression in Sk-Hep1 human liver cells. *Biol Chem*. 392:377-385.

Lin, W.J., and W.C. Yeh. 2005. Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. *Shock*. 24:206-209.

Liu, J., S. Cao, L.M. Herman, and X. Ma. 2003. Differential Regulation of Interleukin (IL)-12 p35 and p40 Gene Expression and Interferon (IFN)-γ–primed IL-12 Production by IFN Regulatory Factor 1. *The Journal of Experimental Medicine*. 198:1265-1276.

Liu, M., S. Guo, J.M. Hibbert, V. Jain, N. Singh, N.O. Wilson, and J.K. Stiles. 2011a. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev*. 22:121-130.

Liu, W., M. Iiizumi-Gairani, H. Okuda, A. Kobayashi, M. Watabe, S.K. Pai, P.R. Pandey, F. Xing, K. Fukuda, V. Modur, S. Hirota, K. Suzuki, T. Chiba, M. Endo, T. Sugai, and K. Watabe. 2011b. KAI1 gene is engaged in NDRG1 gene-mediated metastasis suppression through the ATF3-NFkappaB complex in human prostate cancer. *The Journal of biological chemistry*. 286:18949-18959.

Lo, B.K., M. Yu, D. Zloty, B. Cowan, J. Shapiro, and K.J. McElwee. 2010. CXCR3/ligands are significantly involved in the tumorigenesis of basal cell carcinomas. *Am J Pathol*. 176:2435-2446.

Lohoff, M., D. Ferrick, H.W. Mittrucker, G.S. Duncan, S. Bischof, M. Rollinghoff, and T.W. Mak. 1997. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. *Immunity*. 6:681-689.

Lu, D., J. Chen, and T. Hai. 2007. The regulation of ATF3 gene expression by mitogen-activated protein kinases. *Biochem J*. 401:559-567.

Lu, D., C.D. Wolfgang, and T. Hai. 2006. Activating transcription factor 3, a stress-inducible gene, suppresses Ras-stimulated tumorigenesis. *The Journal of biological chemistry*. 281:10473-10481.

Marsh, B., S.L. Stevens, A.E.B. Packard, B. Gopalan, B. Hunter, P.Y. Leung, C.A. Harrington, and M.P. Stenzel-Poore. 2009. Systemic Lipopolysaccharide Protects the Brain from Ischemic Injury by Reprogramming the Response of the Brain to Stroke: A Critical Role for IRF3. *The Journal of Neuroscience*. 29:9839-9849.

Maruyama, K., M. Fukasaka, A. Vandenbon, T. Saitoh, T. Kawasaki, T. Kondo, Kazunari K. Yokoyama, H. Kidoya, N. Takakura, D. Standley, O. Takeuchi, and S. Akira. 2012. The Transcription Factor Jdp2 Controls Bone Homeostasis and Antibacterial Immunity by Regulating Osteoclast and Neutrophil Differentiation. *Immunity*. 37:1024-1036.

Matsuyama, T., T. Kimura, M. Kitagawa, K. Pfeffer, T. Kawakami, N. Watanabe, T.M. Kundig, R. Amakawa, K. Kishihara, A. Wakeham, and et al. 1993. Targeted disruption of IRF-1 or

IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell.* 75:83-97.

McDermott, A.M. 2009. The role of antimicrobial peptides at the ocular surface. *Ophthalmic Res.* 41:60-75.

McDermott, A.M., R.L. Redfern, and B. Zhang. 2001. Human beta-defensin 2 is up-regulated during re-epithelialization of the cornea. *Curr Eye Res.* 22:64-67.

McDermott, A.M., R.L. Redfern, B. Zhang, Y. Pei, L. Huang, and R.J. Proske. 2003. 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.* 44:1859-1865.

Means, T.K., F. Hayashi, K.D. Smith, A. Aderem, and A.D. Luster. 2003. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J Immunol.* 170:5165-5175.

Miao, E.A., C.M. Alpuche-Aranda, M. Dors, A.E. Clark, M.W. Bader, S.I. Miller, and A. Aderem. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol.* 7:569-575.

Miao, E.A., E. Andersen-Nissen, S.E. Warren, and A. Aderem. 2007. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol.* 29:275-288.

Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi. 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell.* 54:903-913.

Mizel, S.B., and J.A. Snipes. 2002. Gram-negative Flagellin-induced Self-tolerance Is Associated with a Block in Interleukin-1 Receptor-associated Kinase Release from Toll-like Receptor 5. *Journal of Biological Chemistry.* 277:22414-22420.

Mo, P., H. Wang, H. Lu, D.D. Boyd, and C. Yan. 2010. MDM2 Mediates Ubiquitination and Degradation of Activating Transcription Factor 3. *Journal of Biological Chemistry*. 285:26908-26915.

Mohan, K., E. Cordeiro, M. Vaci, C. McMaster, and T.B. Issekutz. 2005. CXCR3 is required for migration to dermal inflammation by normal and in vivo activated T cells: differential requirements by CD4 and CD8 memory subsets. *European journal of immunology*. 35:1702-1711.

Muller, M., S. Carter, M.J. Hofer, and I.L. Campbell. 2010. Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity--a tale of conflict and conundrum. *Neuropathol Appl Neurobiol*. 36:368-387.

Nahid, M.A., M. Satoh, and E.K. Chan. 2011a. MicroRNA in TLR signaling and endotoxin tolerance. *Cellular & molecular immunology*. 8:388-403.

Nahid, M.A., M. Satoh, and E.K. Chan. 2011b. MicroRNA in TLR signaling and endotoxin tolerance. *Cell Mol Immunol*.

Narayan, V., M. Eckert, A. Zylicz, M. Zylicz, and K.L. Ball. 2009. Cooperative Regulation of the Interferon Regulatory Factor-1 Tumor Suppressor Protein by Core Components of the Molecular Chaperone Machinery. *Journal of Biological Chemistry*. 284:25889-25899.

Nassif, K.F. 1996. Ocular surface defense mechanisms. Little Brown & Co. 35-41 pp.

Negishi, H., Y. Fujita, H. Yanai, S. Sakaguchi, X. Ouyang, M. Shinohara, H. Takayanagi, Y. Ohba, T. Taniguchi, and K. Honda. 2006. Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. *Proc Natl Acad Sci U S A*. 103:15136-15141.

Negishi, H., Y. Ohba, H. Yanai, A. Takaoka, K. Honma, K. Yui, T. Matsuyama, T. Taniguchi, and K. Honda. 2005. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proceedings of the National Academy of Sciences of the United States of America*. 102:15989-15994.

Nguyen, H., L. Teskey, R. Lin, and J. Hiscott. 1999. Identification of the secretory leukocyte protease inhibitor (SLPI) as a target of IRF-1 regulation. *Oncogene.* 18:5455-5463.

Nish, S., and R. Medzhitov. 2011. Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. *Immunity.* 34:629-636.

O'Neill, L.A., and A.G. Bowie. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol.* 7:353-364.

Ogasawara, K., S. Hida, N. Azimi, Y. Tagaya, T. Sato, T. Yokochi-Fukuda, T.A. Waldmann, T. Taniguchi, and S. Taki. 1998. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature.* 391:700-703.

Ohmori, Y., and T.A. Hamilton. 1997. IL-4-induced STAT6 suppresses IFN-gamma-stimulated STAT1-dependent transcription in mouse macrophages. *J Immunol.* 159:5474-5482.

Ohteki, T., C. Maki, and S. Koyasu. 2001. Overexpression of Bcl-2 differentially restores development of thymus-derived CD4-8+ T cells and intestinal intraepithelial T cells in IFN-regulatory factor-1-deficient mice. *J Immunol.* 166:6509-6513.

Ohteki, T., H. Yoshida, T. Matsuyama, G.S. Duncan, T.W. Mak, and P.S. Ohashi. 1998. The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of natural killer 1.1+ T cell receptor-alpha/beta+ (NK1+ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J Exp Med.* 187:967-972.

Pan, Y., H. Chen, F. Siu, and M.S. Kilberg. 2003. Amino acid deprivation and endoplasmic reticulum stress induce expression of multiple activating transcription factor-3 mRNA species that, when overexpressed in HepG2 cells, modulate transcription by the human asparagine synthetase promoter. *The Journal of biological chemistry.* 278:38402-38412.

Penninger, J.M., C. Sirard, H.W. Mittrucker, A. Chidgey, I. Kozieradzki, M. Nghiem, A. Hakem, T. Kimura, E. Timms, R. Boyd, T. Taniguchi, T. Matsuyama, and T.W. Mak. 1997. The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8+ thymocytes. *Immunity.* 7:243-254.

Pepose, J.S., G.N. Holland, and K.R. Wilhelmus. 1996. Ocular infection & immunity. Mosby, St. Louis. xl, 1552 p. pp.

Pion, E., V. Narayan, M. Eckert, and K.L. Ball. 2009. Role of the IRF-1 enhancer domain in signalling polyubiquitination and degradation. *Cellular Signalling*. 21:1479-1487.

Platanias, L.C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. 5:375-386.

Prince, A. 2006. Flagellar activation of epithelial signaling. *Am J Respir Cell Mol Biol*. 34:548-551.

Quaranta, M.G., A. Napolitano, M. Sanchez, L. Giordani, B. Mattioli, and M. Viora. 2007. HIV-1 Nef impairs the dynamic of DC/NK crosstalk: different outcome of CD56(dim) and CD56(bright) NK cell subsets. *Faseb J*. 21:2323-2334.

Ramos, H.C., M. Rumbo, and J.C. Sirard. 2004. Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends Microbiol*. 12:509-517.

Rhee, S.H., E. Im, and C. Pothoulakis. 2008. Toll-like receptor 5 engagement modulates tumor development and growth in a mouse xenograft model of human colon cancer. *Gastroenterology*. 135:518-528.

Rhee, S.H., H. Kim, M.P. Moyer, and C. Pothoulakis. 2006. Role of MyD88 in Phosphatidylinositol 3-Kinase Activation by Flagellin/Toll-like Receptor 5 Engagement in Colonic Epithelial Cells. *Journal of Biological Chemistry*. 281:18560-18568.

Rosenberger, C.M., A.E. Clark, P.M. Treuting, C.D. Johnson, and A. Aderem. 2008. ATF3 regulates MCMV infection in mice by modulating IFN-γ expression in natural killer cells. *Proceedings of the National Academy of Sciences*. 105:2544-2549.

Roussel, L., S. Robins, A. Schachter, J. Berube, Q. Hamid, and S. Rousseau. 2011. Steroids and extracellular signal-regulated kinase 1/2 activity suppress activating transcription factor 3 expression in patients with severe asthma. *The Journal of allergy and clinical immunology*. 127:1632-1634.

Ruan, X., J. Chodosh, M.C. Callegan, M.C. Booth, T.D. Lee, P. Kumar, M.S. Gilmore, and H.A. Pereira. 2002. Corneal expression of the inflammatory mediator CAP37. *Invest Ophthalmol Vis Sci*. 43:1414-1421.

Santaolalla, R., M. Fukata, and M.T. Abreu. 2011. Innate immunity in the small intestine. *Curr Opin Gastroenterol*. 27:125-131.

Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, and S. Akira. 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol*. 6:1087-1095.

Savitsky, D., T. Tamura, H. Yanai, and T. Taniguchi. 2010a. Regulation of immunity and oncogenesis by the IRF transcription factor family. *Cancer Immunol Immunother*. 59:489-510.

Savitsky, D., T. Tamura, H. Yanai, and T. Taniguchi. 2010b. Regulation of immunity and oncogenesis by the IRF transcription factor family. *Cancer immunology, immunotherapy : CII*. 59:489-510.

Shaulian, E., and M. Karin. 2001. AP-1 in cell proliferation and survival. *Oncogene*. 20:2390-2400.

Shaulian, E., and M. Karin. 2002. AP-1 as a regulator of cell life and death. *Nat Cell Biol*. 4:E131-136.

Shultz, D.B., M.R. Rani, J.D. Fuller, R.M. Ransohoff, and G.R. Stark. 2009. Roles of IKK-beta, IRF1, and p65 in the activation of chemokine genes by interferon-gamma. *J Interferon Cytokine Res*. 29:817-824.

Sierro, F., B. Dubois, A. Coste, D. Kaiserlian, J.P. Kraehenbuhl, and J.C. Sirard. 2001. Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proc Natl Acad Sci U S A*. 98:13722-13727.

Song, J., and S.N. Abraham. 2008. Innate and adaptive immune responses in the urinary tract. *Eur J Clin Invest*. 38 Suppl 2:21-28.

Souza-Fonseca-Guimaraes, F., M. Adib-Conquy, and J.M. Cavaillon. 2012. Natural killer (NK) cells in antibacterial innate immunity: angels or devils? *Mol Med*. 18:270-285.

Stirnweiss, A., A. Ksienzyk, K. Klages, U. Rand, M. Grashoff, H. Hauser, and A. Kroger. 2010. IFN regulatory factor-1 bypasses IFN-mediated antiviral effects through viperin gene induction. *J Immunol*. 184:5179-5185.

Strieter, R.M., S.L. Kunkel, D.A. Arenberg, M.D. Burdick, and P.J. Polverini. 1995. Interferon gamma-inducible protein 10 (IP-10), a member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochem Biophys Res Commun*. 210:51-57.

Sun, L., and Z.J. Chen. 2004. The novel functions of ubiquitination in signaling. *Curr Opin Cell Biol*. 16:119-126.

Takaoka, A., H. Yanai, S. Kondo, G. Duncan, H. Negishi, T. Mizutani, S. Kano, K. Honda, Y. Ohba, T.W. Mak, and T. Taniguchi. 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature*. 434:243-249.

Tallant, T., A. Deb, N. Kar, J. Lupica, M.J. de Veer, and J.A. DiDonato. 2004. Flagellin acting via TLR5 is the major activator of key signaling pathways leading to NF-kappa B and proinflammatory gene program activation in intestinal epithelial cells. *BMC Microbiol*. 4:33.

Tamura, T., H. Yanai, D. Savitsky, and T. Taniguchi. 2008a. The IRF family transcription factors in immunity and oncogenesis. *Annu Rev Immunol*. 26:535-584.

Tamura, T., H. Yanai, D. Savitsky, and T. Taniguchi. 2008b. The IRF family transcription factors in immunity and oncogenesis. *Annual review of immunology*. 26:535-584.

Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol*. 19:623-655.

Tattoli, I., M.T. Sorbara, D. Vuckovic, A. Ling, F. Soares, Leticia A.M. Carneiro, C. Yang, A. Emili, Dana J. Philpott, and Stephen E. Girardin. 2012. Amino Acid Starvation Induced

by Invasive Bacterial Pathogens Triggers an Innate Host Defense Program. *Cell Host & Microbe*. 11:563-575.

Thompson, M., D. Xu, and B. Williams. 2009. ATF3 transcription factor and its emerging roles in immunity and cancer. *Journal of Molecular Medicine*. 87:1053-1060.

Uematsu, S., and S. Akira. 2009. Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. *J Gastroenterol*. 44:803-811.

Ueta, M., and S. Kinoshita. 2010. Innate immunity of the ocular surface. *Brain Res Bull*. 81:219-228.

Vartanian, K., and M. Stenzel-Poore. 2010. Toll-like receptor tolerance as a mechanism for neuroprotection. *Transl Stroke Res*. 1:252-260.

Vasquez, R.E., and L. Soong. 2006. CXCL10/gamma interferon-inducible protein 10-mediated protection against Leishmania amazonensis infection in mice. *Infection and Immunity*. 74:6769-6777.

Vijay-Kumar, M., J.D. Aitken, and A.T. Gewirtz. 2008a. Toll like receptor-5: protecting the gut from enteric microbes. *Semin Immunopathol*. 30:11-21.

Vijay-Kumar, M., J.D. Aitken, C.J. Sanders, A. Frias, V.M. Sloane, J. Xu, A.S. Neish, M. Rojas, and A.T. Gewirtz. 2008b. Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. *J Immunol*. 180:8280-8285.

Watanabe, N., J. Sakakibara, A.G. Hovanessian, T. Taniguchi, and T. Fujita. 1991. Activation of IFN-beta element by IRF-1 requires a posttranslational event in addition to IRF-1 synthesis. *Nucleic Acids Res*. 19:4421-4428.

Wesche, H., W.J. Henzel, W. Shillinglaw, S. Li, and Z. Cao. 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*. 7:837-847.

West, A.P., B.A. Dancho, and S.B. Mizel. 2005. Gangliosides inhibit flagellin signaling in the absence of an effect on flagellin binding to toll-like receptor 5. *J Biol Chem*. 280:9482-9488.

West, M.A., and W. Heagy. 2002. Endotoxin tolerance: A review. *Crit Care Med*. 30:S64-S73.

Whitmore, M.M., A. Iparraguirre, L. Kubelka, W. Weninger, T. Hai, and B.R.G. Williams. 2007. Negative Regulation of TLR-Signaling Pathways by Activating Transcription Factor-3. *The Journal of Immunology*. 179:3622-3630.

Willcox, M.D. 2007. Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. *Optom Vis Sci*. 84:273-278.

Wolfgang, C.D., G. Liang, Y. Okamoto, A.E. Allen, and T. Hai. 2000. Transcriptional autorepression of the stress-inducible gene ATF3. *The Journal of biological chemistry*. 275:16865-16870.

Wuest, T.R., and D.J. Carr. 2008. Dysregulation of CXCR3 signaling due to CXCL10 deficiency impairs the antiviral response to herpes simplex virus 1 infection. *J Immunol*. 181:7985-7993.

Xue, M.L., A. Thakur, L. Lutze-Mann, and M.D. Willcox. 2000. Pro-inflammatory cytokine/chemokine gene expression in human corneal epithelial cells colonized by Pseudomonas aeruginosa. *Clin Experiment Ophthalmol*. 28:197-200.

Yamada, H., S. Mizuno, and I. Sugawara. 2002. Interferon regulatory factor 1 in mycobacterial infection. *Microbiol Immunol*. 46:751-760.

Yan, C., D. Lu, T. Hai, and D.D. Boyd. 2005. Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination. *The EMBO journal*. 24:2425-2435.

Yan, L., L. Della Coletta, K.L. Powell, J. Shen, H. Thames, C.M. Aldaz, and M.C. MacLeod. 2011. Activation of the canonical Wnt/beta-catenin pathway in ATF3-induced mammary tumors. *PLoS One*. 6:e16515.

Yang, D., Q. Chen, D.M. Hoover, P. Staley, K.D. Tucker, J. Lubkowski, and J.J. Oppenheim. 2003. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J Leukoc Biol*. 74:448-455.

Young, G.M., D.H. Schmiel, and V.L. Miller. 1999. A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Natl Acad Sci U S A*. 96:6456-6461.

Yu, F.S., M.D. Cornicelli, M.A. Kovach, M.W. Newstead, X. Zeng, A. Kumar, N. Gao, S.G. Yoon, R.L. Gallo, and T.J. Standiford. 2010. Flagellin stimulates protective lung mucosal immunity: role of cathelicidin-related antimicrobial peptide. *J Immunol*. 185:1142-1149.

Yu, F.S., and L.D. Hazlett. 2006. Toll-like receptors and the eye. *Invest Ophthalmol Vis Sci*. 47:1255-1263.

Yu, Y., S. Nagai, H. Wu, A.S. Neish, S. Koyasu, and A.T. Gewirtz. 2006. TLR5-mediated phosphoinositide 3-kinase activation negatively regulates flagellin-induced proinflammatory gene expression. *J Immunol*. 176:6194-6201.

Zaheer, R.S., and D. Proud. 2010. Human rhinovirus-induced epithelial production of CXCL10 is dependent upon IFN regulatory factor-1. *Am J Respir Cell Mol Biol*. 43:413-421.

Zeng, H., A.Q. Carlson, Y. Guo, Y. Yu, L.S. Collier-Hyams, J.L. Madara, A.T. Gewirtz, and A.S. Neish. 2003. Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella. *J Immunol*. 171:3668-3674.

Zhang, C., C. Gao, J. Kawauchi, Y. Hashimoto, N. Tsuchida, and S. Kitajima. 2002. Transcriptional activation of the human stress-inducible transcriptional repressor ATF3 gene promoter by p53. *Biochemical and biophysical research communications*. 297:1302-1310.

Zhang, J., A. Kumar, M. Wheater, and F.S. Yu. 2009. Lack of MD-2 expression in human corneal epithelial cells is an underlying mechanism of lipopolysaccharide (LPS) unresponsiveness. *Immunol Cell Biol*. 87:141-148.

Zhang, J., H. Li, J. Wang, Z. Dong, S. Mian, and F.S. Yu. 2004. Role of EGFR transactivation in preventing apoptosis in Pseudomonas aeruginosa-infected human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 45:2569-2576.

Zhang, J., K. Xu, B. Ambati, and F.-S.X. Yu. 2003a. Toll-like Receptor 5-Mediated Corneal Epithelial Inflammatory Responses to Pseudomonas aeruginosa Flagellin. *Invest. Ophthalmol. Vis. Sci.* 44:4247-4254.

Zhang, J., K. Xu, B. Ambati, and F.S. Yu. 2003b. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to Pseudomonas aeruginosa flagellin. *Invest Ophthalmol Vis Sci.* 44:4247-4254.

Zhang, Z., J.-P. Louboutin, D.J. Weiner, J.B. Goldberg, and J.M. Wilson. 2005. Human Airway Epithelial Cells Sense Pseudomonas aeruginosa Infection via Recognition of Flagellin by Toll-Like Receptor 5. *Infect. Immun.* 73:7151-7160.

Zheng, X., F. Bian, P. Ma, C.S.D. Paiva, M. Stern, S.C. Pflugfelder, and D.-Q. Li. 2010. Induction of Th17 differentiation by corneal epithelial-derived cytokines. *Journal of Cellular Physiology.* 222:95-102.

Zigler, M., G.J. Villares, A.S. Dobroff, H. Wang, L. Huang, R.R. Braeuer, T. Kamiya, V.O. Melnikova, R. Song, R. Friedman, R.M. Alani, and M. Bar-Eli. 2011. Expression of Id-1 is regulated by MCAM/MUC18: a missing link in melanoma progression. *Cancer Research.* 71:3494-3504.

Zmuda, E.J., L. Qi, M.X. Zhu, R.G. Mirmira, M.R. Montminy, and T. Hai. 2010. The roles of ATF3, an adaptive-response gene, in high-fat-diet-induced diabetes and pancreatic beta-cell dysfunction. *Mol Endocrinol.* 24:1423-1433.

ABSTRACT

THE TRANSCRIPTIONAL REGULATION OF FLAGELLIN-INDUCED INNATE PROTECTION OF THE CORNEA: ROLE OF IRF1 AND ATF3

by

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December 2013

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Pre-exposure of the cornea to TLR5 ligand flagellin induces profound mucosal innate protection against infections by reprogramming gene expression. This study explored the flagellin-induced modifications of transcription factor expression and function, specifically of IRF1 and ATF3 in corneal epithelial cells to elucidate the transcriptional mechanisms underlying the protective function of flagellin on the cornea.

Initially we used Superarray to screen for transcription factors and identified Interferon Regulatory Factor (IRF) 1 and Activating Transcription Factor (ATF) 3 as the most drastically affected genes by flagellin pretreatment in *P. aeruginosa* challenged human corneal epithelial cells (CEC). However, flagellin pretreatment had opposite effects on IRF1 (inhibition) and ATF3 (enhancement) gene expression in response to *P. aeruginosa*, and other IRFs were not affected. To find the functional target gene of IRF1, we knocked-down IRF1 using siRNA and identified the pleiotropic chemokine CXCL10, but not IL12-p35 or iNOS, as a specific target.

We then attempted to understand the role of IRF1 and CXCL10 in mucosal innate protection against infections *in vivo.* Flagellin augmented the *P. aeruginosa*-induced expression of CXCL10 in CECs in WT, but not in IRF1-/- mice at 6 hpi. IRF1 deficiency markedly increased the severity of *P. aeruginosa* keratitis and significantly attenuated flagellin-elicited protection

compared to WT controls at 3 dpi. CXCL10 neutralization in the cornea of WT mice displayed similar pathogenesis to that of IRF1-/- mice. To understand the regulation of CXCL10 expression *in vivo,* we used neutralizing antibodies to target IFNγ and its producer NK cells. The neutralization of IFNγ receptor or NK cells prevented flagellin-augmented IRF1 and CXCL10 expression and increased the susceptibility to *P. aeruginosa* infection in mouse corneas. Together, our results indicate that IRF1 plays a role in the corneal innate immune response by regulating CXCL10 expression and that IFNγ-producing NK cells induces epithelial expression of IRF1, thus significantly contributing to the protection of the cornea from *P. aeruginosa* infection.

Next we sought understand the functional role of ATF3 in mucosal innate protection against infections *in vivo.* Consistent with *in vitro* results, flagellin augmented the *P. aeruginosa*-induced epithelial expression of ATF3 in CECs in WT at 6 hpi. ATF3 deficiency markedly increased the severity of *P. aeruginosa* keratitis, indicated by significantly increased bacterial count, neutrophil infiltration and CXCL2 expression. In addition, ATF3-null mice displayed compromised flagellin-induced corneal protection compared to PBS-pretreated ATF3-/- littermates at 3 dpi. Together, our results indicate that ATF3 plays a role in the corneal innate immune response by regulating inflammation and bacterial clearance, and ATF3 is indispensible to maintain the protective properties of flagellin against bacterial keratitis.

In conclusion, flagellin pretreatment of corneal epithelial cells reprograms the expression of epithelial IRF1 and ATF3, via different mechanisms, resulting in the enhancement of the innate immune response of the cornea. Both transcription factors play a critical role in protecting the cornea from bacterial invasion and regulating inflammation, and may serve as important markers for the development of therapeutic drugs to treat and/or prevent bacterial keratitis.

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GS Yoon, N Gao, A Kumar, J Li, X Xiao, TJ Standiford, and FS X. Yu. *Interferon regulatory factor-1 in flagellin-induced reprogramming: potential protective role of CXCL10 in cornea innate defense against Pseudomonas aeruginosa infection.* 2013 in submission

N Gao, **GS Yoon**, XW Liu, XF Mi, TJ Standiford, and F-S X. Yu. *Genome-wide transcriptional analysis of flagellin induced reprogramming in mouse corneal epithelial cells in response to Pseudomonas aeruginosa: involvement of S100A8/A9.* Mucosal Immunology, Jan 2013 Epub

N Gao, J Yin, **GS Yoon**, QS Mi, FS Yu. *Dendritic cell-epithelium interplay is a determinant factor for corneal epithelial wound repair.* American Journal of Pathology 2011 179(5): 2243-53

Yu FS, Cornicelli MD, Kovach MA, Newstead MW, Zeng X, Kumar A, Gao N, **Yoon GS**, Gallo RL, Standiford TJ. *Flagellin stimulates protective lung mucosal immunity: role of cathelicidin-related antimicrobial peptide.* Journal of Immunology 2010 185(2):1142-9