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Vip And Host Immunity

Xiaoyu Jiang
Wayne State University,

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by

XIAOYU JIANG

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DEDICATION

This work is dedicated to my family,

for their endless love, support and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

1.1 Host immunity

The immune system is an elaborate and dynamic regulatory and communication network composed of many kinds of molecules, cells, tissues and organs responsible for host resistance against infections initiated by microbial pathogens. The immune system is subdivided into two arms, the innate and the adaptive.

1.1.1 Innate Immunity

The innate immune system is the first line of defense against invading microbial pathogens. Such pathogens first encounter an anatomical barrier which includes skin and internal epithelial layers, as well as the chemicals they secrete. Once the anatomical barrier is breached, cellular and humoral components of the innate immune system are engaged to eliminate the foreign body. The major cell types involved in innate immunity include: neutrophils, macrophages, eosinophils, basophils, mast cells, natural killer cells and dendritic cells. These cells identify pathogen-associated molecular patterns (PAMPs) through receptors on their surface that are called pattern-recognition receptors (PRRs) (Lee et al., 2012).

PRRs are classified based on their ligand function, localization and/or evolutionary relationships. Based on their functions, PRRs can be classified into three families: secreted, endocytic and signaling PRRs. Secretory PRRs, including mannan binding lectin and C reactive protein, are secreted into the plasma and recognize bacterial cell components which enable them to activate

the complement pathway and phagocytic cells. Mannan binding lectin is produced in the liver and secreted into the bloodstream where it binds to carbohydrates on the surface of pathogens and activates the lectin pathway of the complement system (Worthley et al., 2005). The endocytic PRRs include mannose receptor, scavenger receptor and opsonin receptor. They are expressed at the surface of phagocytic cells, and function to mediate the recognition, engulfment and destruction of microbes and/or microbial moieties without intracellular signaling. Signaling PRRs, on the other hand, include Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) and retinoic acid inducible gene I (RIG)-1-like helicases (RLH) which are involved in cell activation in response to diverse microbial moieties. Signaling PRRs are found on the cell surface (e.g., TLR2, TLR4, TLR5, TLR1/2, and TLR2/6), associated with the endosomal membrane (e.g., TLR3, TLR7, TLR8 and TLR9) or in the cytoplasm (NOD and caspase activating and recruitment domain-containing proteins). They recognize specific microbial ligands including bacterial lipopolysaccharide (LPS), flagellin and lipopeptides (Jeannin et al., 2008). Upon activation, these receptors bind and activate the adaptor molecules to trigger myeloid differentiation primary response gene (88) product (MyD88) dependent and independent signaling pathways. This in turn subsequently activates the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which regulates immune gene responses and inflammation (Kawai and Akira, 2010). These together allow an immediate response against microbial infection and contribute to the initiation of long-lasting

adaptive immunity.

1.1.2 Adaptive Immunity

The adaptive immune system is composed of highly specialized, systemic cells, including macrophages, dendritic cells and T and B cells and associated processes that eliminate or prevent pathogen growth. Antigen presenting cells (APCs, including dendritic cells, B cells, and macrophages) initially process and present antigen. Several types of T cell subgroups can be activated by the antigens which are processed and presented by APCs, and each type of T cell is especially equipped to deal with each unique virulent factor associated with bacterial and/or viral pathogens. There are two main subsets of T cells, known as $CD4^+$ and $CD8^+$ T cells, that are distinguished by associated cell surface molecules. T cells that are positive for CD4 are also known as T helper cells, and this type of cell is considered as being the most prolific cytokine producers (Wang et al., 2008). This subset of T cells can be further subdivided into Th1 and Th2 groups, and the cytokines they produce are known as Th1 and Th2 type cytokines. Th1 type cytokines induce pro-inflammatory responses and activate macrophages to induce the generation of cytotoxic T cells leading to a cell mediated immune response. Interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) are the primary Th1 type cytokines (Vesosky et al., 2006). The Th2-type cytokines, including interleukins 4, 5, 10, and 13, are associated with anti-inflammatory responses and B cell activation leading to humoral immune responses. Moreover, Th2 cytokines are capable of counteracting and/or balancing Th1 cytokine mediated immune responses (Berger, 2000). $CD8^+$ T

cells, which are also called cytotoxic T cells, are involved in killing cancer cells as well as cells infected by either virus or bacterial microbes. These cells express $CD8^+$ glycoprotein and recognize their target by binding to antigen associated MHC class I molecules.

When helper T cells recognize the antigen presented on the surface of the B cell, they become activated, which in turn activates the B cells (Parker, 1993). Activated individual T and B cells are thus selected to undergo clonal expansion in order to produce sufficient numbers of clones that differentiate into effector T and B cells, thereby removing pathogens via either cell or humoral mediated immune defenses (Braciale et al., 2012).

Cell mediated immunity is an immune response that involves the activation of macrophages and natural killer cells, the production of antigen specific T cells, and the release of various cytokines in response to antigen stimulation. Cell mediated immunity is directed primarily against microbes that survive in phagocytes and microbes that infect non-phagocytic cells. On the other hand, for humoral or antibody mediated immunity, B cells differentiate into plasma cells that secrete specific antibodies. In mammals there are five types of antibodies: IgA, IgD, IgE, IgG, and IgM, each with different biological properties and capabilities to recognize different kinds of antigens. Based on those specific antibodies, each activated B cell can recognize a unique antigen and neutralize specific pathogens (Janeway, 2001).

1.2 Inflammation

Inflammation is the first response of the immune system to a wound or

infection and may then activate the innate immune cascade. It is characterized by redness, heat, swelling, pain and dysfunction of the involved site. The host reaction is protective as the purpose is to eliminate injurious stimuli (pathogens, damaged cells, or irritants), as well as to clean up dead and dying cells and initiate the healing process in the inflamed tissue site (Sadik et al., 2011). Although inflammation plays an important role in isolating the infected area, mobilizing effector cells, releasing molecules to the site and promoting healing, improperly regulated inflammation may lead to tissue damage and enhanced host disease.

1.2.1 Acute Inflammation

Inflammation may be classified into either acute or chronic in type, each with different features. Its principal signs, as detailed above, include pain and loss of function. It can be caused by mechanical trauma, chemical injuries or infection, all of which induce tissue injury. The injured tissue will then trigger an immediate, early and acute inflammatory response by releasing inflammatory mediators to nearby blood vessels. These mediators begin to function within a relatively short time period (minutes to hours) and induce increased blood flow (due to blood vessel dilation) accompanied by vascular endothelial cell swelling and retraction, both of which lead to increased vascular permeability (Ryan and Majno, 1977). Another function of these mediators is to chemoattract neutrophils to the infected tissue site. Circulating neutrophils first roll along the swollen vascular endothelial cell and adhere to these cells. This process is mediated by a family of molecules called selectins. Adjacent neutrophils adhere to each other,

aggregate and undergo shape changes. When emigration begins, the neutrophils actively migrate through the vessels into the damaged tissues (Armstrong et al., 2004).

1.2.2 Chronic Inflammation

If the sequelae of acute inflammation are not resolved in a timely fashion, but rather persist, continuing tissue necrosis and damage occurs and acute inflammation may become more long term, or chronic. Moreover, some pathogens tend to directly induce chronic rather than acute inflammation. Many of the features of acute inflammation continue as the inflammation becomes chronic inflammation (lasting days to years), including increased blood flow and vascular permeability leading to leukocyte infiltration and tissue damage. During the period of chronic inflammation, an induced progressive shift of immune cell types (from neutrophils to macrophages) at the inflammation site will lead to simultaneous destruction and healing of the inflamed tissue with accompanying fibrosis and angiogenesis (Rieder et al., 2007). Macrophages, the primary cell type of chronic inflammation, are derived from monocytes and reach the injury site within 24-48 hours. These cells are activated to fulfill immunological and secretory functions, including phagocytosis of pathogens and cellular debris, while at the same time, releasing cytokines. The released cytokines activate lymphocytes and other immune cells in response to pathogen stimulation (Fujiwara and Kobayashi, 2005).

Both acute and chronic inflammation are tightly regulated by coordinated functions of cytokines, chemokines, growth factors and several other processes.

Chemokines provide directional cues for movements of leukocytes which release cytokines at the inflammatory site to regulate the inflammatory process (Luster, 1998). For example, IL-33, a member of the IL-1 superfamily, signals through ST2 and reduces corneal inflammation through upregulation of Th2 type cytokines and anti-inflammatory cytokine production (Hazlett et al., 2010).

1.2.3 Inflammatory Cells

There are several types of cells involved in both acute and chronic inflammatory responses. Langerhans cells are dendritic cells which are localized at many mucosal sites. These cells play an important role in initiation of the immune response in bacterial, viral, and parasitic infection, particularly in the eye (Hazlett et al., 2002). In the skin, these epidermal dendritic cells also regulate immune reactions in both sterile inflammation and infectious disease (Seiffert et al., 2002). During infection, local Langerhans cells take up and process the invading microbial antigens to become fully activated APCs. Activated Langerhans cells are then capable of presenting processed antigens to T cells. Under normal conditions, the corneal epithelium is devoid of MHC class II positive Langerhans cells (Jager, 1992), but possesses its own bone marrow derived cells, including cornea epithelial Langerhans cells and anterior stroma dendritic cells, which can function as APC after antigen stimulation (Hamrah and Dana, 2007). Moreover, in the peripheral conjunctiva, resident Langerhans cells are present with antigen presenting capacity (Hazlett et al., 2002).

Activated Langerhans cells may trigger an inflammatory response by releasing chemokines to chemoattract neutrophils which are the main cell type

participating in acute inflammatory processes (Tamarozzi et al., 2011). During the acute phase of inflammation, particularly after bacterial infection, neutrophils are one of the first-responders among inflammatory cells to infiltrate and accumulate at the inflammatory site. Since the lifespan of neutrophils is relative short (approximately 10 hours), they need to be replenished quickly by more infiltrating cells. Because the inflammatory mediators (such as histamine) released by damaged tissues increase the vessel permeability, the aggregated neutrophils emigrate from vessels into their target tissues down a concentration gradient of the chemotactic factors (Kobayashi, 2006). Neutrophil chemotactic factors include complement component (C5a), cytokines, chemokines and leukotriene B4 (LTB4) (Elner et al., 1990). Chemotactic mediators bind to surface receptors on neutrophils to induce calcium influx and, in turn trigger, assembly of cytoskeletal contractile elements which initiate neutrophil migration (Cronstein et al., 1990). As soon as migration to the target tissue is complete, neutrophils bind to what is recognized as abnormal (e.g., bacterial and virus particles) and extend pseudopodia to surround them. Pseudopodia then fuse to completely enclose these structures to form a cytoplasmic phagosome which fuses with neutrophilic granules. These granules discharge their contents which are exposed and lysed by lysosomal enzymes. If the particles are bacteria, neutrophils kill and destroy them by both oxidative and nonoxidative mechanisms. Important oxidative mechanisms involve the release of hydrogen peroxide generated by the NADPH oxidase system and hypochlorous acid generated by myeloperoxidase (Rosen, 2004). Important nonoxidative molecules include anti-microbial peptides (e.g.,

defensins), lysozyme, neutral serine proteases, and bactericidal/permeability increasing protein (BPI). Moreover, recent studies show that neutrophils are capable of killing bacteria extracellularly. Upon activation, neutrophils secret granule containing proteins and chromatins into the extracellular space and form extracellular fibers or neutrophil extracellular traps (NETs). These fibers bind to both Gram-positive and Gram-negative bacteria, degrading virulence factors, and killing the bacteria (Brinkmann et al., 2004).

The primary cell type of chronic inflammation is the macrophage. Macrophages are derived by the differentiation of monocytes and participate in both innate and adaptive immune responses. Macrophages regulate the immune system through initiating and directing the immune response, including phagocytosis, antigen processing/presentation, cytokine production, generation of reactive oxygen species and nitric oxide release. When monocytes migrate to the injured tissue attracted by chemotactic factors, they will differentiate into macrophages. These recruited cells as well as macrophages at the tissue site (fixed macrophages), ingest the invading pathogen to form a phagosome which fuses with lysosomes within the macrophage cytoplasm. The fusion product, the phagolysosome, contains enzymes and toxic peroxides to digest and kill the pathogen. After processing the pathogen, the macrophage will integrate the antigen into the cell membrane and attach it to MHC class II molecules which present the antigen to helper T cells (Unanue, 1984). The digested microbial products, such as LPS, signal through PRRs (e.g., TLR4) or other surface receptors to stimulate and activate the macrophages to secret both pro- and anti-

inflammatory cytokines (Daniels et al., 2000). Unfortunately, sustained production of pro-inflammatory cytokines released by macrophages may induce chronic inflammation leading to increased tissue damage.

1.3 Corneal Innervation

The cornea is one of the most densely innervated tissues of the body (Muller et al., 2003). Corneal nerves, composed primarily of sensory nerve fibers, are found in many species, including the mouse (de Castro et al., 1998) and human (Al-Aqaba et al., 2010; McKenna and Lwigale, 2010). In addition, sympathetic (Marfurt and Ellis, 1993) and parasympathetic nerves (Marfurt et al., 1998), which respectively originate from the superior cervical and ciliary ganglia, contribute moderately to the innervation of the cornea. At the periphery, nerve bundles enter the cornea in a radial fashion parallel to the corneal surface and then subdivide into smaller side branches (Muller et al., 2003). These nerves are important not only for overall corneal health, particularly, for the epithelial cells they innervate (Baker et al., 1993), but also are essential in neuroimmunoregulation of the tissue.

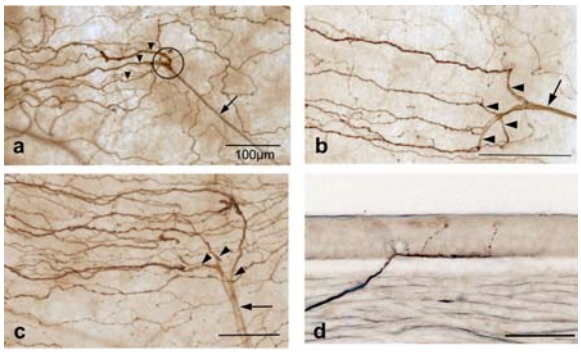

Figure 1. Stromal nerves (human cornea) penetrate through Bowman's membrane in anterior-cornea whole mounts (a–c) and in a 30 µm-thick perpendicular section (d). (From Marfurt et al., 2010).

1.4 Neuroimmunoregulation

Neuroimmunoregulation is the bidirectional communication between the neuroendocrine and immune systems (Weigent and Blalock, 1987). It is required

for immune system homeostasis and optimal host defense (Downing and Miyan, 2000). Neuropeptides, small protein-like molecules, are used by neurons to communicate with each other, and as such, play an important role in these processes (Scharrer, 1987). Of these peptides, two, namely substance P (SP) and vasoactive intestinal peptide (VIP) are the main focus of ongoing studies in the laboratory. SP is a neuropeptide secreted by nerves and inflammatory cells and responsible for a pro-inflammatory type of response (O'Connor et al., 2004); whereas VIP is an anti-inflammatory neuropeptide that affects the immune response antithetically (Delgado et al., 2004).

1.5 VIP

VIP, a 28 amino acid neuropeptide (Figure 2), is a member of the secretin superfamily that includes secretin, growth hormone releasing peptide (GHRP) and pituitary adenylate cyclase activating peptide (PACAP) (Smalley et al., 2009). VIP is synthesized from a precursor molecule (prepro-VIP, 170 amino acids) which contains peptide histidine methionine (PHM; in human tissues) or peptide histidine isoleucine (PHI; its counterpart in other mammalian species) (Delgado et al., 2004). After cleavage by several types of signal peptidases (Bloom et al.,

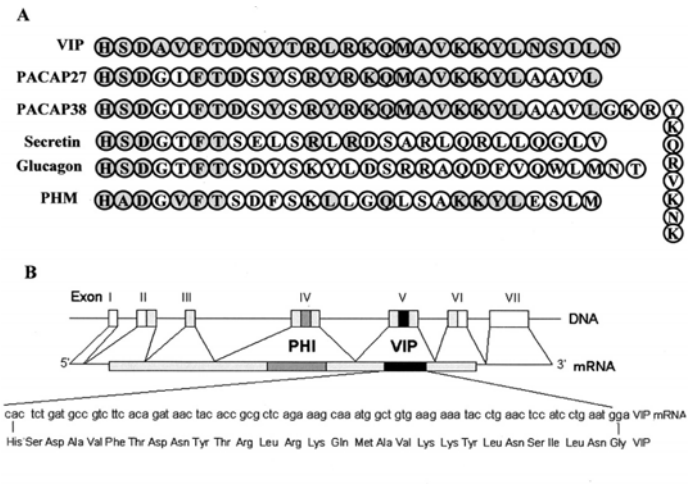

Figure 2. VIP structure: protein and gene. A. sequence of VIP family peptides. B. VIP gene structure (Delgado et al., 2004).

1983; Delgado et al., 2004; Itoh et al., 1983), VIP is delivered by neurons to immune organs and lymphoid tissues in the heart, gastrointestinal tract, lungs, and the kidney (Henning and Sawmiller, 2001). VIP is also detected ocularly in human aqueous humor (Koh et al., 2005), and corneal endothelium (Koh and Waschek, 2000) and also is present in both human and mouse corneal nerves (parasympathetic fibers) (Muller et al., 2003). VIPergic fibers have been identified in the normal corneal epithelium and show more immunostaining after infection in the stroma of BALB/c vs B6 mice (Szliter et al., 2007).

VIP functions by binding to one of three receptors to activate the following two pathways: one cAMP-dependent and the other one cAMP-independent, as are shown in Figure 3 (Chorny et al., 2006). Two

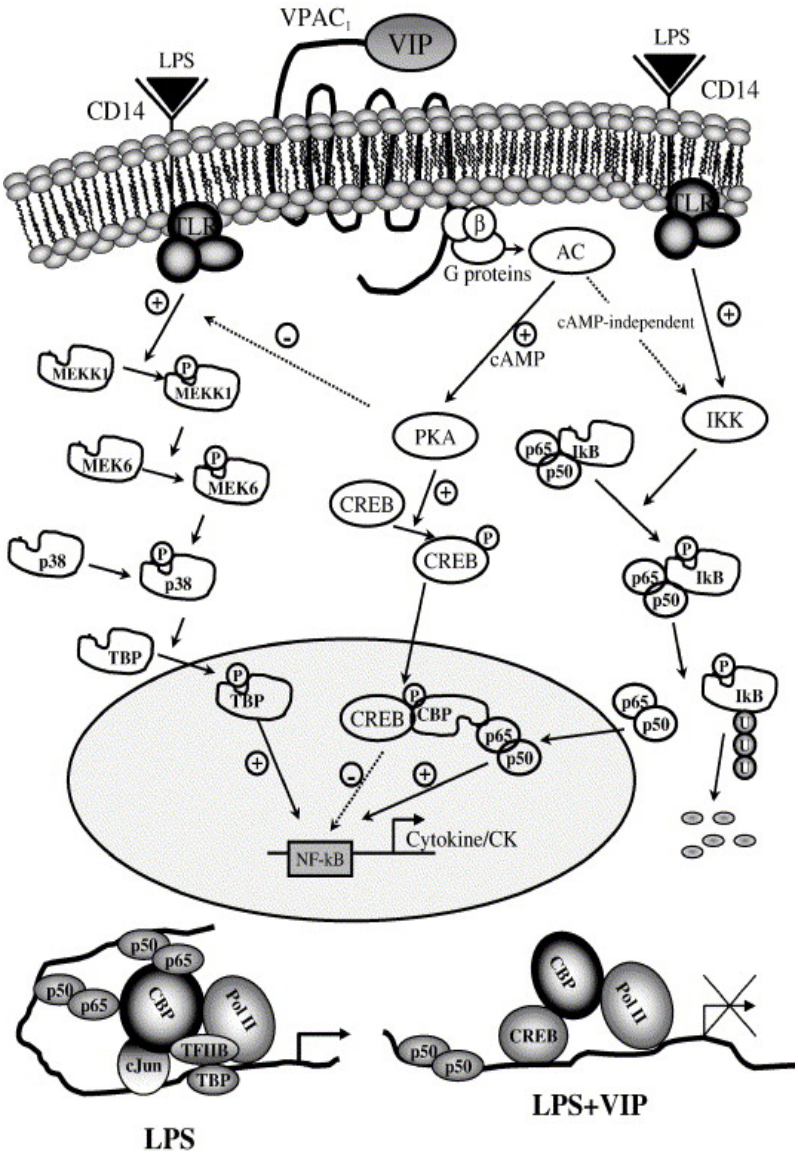

Figure 3. VIP signaling: cAMP dependent and independent pathways (Chorny et al., 2006).

VIP receptors are VIP-shared type 2 (VPAC1 and VPAC2) receptors (Jamen et al., 2000) and a third one is a PACAP-preferring type 1 (PAC1) which shows lower affinity for VIP (Laburthe and Couvineau, 2002). These VIP receptors are type II, G protein coupled receptors (GPCR), which are detected in several types of immune cells (Cocco et al., 2010), and can trigger a cascade of intracellular events that differ depending on cell and receptor types. Both VPAC1 and VPAC2 mRNA are detected preferentially in $CD4^+$, but also in $CD8^+$ T cells purified from mouse spleen; however, no signal is detected in either unstimulated or LPS-stimulated B cells (Delgado et al., 1996). VPAC1 is also constitutively expressed in resting $CD4^+$ and $CD8^+$ T cells purified from human peripheral blood (Lara-Marquez et al., 2001). In the mouse, BALB/c mouse-derived macrophages display significantly higher VIPR1 (VPAC1) mRNA levels when compared with cells from B6 mice in both unstimulated and LPS-stimulated cells, suggesting that the effects of VIP are mainly mediated via VIPR1 on macrophages (Szliter et al., 2007).

The interaction between VIP and its receptors induces a wide variety of biological activities. Among these, VIP displays several non-immune activities, which include: systemic vasodilatation, bronchodilatation, smooth muscle relaxation and neurotrophic effects (Delgado et al., 2004). VIP also accelerates wound healing processes in mechanically-induced damage of human bronchial epithelial cells (Guan et al., 2006), and protects cold-restraint stress-induced gastric lesions and mast cell degranulation (Tuncel et al., 1998). Importantly, VIP has been considered in itself to be a growth factor functioning during mouse

embryonic development (Gressens et al., 1993; Gressens et al., 1997).

The immunological actions of VIP are primarily anti-inflammatory effects in nature (Smalley et al., 2009). Such effects include: inhibition of pro-inflammatory cytokine production and T cell proliferation (Ganea, 1996), inhibition of macrophage chemotaxis and phagocytic activity (De la Fuente et al., 1996) and inhibition of free radical production (Gonzalez-Rey et al., 2007). VIP can promote generation of memory Th2 type T cells (Delgado et al., 2002) and can modulate T cell function in mice, shifting a Th1 to a Th2 type cytokine response (Gutierrez-Canas et al., 2008). In various diseases, VIP reduces production of inflammatory cytokines (TNF-α, IL-12, IL-6, and IL-1β) while stimulating anti-inflammatory cytokines such as IL-10 and IL-1Ra production (Delgado and Ganea, 2008). Most notable to the current dissertation is that previous studies from this laboratory showed that after corneal infection with Pseudomonas aeruginosa (*P. aeruginosa*), exogenous synthetic VIP treatment significantly ameliorated disease and prevented corneal perforation by inhibiting pro-inflammatory cytokines (IFN-γ, IL-1β, TNF-α, and MIP-2), while stimulating anti-inflammatory cytokine (TGF-β1 and IL-10) production (Szliter et al., 2007).

1.6 Growth Factors

Growth factors are putatively involved in many biological activities such as cell growth, proliferation, differentiation, oncogenesis, and wound healing (Turner and Grose, 2010). Classical growth factors include: epidermal growth factor (EGF), keratinocyte growth factor (KGF also known as fibroblast growth factor-7, FGF-7), hepatocyte growth factor (HGF) and vascular endothelial growth factor

(VEGF).

EGF functions through receptor dependent signaling and is well known for its multifaceted actions in development and maintenance of tissue homoeostasis (Lu and Kang, 2010). Amongst its functions, EGF stimulates epithelial growth and differentiation (Cohen, 1962) and enhances wound healing (Brown et al., 1989). EGF is also involved in the regulation of inflammation. As an example, during infection with an enteropathogenic strain of *Escherichia (E.) coli,* oral administration of EGF significantly reduced *E. coli* colonization (Buret et al., 1998). EGF also accelerates corneal epithelial wound healing both *in vivo* and *in vitro* and corneal re-epithelialization in an organ culture wound model (Lu et al., 2001). KGF, or FGF-7, binds to FGFR2 (Rotolo et al., 2008) and is a homeostatic factor with the potential for epithelial protection and repair after various insults (Finch and Rubin, 2004). KGF is also considered as a cytokine which mediates mesenchymal-epithelial interactions (Rubin et al., 1995). Regarding its role in infection, intratracheal treatment with KGF increased clearance of *P. aeruginosa* in an induced lung injury bacterial infection model (Viget et al., 2000), implying its efficacy as an anti-bacterial agent. HGF, which binds to c-met receptor (Bottaro et al., 1991), is a multifunctional polypeptide which regulates diverse cellular processes, including cell survival, proliferation, migration, and differentiation (Liu, 2004). HGF has also been shown to have an anti-inflammatory effect due to its ability to block NF-κB signal pathway transduction and E-selectin expression in human umbilical vein endothelial cells (Gong et al., 2006). Interestingly, the injured cornea releases IL-1 from epithelial cells to induce production of both

HGF and KGF in keratocytes (Suzuki et al., 2003), suggesting a potential beneficial effect of HGF and KGF in corneal wound healing. Another type of growth factor, VEGF (VEGF-A), as a classical growth factor, can influence cell proliferation, migration and survival (Penn et al., 2008), but it also can signal through its receptors VEGFR1 and VEGFR2 to control vasculogenesis (Rini and Small, 2005; Shibuya and Claesson-Welsh, 2006). In general, binding of VEGF-A to VEGFR1 has anti-angiogenic effects by negative regulation of VEGFR2. If levels of VEGFR2 are reduced by an antagonist (Tille et al., 2001) or in VEGFR2 knock out mouse embryos (Nicosia, 1998), reduced or lack of VEGFR2 inhibits VEGF induced vascular angiogenic effects. It has been shown that VIP increases VEGF (Valdehita et al., 2007) expression and secretion in human cancer cells and nerve growth factor (NGF) expression (Hill et al., 2002) in mouse embryonic neural tube.

1.7 TLRs

TLRs, the best characterized class of PRRs (Janeway and Medzhitov, 1998), play an important role in pathogen recognition and activation of innate immunity, which may lead to activation of adaptive immunity (Medzhitov and Janeway, 2000). Thirteen TLRs are present in humans and mice with the exception that TLR10 is only present in humans and TLRs 11, 12 and 13 are only expressed in mice (Beutler, 2004). TLRs are expressed in a variety of ocular tissues, including cornea, and several other tissues, such as the retina. For example, after infection with *P. aeruginosa*, TLR4 is expressed in the corneal epithelium and stroma (Jiang el al., 2012) and its expression is significantly

increased when compared to the normal, uninfected cornea (Huang et al., 2006a).

TLR family	Exogenous ligands	Endogenous ligands	Adaptors proteins
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria)	N.D.	MyD88/TIRAP
TLR2	Lipoprotein/lipopeptides (a variety of pathogens), PGN (Gram-positive bacteria), lipoteichoic acid (Gram-positive bacteria), lipoarabinomannan (mycobacteria), atypical LPS (*Leptospira interrogans* and *Porphyromonas gingivalis*), phenol-soluble modulin (*Staphylococcus epidermidis*), glycoinositolphospholipids (*Trypanosoma Cruzi*), glycolipids (*Treponema maltophilum*), porins (Neisseria), zymosan (fungi)	HsP70	MyD88/TIRAP
TLR3	Double-stranded RNA (virus)	mRNA	TRIF
TLR4	LPS (Gram-negative bacteria), fusion protein (virus), HSP (*Chlamydia pneumoniae*), taxol (plant)	HsP60, fibrinogen, fibronectin, oligosaccharides of HA	MyD88,TIRAP, TRIF, TRAM
TLR5	Flagellin (bacteria)		MyD88
TLR6	Di-acyl lipopeptides (mycoplasma), zymosan (fungi), lipoteichoic acid (gram-positive bacteria), soluble tuberculosis factor	N.D.	MyD88/TIRAP
TLR7	GU rich single-stranded RNA (virus), imidazole quinolines and loxoribine (synthetics)	N.D.	MyD88
TLR8	GU rich single-stranded RNA (virus), imidazole quinolines (synthetic)	N.D.	MyD88
TLR9	DNA (bacteria, virus), CpG ODNs (synthetic)	Chromatin immune complexes	MyD88
TLR10	N.D.	N.D.	N.D.
TLR11	Profilin-like protein (toxoplasma), uropathogenic bacteria	N.D.	MyD88
TLR12	N.D.	N.D.	N.D.
TLR13	N.D.	N.D.	N.D.

HSP: heat-shock protein; PGN: peptidoglycan; LPS: lipopolysaccharide; HA: hyaluronic acid; ODNs: ligodeoxynucleotides; N.D.: not determined.

Table 1. Toll-like receptors, ligands and adaptor molecules (Gomariz et al., 2007).

TLRs differ from each other in ligand specificities, expression patterns, and target genes (Dowling et al., 2008). These receptors recognize highly conserved PAMPs, including bacterial cell-surface LPS, lipoproteins, flagellin, double-stranded RNA of viruses or the unmethylated CpG islands of bacterial and viral DNA. In addition, TLR recognition is ligand specific, as summarized in Table 1.

Based on their functions, TLRs can be divided into pro-inflammatory and anti-inflammatory types. Pro-inflammatory TLRs function predominately as homodimers, with some exceptions, as is the case for TLR1, TLR2 and TLR6. TLRs also require co-receptors for optimal ligand sensitivity. For example TLR4 requires myeloid differentiation protein 2 (MD-2) to recognize LPS (Chaudhuri et

al., 2005). Moreover, the delivery of LPS to MD-2 can be facilitated by CD14 and LPS binding protein (LBP) (Kumar and Yu, 2006). Upon activation, adaptor molecules, such as MyD88, TIRAP (also called MAL), TRIF and TRAM, are recruited by TLR for signal amplification. TLRs can also amplify their signals by a MyD88-independent pathway which requires TRIF, TBK1 and IRF3 to amplify signaling (Takeda and Akira, 2004). These proteins activate downstream molecules within the cell leading to NF-κB activation and consequently, induction of genes that regulate the inflammatory response (Zhang and Ghosh, 2001). TLRs also are involved in regulation of anti-inflammatory responses. In this regard, the negative regulators of TLR signaling, including single Ig IL-1R-related molecule (SIGIRR) and ST2, have been identified and characterized. SIGIRR and ST2 sequester the recruitment of adaptor molecules including MyD88 and IL-1 receptor-associated kinase (Sweet et al., 2001). They also inhibit the formation of pro-inflammatory TLR signaling complexes and regulate pro-inflammatory cytokine and chemokine production (O'Neill, 2003).

TLR signaling is tightly regulated, since prolonged or excessive activation of TLRs may lead to uncontrolled inflammation in the host (Shibolet and Podolsky, 2007). In this regard, in a recent study from this laboratory, murine beta-defensin-2 (mBD2) was found to negatively regulate TLR2, TLR4 and TLR9 signaling, and promoted the resistance response to corneal infection with *P. aeruginosa* (Wu et al., 2009a). Other studies showed that numerous proteins, such as IL-1R-associated kinase M (IRAK-M), A20 and Tollip, also negatively regulate TLR signaling (Whitmore et al., 2007). Interestingly, in other studies, intraperitoneal

administration of VIP (1 nM) down-regulated TLR-2 and TLR-4 expression in colonic extracts in a model of Crohn's disease and TLR-4 expression on the surface of macrophages, dendritic cells and lymphocytes within the mesenteric lymph nodes (Gomariz et al., 2005).

1.6 Overview and Significance

In the United States, microbial keratitis is the most frequent complication for extended wear contact lens users. Its incidence among this group is 25,000-30,000 cases each year, with treatment estimated at between $15 and $30 million, an extensive medical and economic impact (Khatri et al., 2002). *P. aeruginosa*, a common Gram-negative bacteria associated with microbial keratitis, accounts for approximately 70% of the culture-proven cases in contact lens users (Schein et al., 1989).

P. aeruginosa is an opportunistic pathogen that produces lipopolysaccharide (LPS/endotoxin) and numerous other virulence factors, including but not limited to bacterial toxins which contribute to the progression of microbial keratitis (Hazlett, 2004). However, the prognosis of the disease also is influenced by host immunity, the inflammatory response, and successful treatment with antibiotics (Hazlett, 2004). Clinically, the disease progresses rapidly and is characterized by a suppurative stromal infiltrate, inflammatory epithelial edema, ocular pain and redness, stromal ulceration, decreased vision, and oftentimes, corneal perforation (Hazlett, 2007).

In order to better understand disease pathogenesis, animal models of *P. aeruginosa*-induced corneal infection have been established and include topical

application of the bacteria after wounding, intrastromal inoculation, or placement of a contaminated contact lens or suture on the cornea (Hazlett LD, 2004). Of particular interest for the current studies is the defined inbred murine model of *P. aeruginosa*-induced corneal infection. C57BL/6 (B6) mice are Th1 responsive to *P. aeruginosa* infection and are classified as susceptible, since the cornea perforates at 5-7 days post infection (p.i.) (Hazlett et al., 2000). In contrast, BALB/c mice are Th2 responsive to *P. aeruginosa* infection and are classified as resistant, since less destruction of the stroma occurs and the cornea does not perforate at 5-7 days p.i. (Hazlett et al., 2000). Furthermore, a recent study from this laboratory has found that VIP balances Th1/Th2 type cytokine levels, leading to protection against corneal perforation in susceptible B6 mice (Szliter et al., 2007).

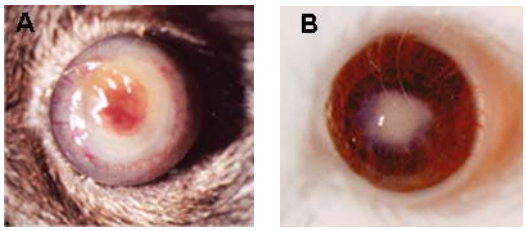

Figure 4. Slit lamp photographs of mouse eyes infected with *P. aeruginosa* at 7 days p.i. A, B6, susceptible strain; B, BALB/c, resistant strain.

In this regard, the goal of the current dissertation is to test the following hypotheses:

1. VIP modulates growth factor expression in microbial keratitis.
2. VIP regulates TLR signaling pathways in microbial keratitis.
3. VIP is not required for growth factors and their receptor expression in the normal cornea, but critical for their regulation in the infected cornea.

Chapter 2

VIP AND GROWTH FACTORS IN THE INFECTED CORNEA

2.1 Abstract

Purpose. VIP is an anti-inflammatory neuropeptide that down-regulates pro-inflammatory cytokines and promotes healing in a susceptible model of *P. aeruginosa* keratitis. Growth factors also play a role in corneal healing and restoration of tissue homeostasis after wounding. However, whether VIP treatment modulates growth factors to promote healing in the infected cornea remains untested and is the purpose of this study. Methods. B6 mice were injected with VIP and mRNA and protein levels, and immunostaining for EGF, FGF, HGF, and VEGF-A were done. Exogenous treatment with a mixture of the growth factors also was tested and levels of cytokines, defensins, and bacterial counts were determined. Results. Real-time RT-PCR, immunostaining, and ELISA data demonstrated that treatment with VIP enhanced levels of EGF, FGF, and HGF during disease, and that VEGF-A, and associated angiogenic molecules also were increased by VIP. Moreover, immunohistochemical studies confirmed that both epithelial and stromal cells participated in growth factor production. Most notably, treatment with a mixture of EGF, FGF, and HGF after disease onset, prevented corneal perforation when compared with controls. This outcome was associated with down-regulation of pro-inflammatory cytokines such as macrophage inflammatory protein-2 (MIP-2), up-regulation of anti-inflammatory cytokines such as TGF-β, and antimicrobials β-defensins 2 and 3, as well as decreased plate counts at 1 day postinfection (p.i.) ($p = 0.0001$).

Conclusions. Collectively, the data provide evidence that VIP treatment modulates growth factors, angiogenic molecules, and defensins in the infected cornea and that this in turn promotes healing and restoration of tissue homeostasis.

2.2 Introduction

An opportunistic, gram-negative pathogen, *P. aeruginosa* is one of the most virulent organisms associated with microbial keratitis and is often associated with contact lens usage (Wilhelmus, 1987). Both bacterial (e.g., lipopolysaccharide) and host factors released from infiltrating cells during infection are thought to contribute to a rapidly progressing liquefactive stromal necrosis (Iglewski et al., 1977; Kernacki et al., 2000; Moon et al., 1988; Pillar and Hobden, 2002). Experimental murine models of the disease have been established: Th1 responder mouse strains (e.g., B6) are susceptible (cornea perforates), whereas Th2 responder strains (e.g., BALB/c) are resistant (cornea heals) (Hazlett et al., 2000).

In previous studies using these murine models, we provided evidence that an anti-inflammatory neuropeptide, VIP given exogenously, promotes resistance against *P. aeruginosa* corneal infection by regulation of cytokine production and subsequent alteration of the host inflammatory cell response (Szliter et al., 2007). Neuropeptides, such as VIP are small protein-like molecules used by neurons to bidirectionally communicate with the cells of the immune system (Weigent and Blalock, 1987). In the eye, VIP can be detected in corneal nerves and the aqueous humor in both mice and humans (Delgado et al., 2004). Others also

have shown previously that VIP reduces pro-inflammatory cytokine production, limits cell-mediated immunity, and inhibits macrophage and T cell proliferation (Delgado et al., 2004); and by down-regulation of IL-6 and TNF-α, the neuropeptide showed a protective effect in models of endotoxemia (Delgado et al., 1999a). Despite these anti-inflammatory effects which have been reported in diverse systems and which clearly modulate disease outcome, the role of VIP in regulation of corneal healing, specifically, control of growth factor production, has not been examined.

Classically, growth factors, which also may be considered as cytokines, bind to their receptors and regulate cell growth, proliferation, migration, and differentiation (Turner and Grose, 2010). Thus, the current studies investigated the potential regulation by VIP of growth factors in *P. aeruginosa*-infected corneas in B6 (susceptible) mice that exhibit lower levels of VIP when compared with resistant (BALB/c) mice (Hazlett et al., 2000). Exogenous VIP treatment was given to B6 mice to determine whether VIP modulates growth factor production and thereby, favors resolution of disease. Data from these studies provide evidence that VIP treatment leads to up-regulation of growth factor production in the cornea after *P. aeruginosa* infection, and that both epithelial and stromal cells are participatory. Furthermore, evidence is provided to show that treatment with a mixture of growth factors, given topically after infection, prevents perforation of the cornea. Mechanistically this is achieved by down-regulation of pro-inflammatory cytokines, up-regulation of anti-inflammatory cytokines, antimicrobials β-defensins 2 and 3, and decreased bacterial plate counts. The

data suggest that VIP modulates growth factor production in the infected cornea and that this in turn, contributes to better disease outcome.

2.3 Materials and Methods

Infection

Eight-week-old female B6 mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with ethyl ether and placed beneath a stereoscopic microscope at ×40 magnification. The cornea of the left eye was wounded (Rudner et al., 2000) and a 5-μL aliquot containing 1.0×10^6 CFU/μL of *P. aeruginosa* (American Type Culture Collection, strain 19,660, Manassas, VA) was topically delivered. Animals were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

Ocular Response to Bacterial Infection

Corneal disease was graded (Hazlett et al., 1987): 0 = clear or slight opacity, partially or fully covering the pupil; +1 = slight opacity, fully covering the anterior segment; +2 = dense opacity, partially or fully covering the pupil; +3 = dense opacity, covering the entire anterior segment; and +4 = corneal perforation or phthisis. After infection, a clinical score was recorded (days 1, 3, 5, and/or 7) for each mouse (n = 5 per group per treatment for two experiments = 20 mice).

VIP Treatment

In vivo treatment with synthetic VIP (Bachem, Torrance, CA) has been described previously (Szliter et al., 2007). In brief, B6 mice received daily IP injections of VIP (5 nM in 100 μL sterile phosphate buffered saline, PBS) at 1 day

before infection (Day = −1) through a maximum of 7 days p.i. Control mice were injected similarly with sterile PBS (100 µL).

Real-time RT-PCR

Total RNA was isolated from an individual cornea of PBS, VIP, or growth factor-treated mice (n = 5 per group per time per treatment for two experiments = 140 mice) using RNA-Stat 60 (TelTest, Friendswood, TX), per the manufacturer's recommendations and quantitated spectrophotometrically (260 nM). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase. The 20-µL reaction mixture contained 200 U of MMLV-reverse transcriptase, 10 U of RNasin, 500 ng of oligo (dT) primers, 10 mM dNTPs, 100 mM DTT, and MMLV reaction buffer (Invitrogen, Carlsbad, CA). After, cDNA was amplified using SYBR Green Master Mix (SABiosciences, Frederick, MD), per the manufacture's recommendation. Briefly, the 20-µL reaction system contained 10 µL of SYBR Green PCR Master Mix, 1.0 µL of 10-µM primer mix, 2 µL of cDNA (diluted 1/10), and diethyl pyrocarbonate water. All primers were purchased from SABiosciences, except for β-actin which was designed using PrimerQuest (Integrated DNA Technologies, Cambridge, MA) whose forward primer is 5'-GAT TAC TGC TCT GGC TCC TAG C-3'; the reverse primer is 5'-GAC TCA TCG TAC TCC TGC TTG C-3'. The primer sequences for MIP-2, TNF-α, TGF-β (Szliter et al., 2007) and β-defensins-2 and 3 (Wu et al., 2009a; Wu et al., 2009b) were provided previously.

Quantitative real-time RT-PCR reactions were performed (MyiQ single color real-time RT-PCR detection system; Bio-Rad, Hercules, CA). Optimal

conditions for PCR amplification of cDNA were established using routine methods (Heid et al., 1996). Relative mRNA levels were calculated after normalization to β-actin.

Immunofluorescent Staining

Normal, uninfected, and infected eyes were enucleated from VIP, PBS, and growth factor-treated B6 mice (n = 5 per group per time for two experiments = 60 mice) at 1 and 5 days p.i. Tissue was immersed in 1X Dulbecco's PBS (Mediatech, Inc., Herndon, VA), embedded (Tissue-Tek OCT compound; Miles, Elkhart, IN), and frozen in liquid nitrogen. Ten-micrometer thick sections were cut, mounted to polylysine-coated glass slides, and incubated in a moist chamber at 37°C overnight. After a 2-minute fixation in acetone, nonspecific staining was blocked with 10 mM sodium phosphate buffer containing 2.5% bovine serum albumin and donkey IgG (1:100) for 30 minutes at room temperature. For immunostaining, sections were incubated for 1 hour each with goat anti-mouse VEGF-A, EGF and/or HGF (R&D, Minneapolis, MN), goat anti-human FGF-7 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse β-defensin 2 (1:50, Santa Cruz) or rabbit anti-mouse β-defensin 3 (1:50, Santa Cruz), followed by a secondary Ab, Alexa Fluor 594 conjugated donkey anti-goat (1:1500, Invitrogen, Eugene, OR) or Alexa Fluor 594 conjugated donkey anti-rabbit Ab (1:1500, Invitrogen; both = red color). Sections were then incubated for 2 minutes with nuclear acid stain (1:20,000, SYTOX Green; Lonza, Walkersville, MD). Controls were similarly treated, but the primary Ab was replaced with the same host IgG. Finally, sections were visualized and digital images captured with a confocal laser

scanning microscope (Leica TCS SP2; Leica Microsystems, Bannockburn, IL).

ELISA

Growth factor protein levels were tested using ELISA kits (R&D). Corneas from PBS and VIP-treated B6 mice were individually collected (n = 5 per group per time per treatment for two experiments = 100 mice) at 1, 5, and 7 days p.i. Corneas were homogenized in 0.5 mL of PBS with 0.1% Tween 20. All samples were centrifuged at 13,000 rpm for 5 minutes and an aliquot of each supernatant was assayed in duplicate for EGF, KGF/FGF-7 (FGF), HGF, and VEGF-A per the manufacturer's instruction. ELISA also was used to test for receptor protein levels for VEGFR1 and VEGFR2. The reported sensitivity of these assays is <0.95 pg/mL for EGF, <156.25 pg/mL for HGF, 15.62 pg/mL for KGF/FGF-7 (FGF), <3.0 pg/mL for VEGF-A, <9.8 pg/mL for VEGFR1, and <27 ng/mL for VEGFR2.

Growth Factor Treatment

Recombinant mouse EGF, KGF/FGF-7 (FGF) and HGF (all from R&D) were dissolved in PBS to a final concentration of either 40 µg/mL (EGF and HGF) or 20 µg/mL (FGF). After mice were infected, 5 µL of a mixture of the three growth factors (2 µL EGF, 0.4 µL FGF, and 2 µL HGF in 0.6 µL PBS) was delivered topically, once on the day of infection, twice at 1 day p.i. and once daily through 4 days p.i. Concentrations for the growth factors were selected and modified after review of the literature (Baldwin and Marshall, 2002; Forster et al., 2008; Fredj-Reygrobellet et al., 1987). Controls received PBS similarly.

Bacterial Plate Counts

Corneas from PBS or growth factor-treated B6 mice were collected (n = 5

per group per time for two experiments = 60 mice) at 1, 3, and 5 days p.i. and the number of viable bacteria were quantitated. Individual corneas were homogenized in sterile water containing 0.85% (wt/vol) NaCl containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on *Pseudomonas* isolation agar (Difco Laboratories, Sparks, MD) in triplicate and plates were incubated overnight at 37°C and bacteria counted. Results are reported as $\log^{10}$ number of CFU per cornea ± SEM.

Statistics

The difference in clinical score between the two groups at each time point was tested by the Mann-Whitney U test. An unpaired, two-tailed Student's t-test was used to determine significance of all other data. For each test, differences were considered significant at $P < 0.05$ and represented as mean ± SD. Each experiment was performed twice for reproducibility and representative data from a typical experiment are shown.

2.4 Results

mRNA Expression of Growth Factors

Whether VIP modulated mRNA expression patterns for selected growth factors was first assessed. The mRNA expression patterns of EGF, FGF, HGF, and EGF receptor (EGFR) are shown in Figures 5A, 5C, 5E, and 5G. Compared with PBS, treatment with VIP significantly increased EGF (Fig. 5A) mRNA expression in normal and infected cornea at 3 days p.i. ($P < 0.05$ and $P < 0.001$). FGF mRNA expression (Fig. 5C) was not significantly different between groups at all times tested. HGF mRNA expression (Fig. 5E) was significantly up-regulated

by VIP in normal, uninfected, and infected corneas at 1 and 3 days p.i. ($P < 0.01$, $P < 0.05$, and $P < 0.01$). EGFR was significantly increased at days 5 and 7 p.i. ($P < 0.01$ for both). There were no significant differences between the two groups for any of the growth factors at other time points tested (Figs. 5A, 5C, 5E, 5G).

ELISA

Protein expression patterns for the three growth factors were assessed after examination of mRNA expression patterns. Protein expression for EGF, FGF, and HGF also is shown in Figures 5B, 5D, and 5F. When compared with PBS, VIP treatment upregulated EGF protein expression (Fig. 5B) later in disease (5 and 7 days p.i.; $P < 0.01$ and $P < 0.01$). Before infection and at 1 day p.i., levels were higher in the PBS treated group ($P < 0.001$ and $P < 0.001$). VIP treatment enhanced protein levels of FGF (Fig. 5D) only at 7 days p.i. ($P < 0.05$). For HGF (Fig. 5F), VIP significantly increased protein expression at 1 and 5 days p.i. ($P < 0.05$ and $P < 0.01$) and remained elevated at 7 days p.i., but was not significant.

mRNA Expression of Angiogenic Molecules

Whether mRNA expression of angiogenic molecules were changed after VIP also was assessed. For VEGF-A (Fig. 6A), treatment with VIP versus PBS significantly increased mRNA levels only in the normal, uninfected cornea ($P < 0.001$); but no difference between the two groups was seen at either 1 or 5 days p.i. For VEGFR1 (Fig. 6C), VIP increased mRNA expression in normal, uninfected cornea ($P < 0.001$), did not differ between groups at 1 day, but decreased levels at 5 ($P < 0.01$) days p.i. VIP increased VEGFR2 mRNA expression (Fig. 6E) in normal, uninfected ($P < 0.05$) cornea but no differences

were seen between groups at 1 or 5 days p.i.

ELISA Analysis

Protein expression patterns for angiogenic molecules were assessed after VIP treatment and compared with controls. VIP versus PBS significantly increased VEGF-A (Fig. 6B) protein expression in the infected cornea at 7 days p.i. ($P < 0.05$). VEGFR1 (Fig. 6D) was not significantly different between groups at all times tested. VEGFR2 protein levels (Fig. 6F) were significantly increased (more than two-fold) by VIP versus PBS at 7 days p.i. ($P < 0.001$). No differences in protein levels were detected between the two groups at the other times tested (Figs. 6B, 6D, 6F). Consistent with these data (Figs. 6G, 6H), a photograph taken with a slit lamp at 7 days p.i., shows greater vascularity in the peripheral cornea of VIP- (Fig. 6H) versus PBS- (Fig. 6G) treated mice.

Immunostaining

Immunohistochemistry was used to allow us to spatially localize EGF, FGF, HGF, and VEGF-A in the corneas of VIP- versus PBS-treated B6 mice at 1 (Figs. 7A–J) and 5 days p.i. (Figs. 8A–J). At 1 day p.i. slightly increased immunostaining was seen for EGF after PBS versus VIP treatment (Figs. 7A, 7B, and insets) in the epithelium and stroma. For FGF, staining was similar between groups and in the epithelium (Figs. 7C, 7D). For HGF, VIP-enhanced stromal immunostaining, while more epithelial staining was seen in the PBS group (Figs. 7E, 7F), VEGF-A staining was similar between groups and seen in both the epithelium and stroma (Figs. 7G, 7H, and insets). Negative controls (primary antibody substituted with species-specific IgG) showed no positive

immunostaining for each factor tested. An example of these negative controls shows lack of positive immunostaining (Figs. 7I, 7J) seen with each of the factors tested.

At 5 days p.i., EGF (Figs. 8A, 8B), FGF (Figs. 8C, 8D), HGF (Figs. 8E, 8F), and VEGF-A (Figs. 8G, 8H) staining of increased intensity was detected in the epithelium and stroma of VIP- versus PBS-treated mice. Negative controls (primary antibody substituted with species-specific IgG) showed no positive immunostaining for EGF, FGF, or VEGF-A staining at either time point tested for either treatment. An example of these negative controls shows typical lack of positive immunostaining seen with each of the factors tested (Figs. 8I, 8J).

Growth Factor Treatment

Because results suggested that VIP exerted some of its healing effects in the infected cornea via modulation (enhancing) of growth factor production, the next study was performed to test that hypothesis. To assess whether the healing effects of VIP were through growth factor modulation, a mixture of EGF, FGF, and HGF was topically applied after infection. Clinical score data (Figure 9A) and photography with a slit lamp (Figs. 9B, 9C) revealed significantly less disease in the growth factor-treated mice at 5 days p.i. ($P < 0.05$ for clinical score). Although most corneas of growth factor-treated mice exhibited a +3 opacity, (only one perforated) all corneas of PBS-treated mice had perforated at 5 days p.i. In addition, RT-PCR analysis of growth factor- versus PBS-treated corneas showed that mRNA levels of the pro-inflammatory cytokine, MIP-2 (Fig. 9D), were significantly decreased ($P < 0.05$), while TGF-β (Fig. 9F; $P < 0.001$), VEGF-A (Fig.

9G; P < 0.001), and β-defensins 2 (Fig. 9H; P < 0.05) and 3 (Fig. 9I; P < 0.001) all were up-regulated. There was no difference between the two treatment groups for TNF-α (Fig. 9E), or for VEGFR1 or VEGFR2 (data not shown).

Immunostaining

To begin to determine the mechanism by which growth factors given topically contributed to corneal defense, we used immunohistochemistry to examine infected corneas for antimicrobial molecules which had been shown by this laboratory to be protective in the infected cornea (Wu et al., 2009a; Wu et al., 2009b). Staining for antimicrobial β-defensins in the cornea is shown in Figures 10A–F. After growth factor versus PBS treatment, increased immunostaining for mBD2 was detected in both the epithelium and stroma at 5 days p.i. (Figs. 10A, 10B). However, negligible staining for mBD3 was observed, with no difference between groups (Figs. 10C, 10D). Controls (primary Ab substituted with species-specific IgG) showed no positive immunostaining for mBD2 or mBD3. These negative controls showed lack of positive immunostaining and appeared similar to staining with nuclear label (SYTOX Green; Lonza; Figs. 10E, 10F).

Bacterial Counts

To determine whether increased β-defensin production correlated with decreased bacterial number in the cornea, bacterial plate counts (Fig. 10G) were used to detect viable bacteria in the infected cornea of mice treated with growth factors versus PBS at 1, 3, and 5 days p.i. When compared with controls, growth factor treatment led to decreased bacterial plate counts at 1 (P = 0.0001), 3 (P = 0.35), and 5 days p.i. (P = 0.09), but was not significant at either of the latter

times.

2.5 Discussion

Previously, this laboratory has provided evidence that VIP, a 28-amino acid, anti-inflammatory neuropeptide, regulates cytokine production in *P. aeruginosa*-induced keratitis, leading to decreased stromal destruction in a B6 mouse model in which the infected cornea normally perforates (Szliter et al., 2007). In this regard, VIP is involved in the regulation of a wide variety of immune reactions and among its numerous anti-inflammatory functions, participates in the maintenance of immune homeostasis (Delgado et al., 2004). In addition, VIP has been considered in itself to be a growth factor (Gressens et al., 1997), whereas other studies have suggested that it does not function independently, but that it may regulate other more classic growth factors such as VEGF (Valdehita et al., 2007) and NGF (Hill et al., 2002). These growth factors can facilitate noninfectious wound healing and may also function as anti-inflammatory cytokines.

The present study indicates that VIP promotes production of EGF, FGF, HGF, and VEGF-A and participates in regulating angiogenesis, all of which contribute to resistance to *P. aeruginosa* keratitis. It is highly likely that these intriguing effects may be indirect, through facilitating wound healing, and/or epithelial barrier function, for example. It also is possible that the effect of VIP and growth factors may modulate inflammation, balancing its required contribution to the healing process, with its down-regulation which is permissive to healing. In this regard, the present study has shown that both epithelial and

stromal cells participate in growth factor production and are modulated by VIP treatment. Unfortunately, there is a dearth of other studies concerning the role of VIP regulating many of the classic growth factors which have been studied herein; thus, the effects of VIP on EGF, HGF, and FGF production are initially characterized in the present study. Despite this, previous investigators have shown that VIP can directly activate the EGFR (Bertelsen et al., 2004) which is confirmed at the mRNA level in the present study described herein. In addition, we found that VIP increases EGF expression at the mRNA and protein levels and by immunostaining at mid-to-later times p.i. However, early after infection, EGF mRNA levels were similar between the two groups, but unexpectedly, protein and immunostaining was slightly increased in the PBS- versus VIP-treated group initially, suggesting that VIP has a complex regulatory function for this growth factor. In addition, investigators reported that either EGF or VIP inhibited the secretion of several pro-inflammatory cytokines, such as IL-1 and IL-8, and also reduced adhesion of neutrophilic leukocytes to bronchial epithelial cells in vitro (Zhang et al., 2006). We reported previously that VIP significantly reduced pro-inflammatory cytokine production in microbial keratitis, including IL-1 and MIP-2 (murine homolog of IL-8) levels (Szliter et al., 2007), but the present study shows that this effect may have been enhanced indirectly through VIP modulation of EGF. In fact, in another study it was reported that during infection with an enteropathogenic strain of *Escherichia coli* oral administration of EGF significantly reduced *E. coli* colonization (Buret et al., 1998). These findings are consistent with a past study which has shown that VIP reduces *P. aeruginosa*

corneal bacterial load and with the present study which suggests that this effect may have been at least in part, due to up-regulation of EGF at later times p.i. via VIP administration.

Another growth factor, KGF/FGF-7 also participates in disease resolution through reduction of bacterial load. In this regard, clearance of *P. aeruginosa* was increased by intratracheal treatment with KGF in an induced lung injury bacterial infection model (Viget et al., 2000). KGF also increased antimicrobials β-defensin 2 and cathelicidin (LL-37) mRNA levels which were associated with reduced bacterial load in *P. aeruginosa* infection in a skin model (Erdag et al., 2004). These data are consistent with the present study, in that VIP increased FGF (KGF/FGF-7) expression and when used in a growth factor cocktail, applied to the cornea after infection, increased β-defensins 2 (mRNA and protein) and 3 (mRNA). These data compliment prior work from this laboratory in which we showed that in the cornea, both defensins are protective in a murine (BALB/c, resistant) model of *P. aeruginosa*-induced microbial keratitis (Wu et al., 2009a; Wu et al., 2009b); this inbred strain also was shown to have increased levels of VIP when compared with B6 mice after infection (Szliter et al., 2007).

VIP also increased HGF expression at both mRNA and protein levels (ELISA and immunostaining). This growth factor has anti-inflammatory properties (Gong et al., 2006), in that it can target vascular endothelial cells and disrupt NF-κB signaling (Giannopoulou et al., 2008), critical to regulating inflammation. We also have reported that VIP treatment of susceptible B6 mice, decreased pro-inflammatory cytokines, including but not limited to IFN-γ, IL-1β, TNF-α, and MIP-

2 and enhanced protective cytokines such as TGF-β and IL-10 (Szliter et al., 2007). TGF-β, in addition to its regulation of immune responses, is also profibrotic (Li et al., 2006), which may reflect increased wound healing in the VIP- and/or growth factor-treated mice in which stromal destruction is diminished. Because the present study has shown that HGF levels are enhanced after VIP treatment in B6 mice, it is not inappropriate to suggest that the observed protective effects of VIP could be affected by HGF enhancement. Immunostaining for VEGF-A was observed in both epithelium and stroma at 5 days p.i., confirming the ELISA data and providing information regarding protein localization. High expression of VEGF-A mRNA in the newly formed epidermis after wounding (Hong et al., 2004) is somewhat consistent with our data, which shows a more modest effect. Elevated expression of VEGF-A may be indicative of its exerting its growth factor functions to induce healing, cell division, migration, cell survival, and proliferation (Rini and Small, 2005). In addition to its growth factor properties, VEGF-A can also regulate angiogenesis (Nicosia, 1998; Tille et al., 2001). Data from the present study show that VIP upregulates VEGFR2 protein levels more than two-fold at 5 days p.i. These data indicate that VIP, via up-regulation of VEGFR2 has an angiogenic effect that is beneficial to the avascular cornea for disease resolution, (confirmed by photography using a slit lamp). The data are also consistent with the tenet that the cornea must balance its usually high threshold for resisting vascular ingrowth (corneal hemangiogenesis) with an ability to react to sight-threatening injuries (e.g., *P. aeruginosa*), with a robust and rapid angiogenic response, required to enhance

immune defenses (Cursiefen et al., 2006; Streilein, 2003). We hypothesize that inflammation is reduced through the actions of VIP on pro-inflammatory cytokines and chemokines, but also, with increased VEGF-A and R2 protein levels, as reported herein, immune cells are better able to enter the cornea, more rapidly participate in bacterial disposal, thus limiting bacterial-induced stromal damage; this is followed by healing through the role of VIP in promoting anti-inflammatory cytokines (Szliter et al., 2007), and modulating growth factor production as shown herein. These data also are consistent with other studies using a rat sponge model for quantitative assessment of angiogenesis which have shown that VIP treatment improved angiogenesis in sponge sections (Hu et al., 1996).

In vitro studies have shown that exogenous application of EGF can stimulate corneal epithelial cell migration (Nishida et al., 1992) which may have a positive effect in microbial keratitis, contributing to restoration of epithelial barrier function.

In addition, other studies have shown that topical application of EGF and FGF can promote wound healing in several types of corneal wound models, (Baldwin and Marshall, 2002; Forster et al., 2008; Fredj-Reygrobellet et al., 1987; Nishida et al., 1992) consistent with the lack of perforation in VIP-treated mice in which these growth factors are elevated. In this regard, topical treatment with a growth factor cocktail containing EGF, FGF, and HGF provided evidence that at least some of the effects seen with VIP may be evoked through these growth factors. For example, in growth factor-treated eyes, only one of five versus all 5 corneas of PBS-treated eyes perforated; MIP-2, a chemokine that attracts

neutrophils (PMN) into the cornea2 mRNA was reduced; while TGF-β, an anti-inflammatory cytokine, was up-regulated as shown before with VIP treatment (Szliter et al., 2007). Growth factor treatment also significantly increased β-defensins 2 and 3 mRNA expression at 5 days p.i. and β-defensin 2 staining was enhanced at 5 days p.i. in both epithelium and stroma. These data are consistent with a previous study showing that KGF increased β-defensin 2 expression levels in a skin model expressing KGF/FGF-7 (Erdag et al., 2004). Most importantly, after growth factor treatment, bacterial counts also were reduced at 1, 3, and 5 days p.i., but were significant only at 1 day p.i. These data suggest that there is a delay in bacterial growth in the growth factor-treated cornea which may reflect less stromal damage which could be permissive to bacterial growth.

Overall, this study provides evidence that VIP modulates growth factors, angiogenic molecules (in both epithelium and stroma), β-defensin production, and bacterial counts and that many of the effects observed with VIP treatment can be compensated for through exogenous growth factor delivery.

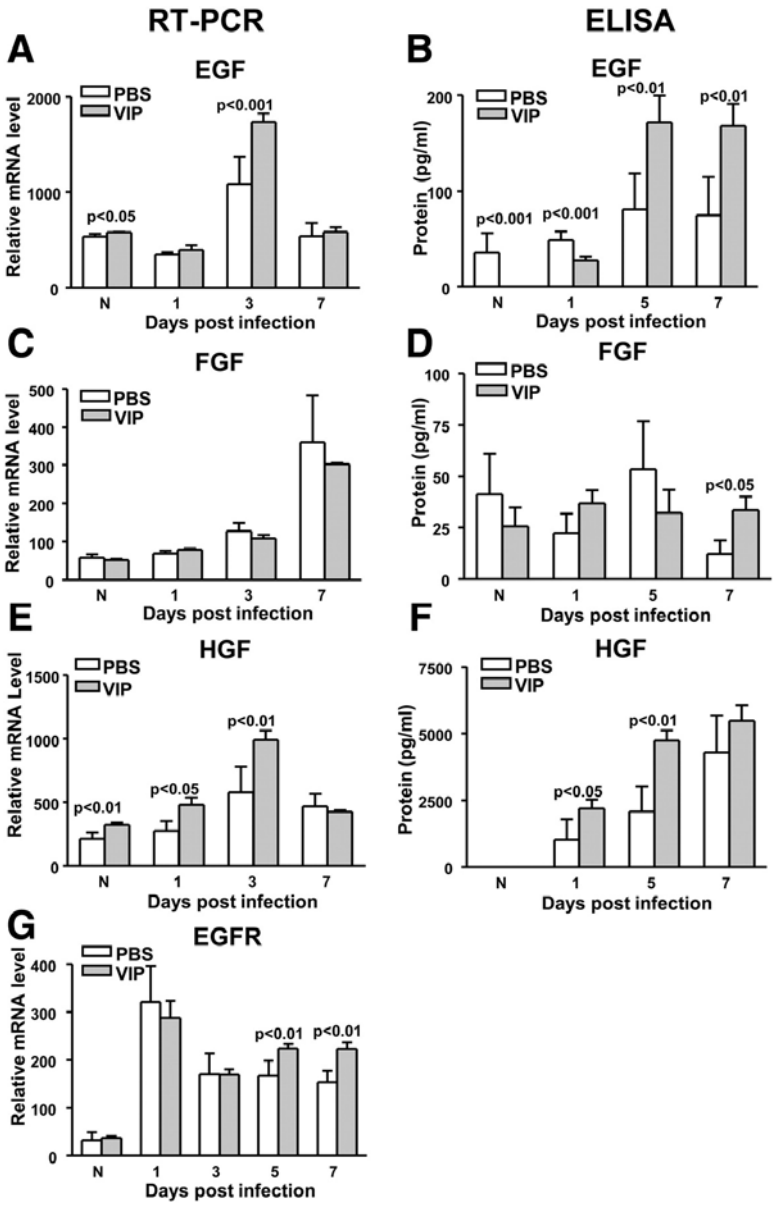

Figure 5. mRNA and protein expression patterns after VIP. EGF (A) mRNA levels were significantly increased in VIP- versus PBS-treated B6 mice in the normal, uninfected cornea and at 3 days p.i. mRNA levels of FGF (C) were unchanged between the two groups. HGF mRNA expression levels (E) were significantly increased by VIP in normal, uninfected cornea and at and 1 and 3 days p.i. EGFR mRNA levels (G) were increased after VIP treatment at 5 and 7 days p.i. (n = 5 per group per time per treatment for two experiments = 80 mice).

ELISA analysis: When compared with PBS, VIP significantly increased EGF protein expression (B) at 5 and 7 days p.i., but earlier in the normal cornea and at 1 day p.i., EGF protein levels were lower after VIP treatment. For FGF (D), VIP increased protein levels only at 7 days p.i. For HGF (F), VIP significantly increased protein levels at 1 and 5 days p.i. No significant changes were seen at other times tested for all assays (n = 5 per group per time per treatment for two experiments = 60 mice). Results for mRNA and protein data are mean ± SD.

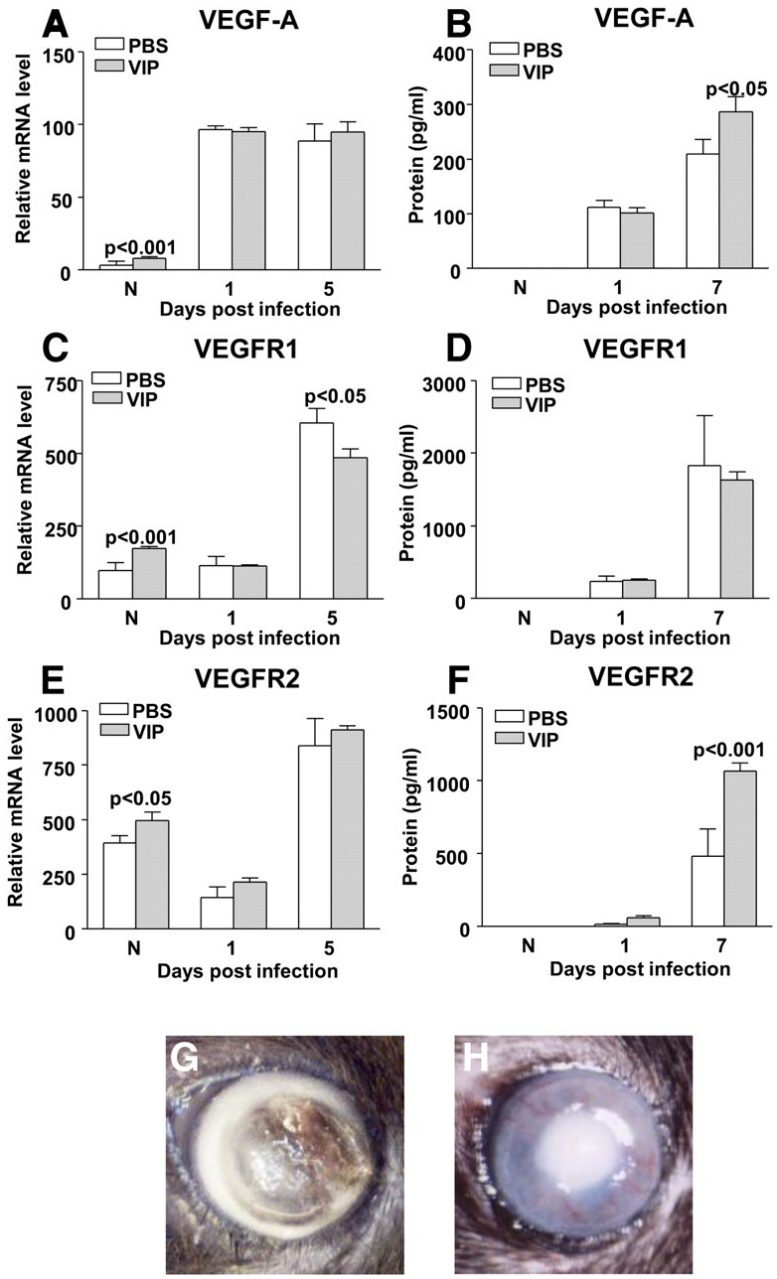

Figure 6. VEGF mRNA, protein, and photography with a slit lamp to document VIP regulation. VIP versus PBS modestly elevated mRNA expression of VEGF-A (A) only in the normal, uninfected cornea. VEGFR1 (C) mRNA levels were increased by VIP in the normal, uninfected cornea, but levels decreased at 5 days p.i. VIP treatment increased VEGFR2 (E) in the normal cornea No significant changes were seen at other times tested for all assays (n = 5 per

group per time per treatment for two experiments = 40 mice). ELISA analysis: For VEGF-A (B), VIP versus PBS increased protein levels at 7 days p.i. For VEGFR1 (D), levels were unchanged at all times tested. For VEGFR2 (F), protein levels were increased only at 7 days p.i. Photography using a slit lamp showed that VIP treatment decreased opacity and increased peripheral corneal vascularization when compared with PBS (D) treatment (magnification, ×15; n = 5 per group per time per treatment for two experiments = 40 mice). Results for mRNA and protein data are mean ± SD. (G, H) Photographs reprinted with permission from Szliter EA, Lighvani S, Barrett RP, Hazlett LD. Vasoactive intestinal peptide balances pro- and anti-inflammatory cytokines in the *Pseudomonas aeruginosa*-infected cornea and protects against corneal perforation. *J Immunol.* 2007;178:1105–1114.

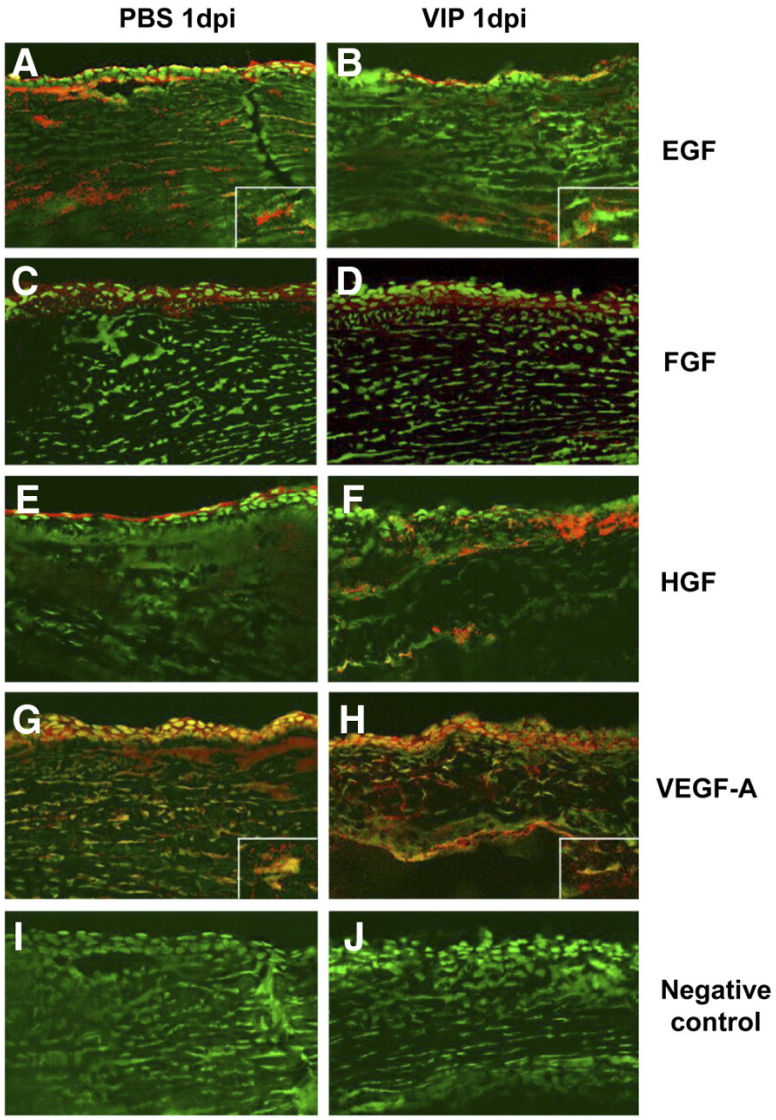

Figure 7. Immunostaining at 1 day p.i. for growth factors and VEGF-A. EGF immunostaining was slightly increased in PBS- (A) versus VIP- (B) treated mice (stromal as shown in the insets and epithelial). For FGF, epithelial staining was seen and similar between groups (C, D). For HGF VIP enhanced stromal immunostaining, while more epithelial staining was seen in the PBS group (E, F). For VEGF-A, staining was similar between groups in both the epithelium and stroma (G, H, and insets). An example of a negative control (primary antibody substituted with species-specific IgG) shows lack of positive immunostaining (I, J)

seen with each of the factors tested. Green: Sytox green nuclear label; red: Alexa Fluor 594 secondary antibody staining; n = 5 per group per treatment for two experiments = 20 mice).

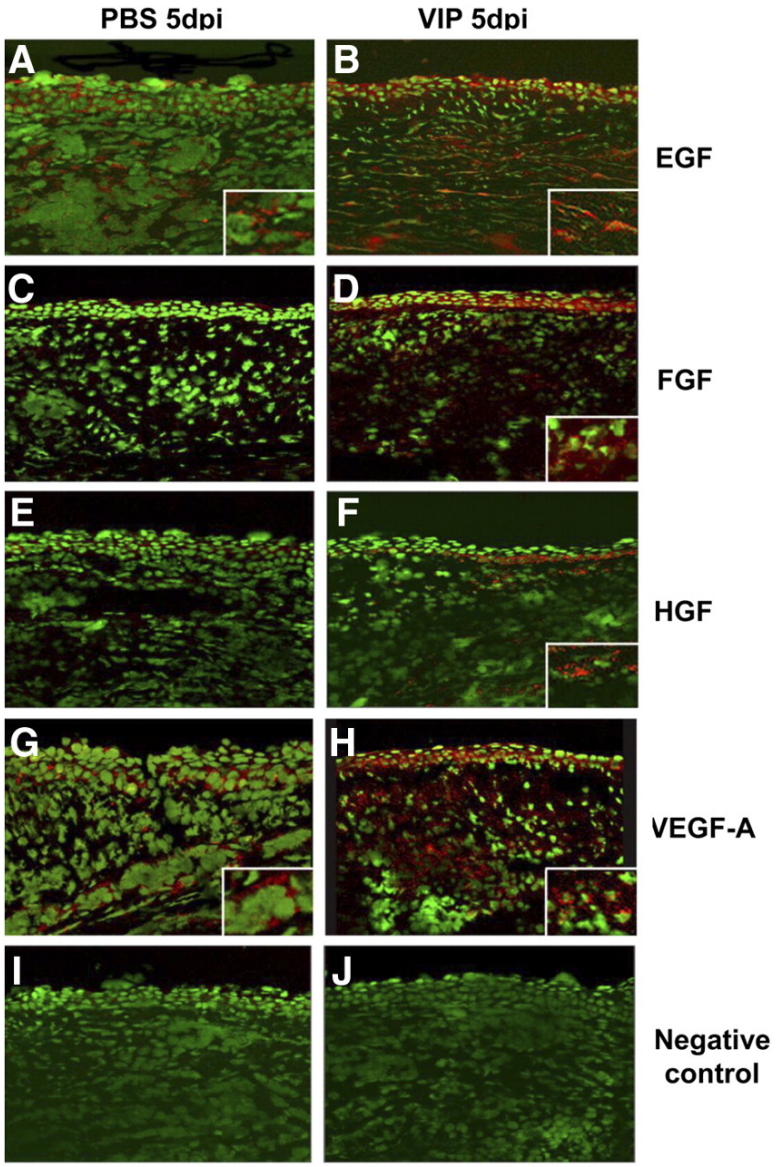

Figure 8. Immunostaining at 5 days p.i. for growth factor and VEGF-A. At this time, EGF, FGF, HGF, and VEGF-A epithelial and stromal staining was more intense in VIP- (B, D, F, H) versus PBS- (A, C, E, G) treated corneas. An example of a negative control (primary antibody substituted with species-specific IgG) shows lack of positive immunostaining (I, J) seen with each of the factors tested. Green: Sytox green nuclear label; red: Alexa Fluor 594 secondary antibody staining. Magnification, ×100; n = 5 per group per treatment for two

experiments = 20 mice.

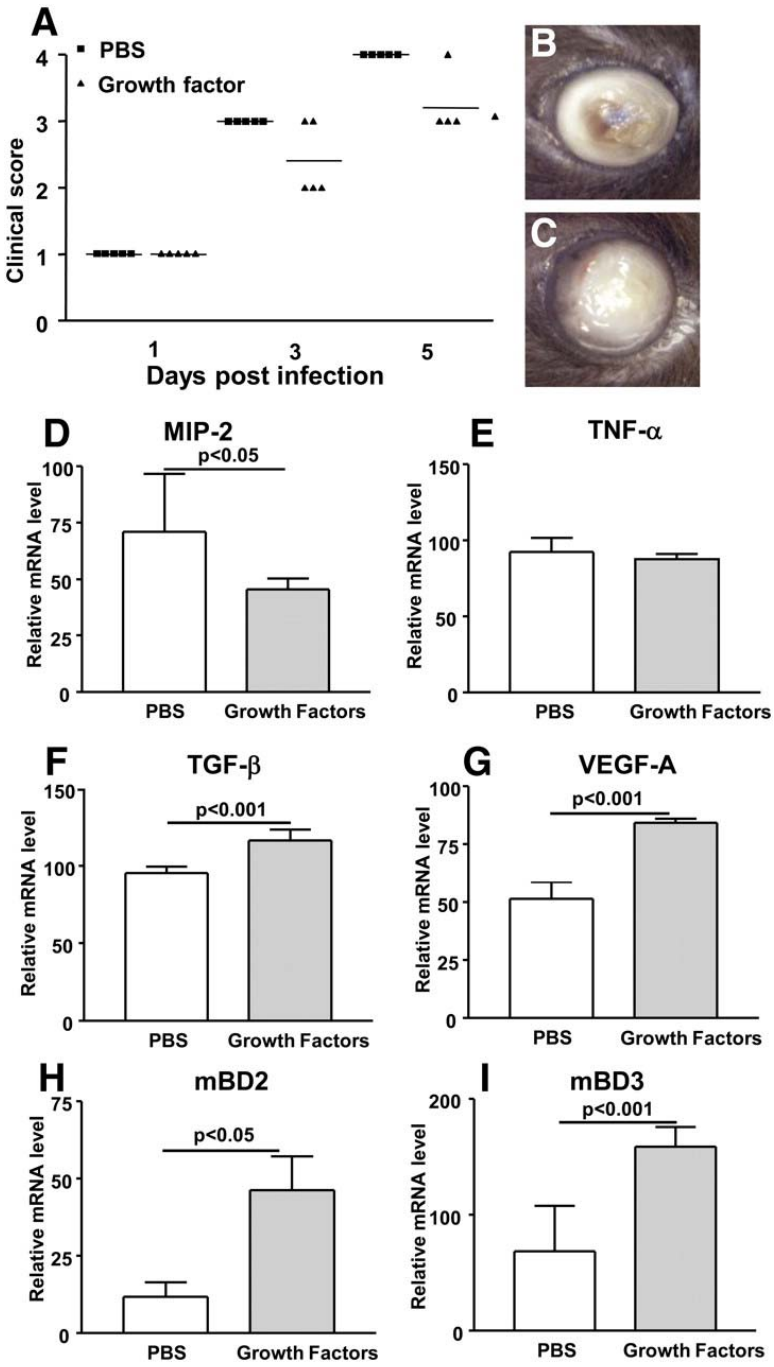

Figure 9. Topical growth factor treatment and assessing disease response by clinical evaluation and RT-PCR. Clinical scores (A) indicated statistically significant differences at 5 ($P < 0.05$) days p.i. between groups. Photographs taken with a slit lamp at 5 days p.i. showed a worsened disease response (perforation) when comparing control (B) versus growth factor (C) treatment.

Magnification, ×6 (n = 5 per group per treatment for two experiments = 20 mice). mRNA levels (D–I) showed a significant decrease in MIP-2 (D), no change in TNF-α (E), and increased TGF-β (F), VEGF-A (G), and β-defensins 2 (H) and 3 (I) in growth factor versus control treated eyes (n = 5 per group per treatment for two experiments = 20 mice). Results are shown as mean ± SD.

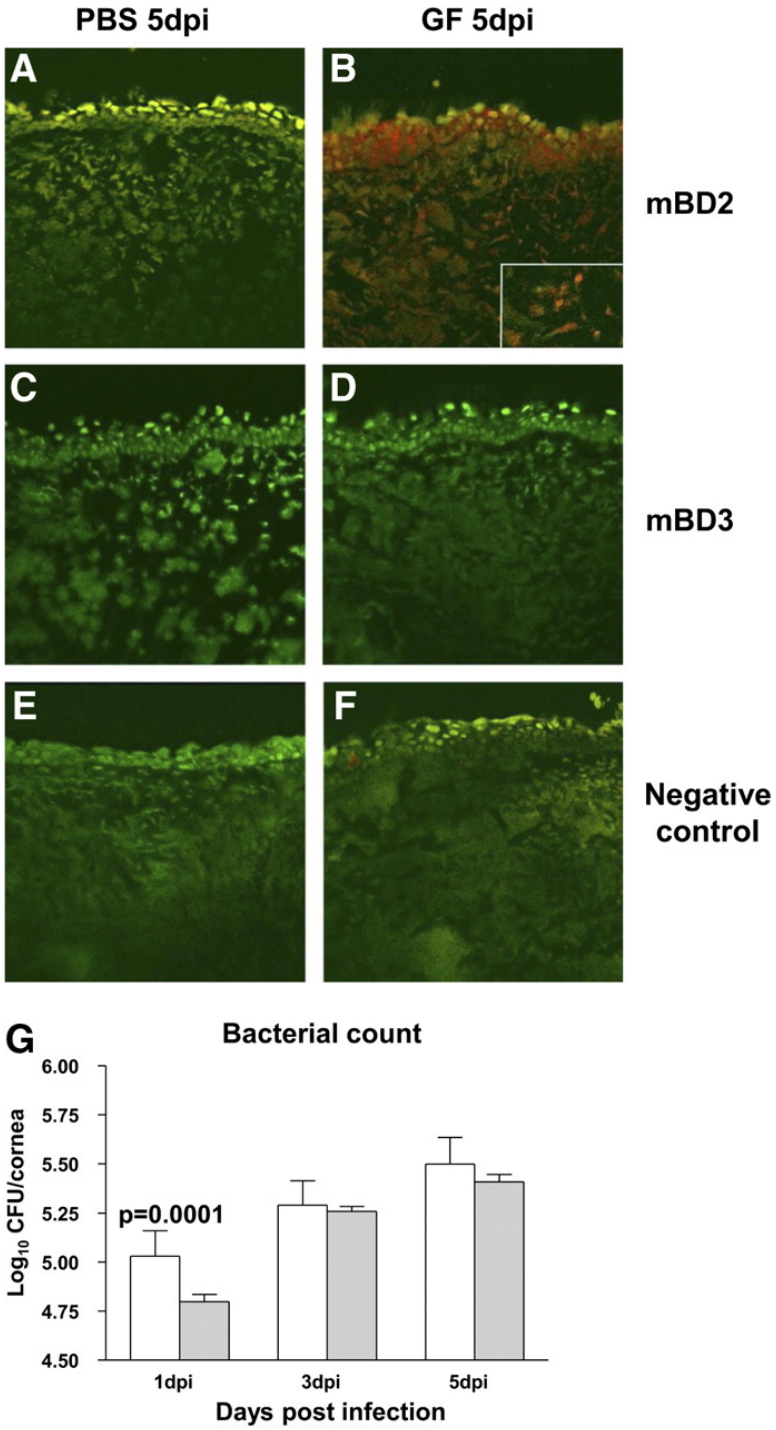

Figure 10. Immunostaining and bacterial counts after topical growth factor treatment. At 5 days p.i., β-defensin 2 epithelial and stromal staining was more intense in growth factor- (B and inset) versus PBS- (A) treated corneas. At this time, negligible staining for β-defensin 3 was seen after either growth factor (D)

or PBS (C) treatment. Controls, in which the primary Ab was replaced by IgG, were negative for immunostaining (E, F) for both defensins. Green: Sytox green nuclear label; red: Alexa Fluor 594 secondary antibody staining (n = 5 per group per treatment for two experiments = 20 mice). (G) Bacterial counts: Growth factor (grey filled bar) versus PBS (unfilled bar) treatment reduced bacterial plate counts at 1, 3, and 5 days p.i., but were significant only at 1 day p.i. (n = 5 per group per treatment for two experiments = 60 mice). Results are shown as mean ± SD.

Chapter 3

VASOACTIVE INTESTINAL PEPTIDE DOWNREGULATES PROINFLAMMATORY TLRS WHILE UPREGULATING ANTI-INFLAMMATORY TLRS IN THE INFECTED CORNEA

3.1 Abstract

TLRs recognize microbial pathogens and trigger an immune response, but their regulation by neuropeptides, such as VIP, during *P. aeruginosa* corneal infection remains unexplored. Therefore, B6 mice were injected i.p. with VIP, and mRNA, protein, and immunostaining assays were performed. After VIP treatment, PCR array and real-time RT-PCR demonstrated that pro-inflammatory TLRs (Chuk, IRAK1, TLR1, TLR4, TLR6, TLR8, TLR9, and TRAF6) were down-regulated, whereas anti-inflammatory TLRs (SIGIRR and ST2) were up-regulated. ELISA showed that VIP modestly down-regulated phosphorylated IKKα but up-regulated ST2 ~2-fold. SIGIRR was also up-regulated, whereas TLR4 immunostaining was reduced in cornea; all confirmed the mRNA data. To determine whether VIP effects were cAMP dependent, mice were injected with small interfering RNA for type 7 adenylate cyclase (AC7), with or without VIP treatment. After silencing AC7, changes in mRNA levels of TLR1, TRAF6, and ST2 were seen and unchanged with addition of VIP, indicating that their regulation was cAMP dependent. In contrast, changes were seen in mRNA levels of Chuk, IRAK1, 2, TLR4, 9 and SIGIRR following AC7 silencing alone; these were modified by VIP addition, indicating their cAMP independence. In vitro studies assessed the effects of VIP on TLR regulation in macrophages and

Langerhans cells. VIP down-regulated mRNA expression of pro-inflammatory TLRs while up-regulating anti-inflammatory TLRs in both cell types. Collectively, the data provide evidence that VIP down-regulates pro-inflammatory TLRs and up-regulates anti-inflammatory TLRs and that this regulation is both cAMP dependent and independent and involves immune cell types found in the infected cornea.

3.2 Introduction

Keratitis induced by *P. aeruginosa* progresses rapidly and is characterized by a suppurative stromal infiltrate with a marked mucopurulent exudate. Other untoward characteristics include inflammatory epithelial edema, ocular pain and redness, stromal ulceration, and often, decreased vision (Hazlett, 2004). Experimentally, infection with *P. aeruginosa* leads to corneal perforation in strains of mice, such as C57BL/6 (B6; susceptible), whereas less severe disease is seen in similarly challenged BALB/c (resistant) animals (Hazlett et al., 2000). In contrast, treatment of B6 mice with VIP promotes better disease outcome postinfection with *P. aeruginosa*, mainly through regulation of cytokine production and subsequent alteration of the host inflammatory cell response (Szliter et al., 2007). Recent studies from this laboratory provided further information regarding the anti-inflammatory effects of VIP and showed that it modulates keratitis through regulation of growth factors, angiogenic molecules, and β defensins in the infected cornea, contributing to healing (Jiang et al., 2011). However, the role of VIP in the regulation of TLRs has not been well-defined, despite the fact that other studies demonstrated that TLRs play an important role in ocular immune

defense (Yu and Hazlett, 2006). They direct the ocular immune response by differentially regulating a variety of events, including bacterial killing, polymorphonuclear leukocyte infiltration, and cytokine expression at both mRNA and protein levels (Huang et al., 2005; Huang et al., 2007; Huang et al., 2006a; Huang et al., 2006b). In particular, TLR4, a specific receptor for LPS contained in the outer membrane of various Gram-negative bacteria, is required for host resistance against *P. aeruginosa* in the infected cornea (Huang et al., 2006a). In fact, upon activation by *P. aeruginosa*, both TLR4 and TLR5 on corneal macrophages were shown to regulate *P. aeruginosa* keratitis through MyD88-dependent and -independent pathways (Sun et al., 2010). Therefore, it is important to understand which TLRs are regulated by VIP, as well as the mechanisms involved during microbial keratitis. In this regard, studies suggested that VIP can signal through cAMP-dependent or -independent pathways (Chorny et al., 2006), but it is not known whether this is important in VIP regulation of TLRs in other diseases (Gomariz et al., 2007) or bacterial keratitis.

Thus, the current studies investigated the expression of TLR-signaling pathways in *P. aeruginosa*-infected corneas, with or without VIP treatment, to determine whether it regulates their expression to favor disease resolution. Our data provide evidence that VIP treatment down-regulates pro-inflammatory TLRs while up-regulating anti-inflammatory TLRs at both the mRNA and protein levels in the cornea after *P. aeruginosa* infection and that, mechanistically, two transduction pathways, cAMP dependent and independent, are involved. Furthermore, *in vitro* studies suggest that at least two cell types in the cornea

may be responsive, because VIP reduced pro-inflammatory TLRs and increased anti-inflammatory TLRs in both LPS-stimulated elicited peritoneal macrophages and XS52 (Langerhans) cells.

3.3 Materials and Methods

Infection

Eight-week-old female B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with ethyl ether and placed beneath a stereoscopic microscope at ×40 magnification. The cornea of the left eye was wounded (Rudner et al., 2000), and a 5 μl-aliquot containing 1.0×10^6 CFU *P. aeruginosa* (strain 19660; American Type Culture Collection, Manassas, VA) was topically delivered. Animals were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

VIP treatment

Treatment with synthetic VIP was described previously (Szliter et al., 2007) at a dosage reported to be efficacious in a lethal endotoxemia model (Delgado et al., 1999b). In brief, using that dosage, B6 mice received daily i.p. injections of VIP (5 nM in 100 μl sterile PBS) at 1 d before infection (day −1) through a maximum of 7 d postinfection (p.i.). Control mice were injected similarly with sterile PBS (100 μl).

RNA interference

Use of small interfering RNA (siRNA) was described in previous work from this laboratory (Wu et al., 2009a). For the current studies, siRNA for type 7

adenylate cyclase (AC7) or an appropriate scrambled control (Santa Cruz Biotechnology, Santa Cruz, CA) was injected subconjunctivally (5 µl/mouse at a concentration of 8 µM) into the left eye of B6 mice (n = 5/group/time) 1 d before infection, with or without VIP treatment (described above). AC7 (4 µM) was applied topically to the infected corneas (5 µl/mouse/time) once on the day of infection and twice on days 1 and 3 p.i. Corneas from scrambled control, silenced AC7, or silenced AC7 plus VIP-treated (n = 5/group/time) mice were individually collected at 5 d p.i. and processed for RT-PCR (described below). Using a similar assay, the efficacy of silencing AC7 in cornea (n = 5/group/time) was confirmed by real-time RT-PCR (5 d p.i.).

Real-time RT-PCR

Total RNA was isolated from individual corneas (n = 5/group/time) or cells using RNA-Stat 60 (Invitrogen, Carlsbad, CA), per the manufacturer's recommendations, and quantitated spectrophotometrically (260 nm). One microgram of total RNA was reverse transcribed using MMLV reverse transcriptase. The 20-µl reaction mixture contained 200 U MMLV-reverse transcriptase, 10 U RNasin, 500 ng oligodeoxythymidine primers, 10 mM deoxyribonucleotide triphosphate, 100 mM DTT, and MMLV reaction buffer (Invitrogen). cDNA was amplified using SYBR Green Master Mix (SABiosciences, Frederick, MD), per the manufacturer's recommendation. Briefly, the reaction system contained 10 µl SYBR Green PCR Master Mix, 0.5 µM primers, and 2 µl cDNA (diluted 1/10) in diethyl pyrocarbonate water. All primer sets for the PCR array were purchased as a 96-well plate (RT^2 Profiler TLR Signaling Pathway

PCR Array; SABiosciences). The individual primer sets were designed using PrimerQuest (Integrated DNA Technologies, Cambridge, MA). Sequences of primers for β-actin, AC7, conserved helix-loop-helix ubiquitous kinase inhibitor of NF-κB kinase subunit α (Chuk), IRAK1, IRAK2, TLR1, TLR4, TLR6, TLR8, TLR9, TNFR-associated factor (TRAF)6, SIGIRR, and ST2 are shown (Table 2).

ELISA

Protein levels of TLR-associated molecules were tested by ELISA. Corneas from PBS- and VIP-treated B6 mice were individually collected (n = 5/group/time) from normal uninfected and infected mice at 1 and 7 d p.i. For total Chuk (IKKα) and p-IKKα (Cell Signaling Technology, Danvers, MA), corneas were homogenized in 0.5 ml lysis buffer (Cell Signaling Technology) with 1 mM PMSF (Sigma, St. Louis, MO) and protease inhibitor (1 tablet/10 ml; Roche, Indianapolis, IN). For ST2 (R&D Systems, Minneapolis, MN), corneas were homogenized in 0.5 ml PBS with 0.1% Tween 20 and protease inhibitor (as above). All samples were centrifuged at 13,000 rpm for 5 min, and an aliquot of each supernatant was assayed in duplicate for total IKKα, p-IKKα, and ST2, per the manufacturer's instructions.

Western blot

Corneas (n = 5/group/time) were collected from PBS- and VIP-treated normal uninfected and infected B6 mice at 1 and 5 d p.i. Pooled corneas (n = 5/group) were lysed in 250 μl radio immunoprecipitation assay lysis buffer (Santa Cruz Biotechnology) and homogenized for 15 min. Tissue debris was pelleted by centrifugation for 10 min at 12,000 rpm, and protein concentration of the

supernatant was determined by bicinchoninic acid (Bio-Rad) protein assay. Supernatants were separated on 10% SDS-PAGE, after loading an 80-μg sample in each lane; as a control, recombinant murine SIGIRR (15 ng; R&D Systems) was similarly loaded. The electrophoretically separated material was transferred to a supported nitrocellulose membrane (Bio-Rad) and blocked in a 5% solution of nonfat dry milk prepared in 1× PBS and 0.05% Tween 20. Blots were incubated with primary goat anti-mouse SIGIRR Ab (R&D Systems) diluted in PBS overnight (4°C), washed three times for 10 min each with TBST, detected with HRP-conjugated secondary Ab (R&D Systems) diluted 1/1000 in PBS containing 5% nonfat milk, and developed using the ECL method (PerkinElmer, Waltham, MA), per the manufacturer's protocol. The blot was scanned on a FluorChem E imaging system (Cell Biosciences, Santa Clara, CA), and band relative integrated density value was analyzed by AlphaView software (Cell Biosciences).

Immunofluorescent staining

Normal, uninfected and infected eyes were enucleated from VIP- or PBS-treated B6 mice (n = 5/group/time) at 1 and 5 d p.i. Tissue was immersed in 1× Dulbecco's PBS (Mediatech, Herndon, VA), embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Ten-micrometer-thick sections were cut, mounted on polylysine-coated glass slides, and incubated in a moist chamber at 37°C overnight. After a 2-min acetone fixation, nonspecific staining was blocked with 10 mM sodium phosphate buffer containing 2.5% BSA and donkey IgG (1:100) for 30 min at room temperature. For immunostaining,

sections were incubated for 1 h each with goat anti-mouse TLR4 (Santa Cruz Biotechnology), followed by an Alexa Fluor 594-conjugated donkey anti-goat secondary Ab (1:1500, Invitrogen). Sections were incubated for 2 min with SYTOX Green nuclear acid stain (1:20,000; Lonza, Walkersville, MD). Controls were treated similarly, but the primary Ab was replaced with the same host IgG. Finally, sections were visualized (TLR4+ staining = red), and digital images were captured with a Leica TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Bannockburn, IL).

Cell isolation, culture, and treatment

Peritoneal macrophages were elicited and isolated from B6 mice, as described before (Szliter et al., 2007). Briefly, cells were induced into the peritoneal cavity by i.p. injection of 1.0 ml 3% Brewer's thioglycollate medium (Becton Dickinson, Sparks, MD) 5 d before harvest (n = 12 mice). Cells were collected by peritoneal lavage with DMEM and stained with trypan blue (1:1); viable cells (>95%) were counted using a hemacytometer. After a differential cell count, cells were collected and pooled, and 3×10^6 macrophages/well (five wells/treatment) were seeded into six-well plates. Nonadherent cells were removed 24 h later, and isolated macrophages were used for the *in vitro*-stimulation assays described below. Mouse XS52 (Langerhans) cells were cultured in complete RPMI 1640 with 10% FCS (both from Life Technologies, Grand Island, NY) supplemented with 0.5 ng/ml murine rGM-CSF (Life Technologies), as described previously (Huang et al., 2005). These cells (capable of presenting protein Ag to primed CD4+ T cells) were derived from newborn

BALB/c mouse epidermis as stable long-term cell lines (dendritic shaped, $CD45^+$/E-cadherin$^+$ and $B7^-1^-$) (Xu et al., 1995). After differential cell count, 3 × 10^6 XS52 (Langerhans) cells/well (five wells/treatment) were seeded into six-well plates. Both cell types were treated with 1 μg ultrapure LPS (*Escherichia coli* 01111:B4 strain; which only activates the TLR4 pathway; InvivoGen, San Diego, CA), with or without VIP (10^{-9} M). For both macrophage and XS52 (Langerhans) cell experiments, which were repeated similarly once (n = 2 experiments for each cell type), cells from each well were collected, mRNA was extracted and assayed in duplicate by real-time RT-PCR (described above), and the data were analyzed statistically.

Langerhans cell staining and quantitation

Normal, uninfected corneas were harvested at 1 and 3 d p.i. and placed in 0.02 mol/l EDTA (pH 7.2) for 1 h at 37°C for epithelial removal and Langerhans cell staining. Epithelial sheets were fixed in cacodylate-buffered formaldehyde for 20 min at 4°C, washed four times with cold 0.1 mol/l cacodylate buffer, and incubated in ADPase substrate solution containing ADPase buffer, 2% lead nitrate, and ADP (5 mg/ml; Sigma) for 15 min at 37°C. Sheets were washed four times with trismaleate buffer (pH 7.2), developed for 5 min in a 1:10 ammonium sulfide solution, washed three times with buffer, mounted onto slides with glycerol, flattened, and coverslipped (Hazlett et al., 2002). Epithelial sheets were observed and photographed with a Zeiss Axiophot microscope with Axiocam digital imagery (Carl Zeiss; Morgan Instruments, Cincinnati, OH) at ×375, and the number of cells was quantitated/field (n = 12; field size = 270 μm^2) by two

investigators who were blinded to the treatment. Staining of Langerhans cells for ADPase activity was done previously and was shown to provide data similar to immunostaining these cells with surface markers, such as DEC205 (Hazlett et al., 2002).

Statistics

Two-way ANOVA, followed by the Bonferroni posttest, was used for analysis of the data shown in Figs. 11, 12, and 17 (Prism 3.0; GraphPad Software, La Jolla, CA). One-way ANOVA, followed by the Tukey posttest, was used for the data shown in Figs. 14–16 (SPSS, Chicago, IL). For each test, differences were considered significant at $p < 0.05$, and data shown are mean ± SEM. Each experiment was performed at least twice, and combined data are shown, with the exception of Figs. 15 and 16, in which representative data from a single similar experiment are provided. Infection of mice, confocal microscopy, and Langerhans cell quantitative studies were done in a blinded fashion.

3.4 Results

PCR array and real-time RT-PCR

To determine the effects of VIP treatment on the regulation of TLRs in cornea during bacterial keratitis, mRNA levels were tested by RT-PCR (Table 2) and 84 TLR-related genes were profiled by PCR array at 3 d p.i. VIP treatment decreased the expression (>3-fold) of 21 genes compared with PBS-treated controls (Table 3). RT-PCR was used to confirm the array data and to test TLR4 (Huang et al., 2006a) and IRAK1 (Deng et al., 2006; Hacker et al., 2011), which are important in *Pseudomonas*-induced infections, but were not changed on the

PCR array greater >3-fold (TLR4, −1.11 and IRAK1, −2.9). Negative regulators of TLRs (ST2 and SIGIRR), not included on the PCR array, that were shown previously to promote resistance to *P. aeruginosa* keratitis (Huang et al., 2007; Huang et al., 2006b) were also tested.

VIP significantly down-regulated mRNA expression for TLR1 only at 7 d p.i. (Fig. 11A, $p < 0.01$). However, compared with PBS treatment, VIP decreased mRNA levels of TLR4 only at 1 d postinfection (Fig. 11B, $p < 0.001$). VIP also down-regulated mRNA levels of TLR6 at 5 and 7 d p.i. (Fig. 11C, $p < 0.05$ and $p < 0.001$, respectively), for TLR8 only at 7 d p.i. (Fig. 11D, $p < 0.001$), and for TLR9 only at 5 d p.i. (Fig. 11E, $p < 0.001$). Compared with PBS treatment, VIP treatment also decreased mRNA expression of TLR adaptor molecule IRAK1 at 1 and 5 d p.i. (Fig. 11F, $p < 0.001$ and $p < 0.001$, respectively), but no differences were seen between the groups for IRAK2 (Fig. 11G). TRAF6, which interacts with IRAK1 (Cao et al., 1996), also was reduced at the mRNA level by VIP treatment compared with PBS treatment at 5 and 7 d p.i. (Fig. 11H, $p < 0.05$ and $p < 0.001$, respectively). mRNA levels of Chuk, a downstream molecule of the IRAK–TRAF6-signaling pathway, which regulates NF-κB expression (Cao et al., 1996), also was down-regulated by VIP at 1 and 7 d p.i. (Fig. 11I, $p < 0.01$ and $p < 0.001$, respectively). In contrast, VIP treatment increased SIGIRR mRNA levels at 5 and 7 d p.i. (Fig. 11J, $p < 0.001$ for both). However, levels in both groups decreased at 1 d p.i. ST2 mRNA levels also were increased by VIP treatment at all times tested, but it was significant only at 7 d p.i. (Fig. 11K, $p < 0.05$).

ELISA and Western blot

ELISA and/or Western blot was used to selectively confirm the mRNA data for total IKKα (Chuk), p-IKKα, ST2, and SIGIRR. For total IKKα (Fig. 12A), there was no significant difference between PBS and VIP treatment at any time tested. However, compared with PBS treatment, VIP treatment slightly down-regulated p-IKKα protein expression only at 7 d p.i. (Fig. 12B, $p < 0.05$). In contrast, protein levels of ST2 (Fig. 12C) were significantly enhanced by VIP treatment compared with PBS treatment at 7 d p.i. ($p < 0.001$), not different at 1 d p.i., and not detectable in either normal sample. SIGIRR protein (Fig. 12D, 12E) was constitutively expressed at similar levels in normal PBS- and VIP-treated mouse cornea. At 1 d p.i., greater levels of SIGIRR ($p < 0.001$) were observed after VIP treatment, with no difference between the two groups at 5 d p.i.

TLR4 immunostaining

Immunohistochemistry was used to spatially localize TLR4 (red staining) in the cornea of VIP- versus PBS-treated B6 mice at 1 and 5 d p.i. At 1 d p.i., reduced TLR4 staining was seen after VIP treatment (Fig. 13B), compared with PBS treatment (Fig. 13A), in corneal epithelium and stroma. At 5 d p.i., compared with PBS treatment, VIP treatment further reduced TLR4 epithelial and stromal staining (compare Fig. 13D with 13C). Controls in which primary Ab was substituted with species-specific IgG were negative after PBS (Fig. 13E) or VIP (Fig. 13F) treatment at 1 or 5 d p.i. (data not shown).

Silencing AC7

To determine whether the anti-inflammatory effect of VIP on TLRs was cAMP dependent, siRNA was used *in vivo* to knock down AC7; it was selected

because, among the 10 mammalian adenyl cyclase isotypes, AC7 is highly expressed in the immune system (Duan et al., 2010). When tested at 5 d p.i., RT-PCR confirmed that silencing AC7 versus scrambled control treatment significantly decreased AC7 mRNA levels (Fig. 14A, $p < 0.01$) and that silencing plus VIP treatment did not reverse the effect. Furthermore, at this time, silencing AC7 increased mRNA levels of TLR1 (Fig. 14B), whereas TRAF6 (Fig. 14C) and ST2 (Fig. 14D) were decreased ($p < 0.05$, $p = 0.001$, and $p < 0.01$, respectively). Silencing plus VIP injection did not change the effect, indicating that VIP regulation of these TLRs is cAMP dependent. mRNA levels of pro-inflammatory TLRs, including Chuk (Fig. 14E), IRAK1 (Fig. 14F), TLR4 (Fig. 14G), TLR9 (Fig. 14H), and IRAK2 (Fig. 14I), were increased after silencing compared with scrambled control treatment ($p < 0.01$, $p < 0.01$, $p = 0.001$, $p = 0.01$, and $p < 0.05$, respectively); levels of the anti-inflammatory TLR SIGIRR were decreased (Fig. 14J, $p = 0.001$). Silencing plus VIP injection changed the effect (Chuk: $p < 0.001$; IRAK1: $p < 0.05$; TLR4: $p = 0.001$; TLR9: $p < 0.01$; IRAK2: $p < 0.05$; SIGIRR: $p < 0.05$), indicating that VIP regulation of these molecules is cAMP independent. No significant changes in mRNA levels of TLR6 (Fig. 14K) were seen with AC7 siRNA treatment, but VIP plus silencing decreased mRNA levels of TLR6 ($p < 0.05$), suggesting the possibility of its regulation by another adenyl cyclase isoform (Song et al., 2007). No effects were seen for TLR8 (Fig. 14L), suggesting that it is independent of AC7 and VIP regulation.

In vitro studies: macrophages

To provide some indication of which cells may be involved in VIP

regulation of TLRs, peritoneal exudate macrophages and XS52 (Langerhans) cells were tested in an in vitro assay. Compared with media control, ultrapure LPS (only activates the TLR4 pathway) significantly increased TLR1 (Fig. 15A, $p < 0.001$), TLR4 (Fig. 15B, $p < 0.01$), MyD88 (Fig. 15C, $p < 0.001$), IRAK1 (Fig. 15D, $p < 0.01$), and TRAF6 (Fig. 15E, $p < 0.001$) mRNA, with no effect on ST2 mRNA (Fig. 15G). However, SIGIRR mRNA (Fig. 15F, $p < 0.001$) was decreased after LPS stimulation compared with media control stimulation. Compared with LPS alone, LPS plus VIP significantly decreased mRNA levels of TLR1 (Fig. 15A, $p < 0.05$), TLR4 (Fig. 15B, $p < 0.01$), MyD88 (Fig.15C, $p < 0.001$), IRAK1 (Fig. 15D, $p < 0.001$), and TRAF6 (Fig. 15E, $p < 0.001$) but increased ST2 (Fig. 15G, $p < 0.05$); no difference was seen for SIGIRR (Fig. 15F). Cultured cells treated with VIP alone showed no significant difference compared with media-treated controls for any of the molecules tested.

In vitro studies: XS52 (Langerhans) cells

LPS versus media control treatment of XS52 (Langerhans) cells significantly increased mRNA levels of TLR1 (Fig. 16A, $p < 0.001$), TLR4 (Fig. 16B, $p < 0.001$), MyD88 (Fig. 16C, $p < 0.001$), and TRAF6 (Fig. 16E, $p < 0.001$) but decreased ST2 (Fig. 16G, $p < 0.001$). IRAK1 (Fig. 16D) and SIGIRR (Fig. 16F) mRNA levels were unchanged after LPS stimulation compared with media control stimulation. Compared with LPS alone, LPS plus VIP significantly decreased mRNA levels of MyD88 (Fig. 16C, $p < 0.05$) and TRAF6 (Fig. 16E, $p < 0.001$) but increased SIGIRR mRNA expression (Fig. 16F, $p < 0.001$). Compared with LPS alone, LPS plus VIP did not significantly change mRNA levels of TLR1 (Fig. 16A),

TLR4 (Fig. 16B), IRAK1 (Fig. 16D), or ST2 (Fig. 16G). Cultured cells treated with VIP alone did not differ from media-treated controls for any of the molecules tested.

Langerhans cell detection and quantitation

In normal, uninfected eyes, dendritic-shaped Langerhans cells were detected in the peripheral cornea/conjunctiva of PBS-treated (Fig. 17A) and VIP-treated (Fig. 17B) mice; quantitatively, they were slightly decreased (not significantly) after VIP treatment (Fig. 17G). At 1 d p.i., rounded, motile Langerhans cells appeared to be decreased in the conjunctiva and peripheral cornea of VIP-treated mice (Fig. 17D) compared with PBS-treated mice (Fig. 17C), and quantitation showed that the difference was significant ($p < 0.05$, Fig. 17G). By 3 d p.i., the cell number appeared similar in PBS-treated (Fig. 17E) and VIP-treated (Fig. 17F) mice; when quantitated, no difference was seen between the two groups (Fig. 17G).

3.5 Discussion

VIP regulates a wide variety of immune reactions; among its numerous anti-inflammatory functions, it participates in the maintenance of immune homeostasis (Gomariz et al., 2010; Smalley et al., 2009). Previously, our laboratory showed that, in the cornea of B6 mice infected with *P. aeruginosa*, VIP treatment regulates cytokine production through up-regulation of VIP receptor 1 on inflammatory cells, leading to less stromal destruction and prevention of corneal perforation (Szliter et al., 2007). Recent work provided further evidence that similar VIP treatment of B6 mice also has an indirect effect: it modulates

growth factors, angiogenic molecules, and defensins in the infected cornea, which, in turn, promote healing (Jiang et al., 2011). In addition, other studies showed that VIP down-regulates pro-inflammatory TLRs and related molecules in several nonocular disease models (Gomariz et al., 2007). TLRs, important in innate immunity, are critical for recognition of conserved pathogen-associated molecular patterns, through TLR/IL-1R expressed on the surface of various cell types, to trigger cytokine/chemokine production (Chtarbanova and Imler, 2011; Medzhitov and Janeway, 2000). In this regard, previous studies from this laboratory and other investigators showed that TLR1 (Yuan and Wilhelmus, 2010), TLR3 (Johnson et al., 2008), TLR4 (Huang et al., 2006a), and TLR9 (Huang et al., 2005) play important roles in the pathogenesis of experimentally induced keratitis. However, VIP regulation of TLRs during these diseases has not been characterized. Thus, the current study, using a PCR array to profile 84 TLR-related genes, provided an overview of the ability of VIP to provide master regulation of TLRs and signaling-pathway molecules in bacterial keratitis. Twenty-one genes were down-regulated ≥ 3-fold by VIP, and several of them were selected for further confirmation by RT-PCR. The results indicated that VIP reduced pro-inflammatory TLRs and related molecules (TLR1, TLR4, TLR6, TLR8, TLR9, IRAK1, TRAF6, and Chuk), which agreed well with the array data. The array did not contain two anti-inflammatory TLRs important in promoting the resistance response of BALB/c mice to *P. aeruginosa*: SIGIRR (Huang et al., 2006b) and ST2 (Huang et al., 2007); however, when tested by RT-PCR, these genes, in general, were up-regulated by VIP treatment. Immunohistochemistry

data further confirmed that VIP decreased TLR4 protein expression in both epithelial and stromal layers of the cornea. These results also are consistent with previous studies showing that VIP inhibits or even reverses TLR2 (Foster et al., 2007), TLR3 (Lee et al., 2009), and TLR4 (Arranz et al., 2008a; Arranz et al., 2008b) expression or their stimulated signaling pathways in other nonocular disease models.

On ligand binding, TLRs recruit adaptor molecules, such as IRAK and TRAF6, to their intracellular signaling domain, leading to the activation of several kinases, as well as the transcription factor, NF-κB, thereby directly up-regulating immune-response genes (Michelsen et al., 2004). In the current study, VIP treatment in vivo down-regulated IRAK1 and TRAF6 mRNA levels, which is consistent with other studies using synovial fibroblasts isolated from patients with rheumatoid arthritis: compared with LPS alone, LPS stimulation plus VIP treatment inhibited IRAK1 and TRAF6 mRNA and phosphorylated protein expression (Arranz et al., 2008b), thereby acting as a negative regulator of TLR4 signaling.

Another important signaling complex, the IKK complex, is found downstream of IRAK–TRAF6 signaling and is composed of IKKα, IKKβ, and IKKγ (Israel, 2010). The current in vivo study showed that VIP inhibited Chuk/IKKα mRNA expression, but only modestly decreased its activity, compatible with previous *in vitro* studies showing that VIP directly inhibited IKKα activity in human monocytes (Delgado and Ganea, 2001) and microglia cells (Delgado et al., 2008). In microglial cells, VIP inhibition of IKKα reduced Aβ-induced neurodegeneration

by indirectly inhibiting the production of numerous inflammatory and neurotoxic molecules (Delgado et al., 2008). Furthermore, loss or decreased IKKα also was suggested to negatively regulate NF-κB and subsequent gene expression by inhibiting its translocation to the nucleus and, thus, down-regulating inflammation (Anest et al., 2003).

In contrast to other TLRs, SIGIRR (Wald et al., 2003) and ST2 (Liew et al., 2005), members of the TLR–IL-1R superfamily, act as negative regulators of IL-1 and LPS signaling. Previous studies from our laboratory found that SIGIRR (Huang et al., 2006b) and ST2 (Huang et al., 2007) promote natural resistance of BALB/c versus B6 mice against *P. aeruginosa* keratitis and that Ab depletion of SIGIRR increased disease in BALB/c mice. However, it was not tested whether VIP treatment regulates expression of either molecule in susceptible B6 mice. The current study showed that in vivo treatment of infected B6 mice with VIP increased (but not significantly) SIGIRR mRNA levels over PBS-treated controls in normal uninfected eyes; at 1 d p.i., the levels decreased in both groups but remained higher with VIP treatment. In previous work from our laboratory (Huang et al., 2006b) comparing the expression of SIGIRR in BALB/c versus B6 mice after *P. aeruginosa* infection, we also observed a decrease in mRNA expression in both groups at 12 h p.i. (and in B6 mice at 1 d p.i., similar to the current study), which increased in both mouse strains later in disease. SIGIRR protein levels decreased in the two mouse strains at 1 d p.i. and increased later in disease, most significantly in BALB/c mice (Huang et al., 2006b). After *in vivo* LPS challenge, similar consumption of SIGIRR mRNA (protein not tested) was shown

by Wald et al. (Wald et al., 2003); they suggested that this may reflect its functional involvement in decreasing inflammation. We cited this possibility as well but did not prove it functionally (Huang et al., 2006b). The current study suggests that a decrease in SIGIRR protein is not required for functionality, because we did not observe SIGIRR consumption at the protein level in B6 mice treated with VIP; rather, protein levels were increased at 1 d p.i. These data are supported by other studies in which overexpression of SIGIRR (adenoviral vector expressing murine SIGIRR) was shown to ameliorate LPS-induced acute lung injury in mice (Chen et al., 2011); its overexpression in human macrophages and dendritic cells downregulated TLR-induced cytokines, whereas its knock down increased cytokine production following TLR stimulation (Drexler et al., 2010). In contrast, ST2 protein is increased later (7 d p.i.) in disease, suggesting that, after VIP treatment, the molecules could act disparately in time to ameliorate disease or participate in corneal healing.

Previous studies on the immunomodulatory properties of VIP led to the identification of two pathways: one cAMP dependent and the other cAMP independent (Chorny et al., 2006). However, whether VIP regulation of TLRs is cAMP dependent or independent had not been tested. To address this issue, we silenced AC7 using siRNA, because among the 10 adenyl cyclases it is highly expressed in the immune system (Duan et al., 2010). Data showed that VIP regulation of TLR1, TRAF6, and ST2 was cAMP dependent, because silencing plus VIP treatment did not change the effects of silencing alone. These data agree with previous, similarly designed in vitro studies in which a PKA inhibitor

was used to block upstream cAMP pathways (Delgado et al., 1999b; Delgado et al., 1999c). Data from those studies concluded that VIP regulation of both a proinflammatory cytokine TNF-α (Delgado et al., 1999c) and NO (Delgado et al., 1999b) are cAMP dependent. In contrast, the current study showed that regulation of Chuk, IRAK1, IRAK2, TLR4, TLR9, and SIGIRR are cAMP independent, because silencing plus VIP treatment changed the effects of silencing AC7 alone. These data are consistent with in vitro studies showing that VIP can function to regulate molecules, such as NF-κB, in a cAMP-independent manner (Delgado and Ganea, 2001). Unexpectedly, compared with scrambled control treatment, AC7 siRNA did not induce significant changes in TLR6; however, when silencing was combined with VIP treatment, TLR6 levels were decreased, suggesting the possibility that other adenyl cyclase isoforms (Song et al., 2007) participate in TLR regulation by VIP.

Previous studies from our laboratory showed the importance of both macrophages (McClellan et al., 2003) and Langerhans cells (Hazlett et al., 2002) in *Pseudomonas* keratitis. In this regard, VIP functions as a macrophage-deactivating factor and was shown to down-regulate pro-inflammatory cytokine expression (IL-1β and MIP-2) after LPS (not ultrapure) stimulation in murine peritoneal macrophages (Szliter et al., 2007). It also down-regulates pro-inflammatory cytokine production in epidermal Langerhans cells (Kodali et al., 2004), but its role in TLR regulation has not been characterized in these types of cells. Data from the current study showed that, in peritoneal macrophages and XS52 (Langerhans) cells, ultrapure LPS stimulation upregulated TLR1 and TLR4,

but VIP plus LPS downregulated both TLRs only in macrophages. These data agree with those of other investigators who showed that, compared with LPS alone, stimulation of murine macrophages with LPS (not ultrapure) and VIP resulted in less TLR4 expression at the transcriptional level (Arranz et al., 2008a); however, no similar data are available for XS52 (Langerhans) cells or why they respond dissimilarly to macrophages. With regard to TLR1, because other investigators showed that cytokines, such as IFN-γ, can increase TLR1 expression in murine monocytes (Krutzik et al., 2003), we similarly tested for IFN-γ after ultrapure LPS stimulation of both cell types and found elevated levels, which also were decreased by treatment with LPS plus VIP (data not shown). With similar treatment, TLR4 downstream-signaling molecules, including MD88, IRAK1, and TRAF6, also were down-regulated in macrophages, whereas only MyD88 and TRAF6 were down-regulated in XS52 (Langerhans) cells. The current study also showed that, compared with LPS treatment alone, LPS stimulation plus VIP increased ST2 mRNA expression in macrophages, but only SIGIRR was increased in XS52 (Langerhans) cells. These data suggest that VIP disparately regulates TLR on these cells and may reflect the kinetics of pathogen encounter (ocular surface versus stroma) p.i. in the eye. Despite the differential regulation of these cells, it is clear that VIP plus LPS reduces pro-inflammatory TLR expression while promoting anti-inflammatory TLR expression. VIP treatment in vivo also reduced Langerhans cell centripetal migration and, thus, decreased the number of cells in the infected cornea at 1 d p.i. Other investigators also showed that VIP treatment inhibits mouse dendritic cell

migration to adjacent lymph nodes (Weng et al., 2007), limiting infection. All of this is consistent with the anti-inflammatory effects of VIP and with previous studies from our laboratory that showed that the presence of Langerhans cells in the cornea (before infection) exacerbates subsequent disease (Hazlett et al., 2002).

Overall, this study provides evidence that in vivo treatment with VIP down-regulates pro-inflammatory TLRs and increases anti-inflammatory TLRs and related signaling molecule expression via cAMP-dependent and -independent pathways, as well as regulates Langerhans cell number and migration in the infected cornea. In vitro studies using ultrapure LPS, which only activates the TLR4 pathway, confirm the in vivo data and provide evidence that TLR expression in macrophages and XS52 (Langerhans) cells is differentially regulated by VIP to improve disease outcome.

Table 2. Primer sequence of the Toll-like receptors and related molecule for Real-Time PCR amplification

Gene	Primer Sequence	Sense
β-actin	5'-GATTACTGCTCTGGCTCCTAGC-3′	F
	5'-GACTCATCGTACTCCTGCTTGC-3'	R
AC7	5'-TGGGCTTGCCTCATGGTACTA-3'	F
	5'-ACCACAAAGACGACAAACAGG -3'	R
Chuk	5'-GTCAGGACCGTGTTCTCAAGG-3'	F
	5'-GCTTCTTTGATGTTACTGAGGGC-3'	R
IRAK1	5'-CAGAACCACCACAGATCATCATC-3'	F
	5'-GGCTATCCAAGACCCCTTCTTC-3'	R
IRAK2	5'-GGAGTGAAGCAGATGTCGTCCAAGC-3'	F
	5'-GCATCTGAGGCAGAGCTGCATCTCT-3'	R
TLR1	5'-TCTTCGGCACGTTAGCACTG-3'	F
	5'-CCAAACCGATCGTAGTGCTGA-3'	R
TLR4	5'-CGCTTTCACCTCTGCCTTCACTACAG-3'	F
	5'-ACACTACCACAATAACCTTCCGGCTC-3'	R
TLR6	5'-CAACCTTATTGAATGTGACCCTCCAGC-3'	F
	5'-TCATCTCAGCAAACACCGAGTATAGCG-3'	R
TLR8	5'-CACGTGTGACATAAGTGATTTTCG-3'	F
	5'-TTTGATCCCCAGGATTGGAA-3'	R
TLR9	5'-AGCTCAACCTGTCCTTCAATTACCGC-3'	F
	5'-ATGCCGTTCATGTTCAGCTCCTGC-3'	R
TRAF6	5'-GCGAGAGATTCTTTCCCTGAC-3'	F
	5'-TGTATTAACCTGGCACTTCTGG-3'	R
SIGIRR	5'-GTGGCTGAAAGATGGTCTGGCATTG-3'	F
	5'-CAGGTGAAGGTTCCATAGTCCTCTGC-3'	R
ST2	5'-TGACGGCCACCAGATCATTCACAG-3'	F
	5'-TGACGGCCACCAGATCATTCACAG-3'	R

Table 3. Selected TLRs from RT^2 Profiler PCR array

Genes	Fold Difference
	VIP/PBS
Casp8	-3.45
Chuk	-11.78
Csf2	-8.27
Csf3	-5.42
Fos	-18.74
Hmgb1	-13.72
Hspd1	-8.10
Il1a	-3.70
Il6	-3.01
Irak2	-5.27
Myd88	-3.58
Ripk2	-3.31
Ticam2	-3.05
Tlr1	-11.78
Tlr6	-6.36
Tlr8	-7.77
Tlr9	-4.40
Tnfaip3	-14.20
Tnfrsf1a	-3.24
Traf6	-7.61
Ube2n	-5.49

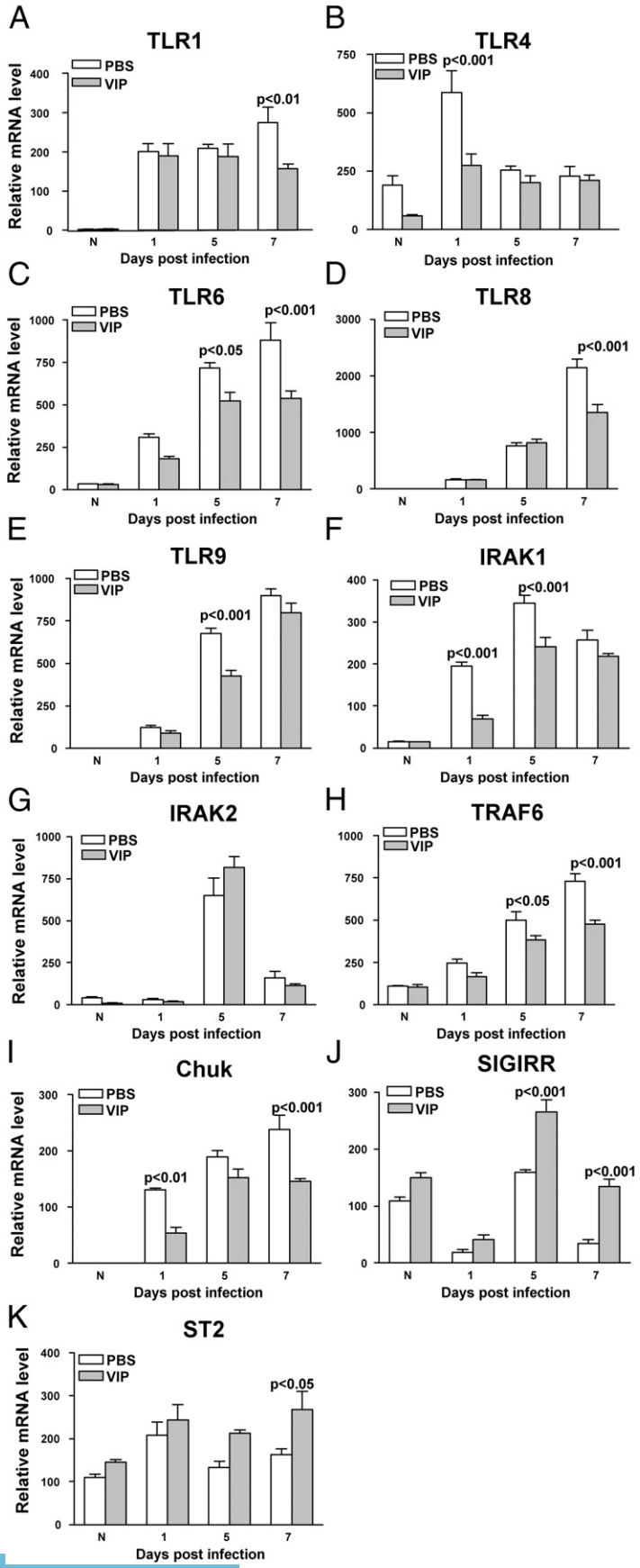

FIGURE 11. RT-PCR for mRNA expression. Compared with PBS treatment, VIP treatment decreased mRNA levels of TLR1 at 7 d p.i. (A), TLR4 at 1 d p.i. (B), TLR6 at 5 and 7 d p.i. (C), TLR8 at 7 d p.i. (D), TLR9 at 5 d p.i. (E), IRAK1 at 1 and 5 d p.i. (F), TRAF6 at 5 and 7 d p.i. (H), and Chuk at 1 and 7 d p.i. (I). VIP versus PBS treatment increased mRNA levels of SIGIRR at 5 and 7 d p.i. (J) and for ST2 at 7 d p.i. (K). (G) No significant change was seen for IRAK 2, nor were any changes seen at other times tested for any of the assays.

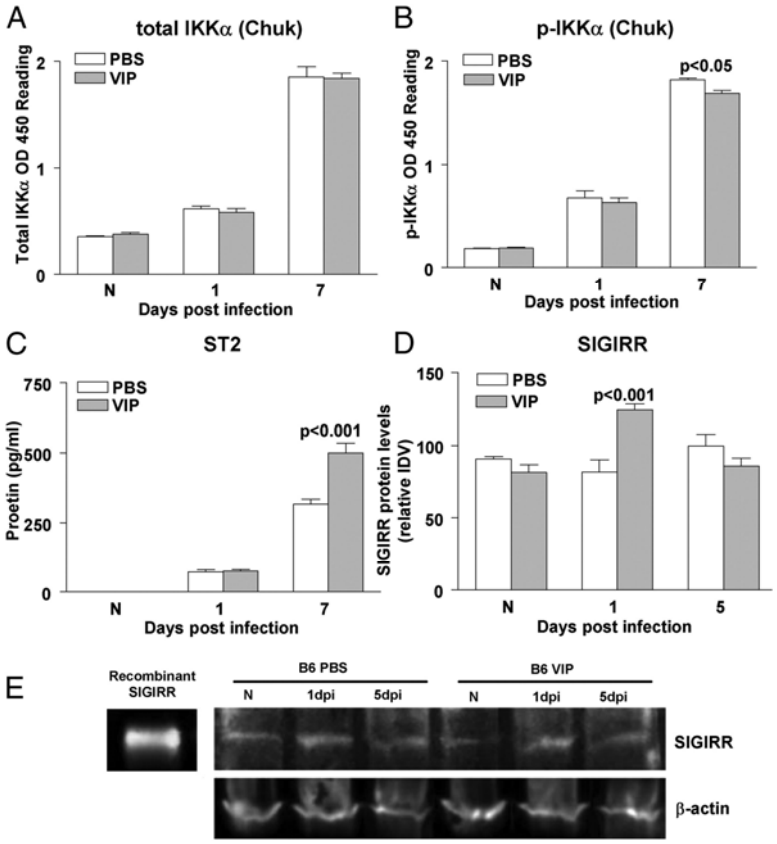

FIGURE 12. ELISA. Compared with PBS treatment, VIP treatment had no effect on total IKKα (A) in normal cornea (N) or infected cornea, but it significantly, although slightly, decreased p-IKKα protein levels at 7 d p.i. (B), with no effect in normal cornea (N) or at 1 d p.i. (C) ST2 protein levels were significantly increased by VIP but only at 7 d p.i. (E) Western blot of SIGIRR protein levels in the uninfected, normal cornea (N) and infected cornea (1 and 5 d p.i.) of PBS- or VIP-treated mice. Equivalent protein was loaded (80 μg/lane) for all lanes except lane 1. Lane 1, Murine rSIGIRR (15 ng); lane 2, PBS-treated normal cornea (N); lane 3, PBS-treated cornea at 1 d p.i.; lane 4, PBS-treated cornea at 5 d p.i.; lane 5, VIP-treated normal cornea (N); lane 6, VIP-treated cornea at 1 d p.i.; lane 7, VIP-treated cornea at 5 d p.i. (D) The intensity of bands was quantitated and normalized to the β-actin control. Data (mean ± SEM integrated density values at

each time point) are significant only at 1 d p.i.

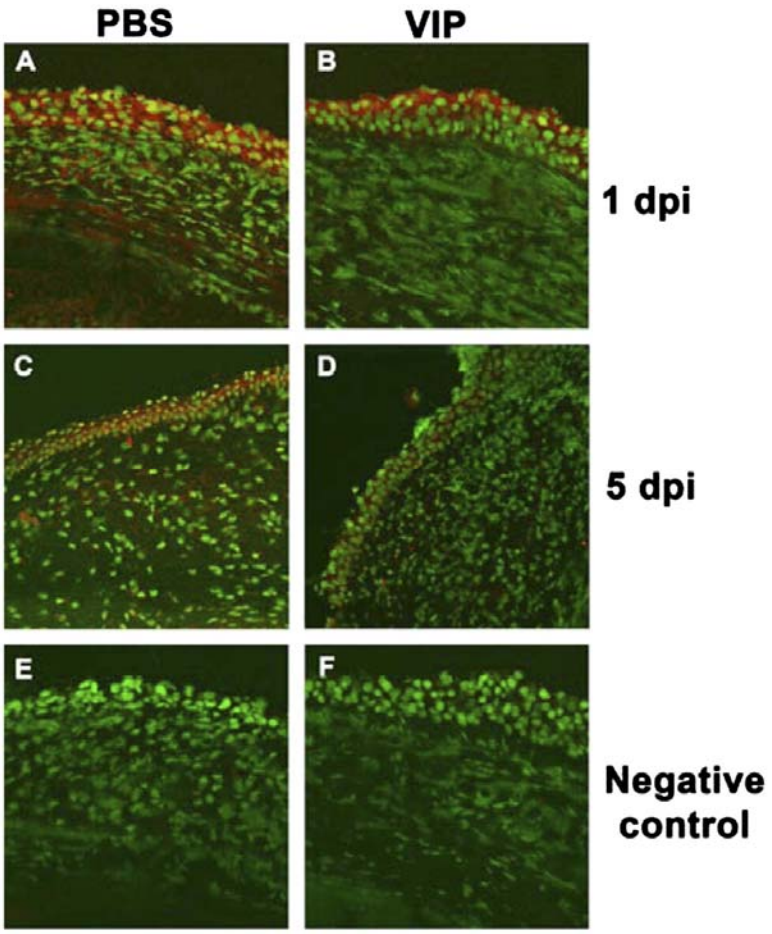

FIGURE 13. TLR4 immunostaining. At 1 and 5 d p.i., TLR4 staining (red) was less intense in the epithelium and stroma after VIP (B, D) treatment compared with PBS (A, C) treatment. PBS-treated (E) and VIP-treated (F) controls (1 d p.i.), in which the primary Ab was replaced by IgG, were negative for TLR4 immunostaining. Original magnification ×100.

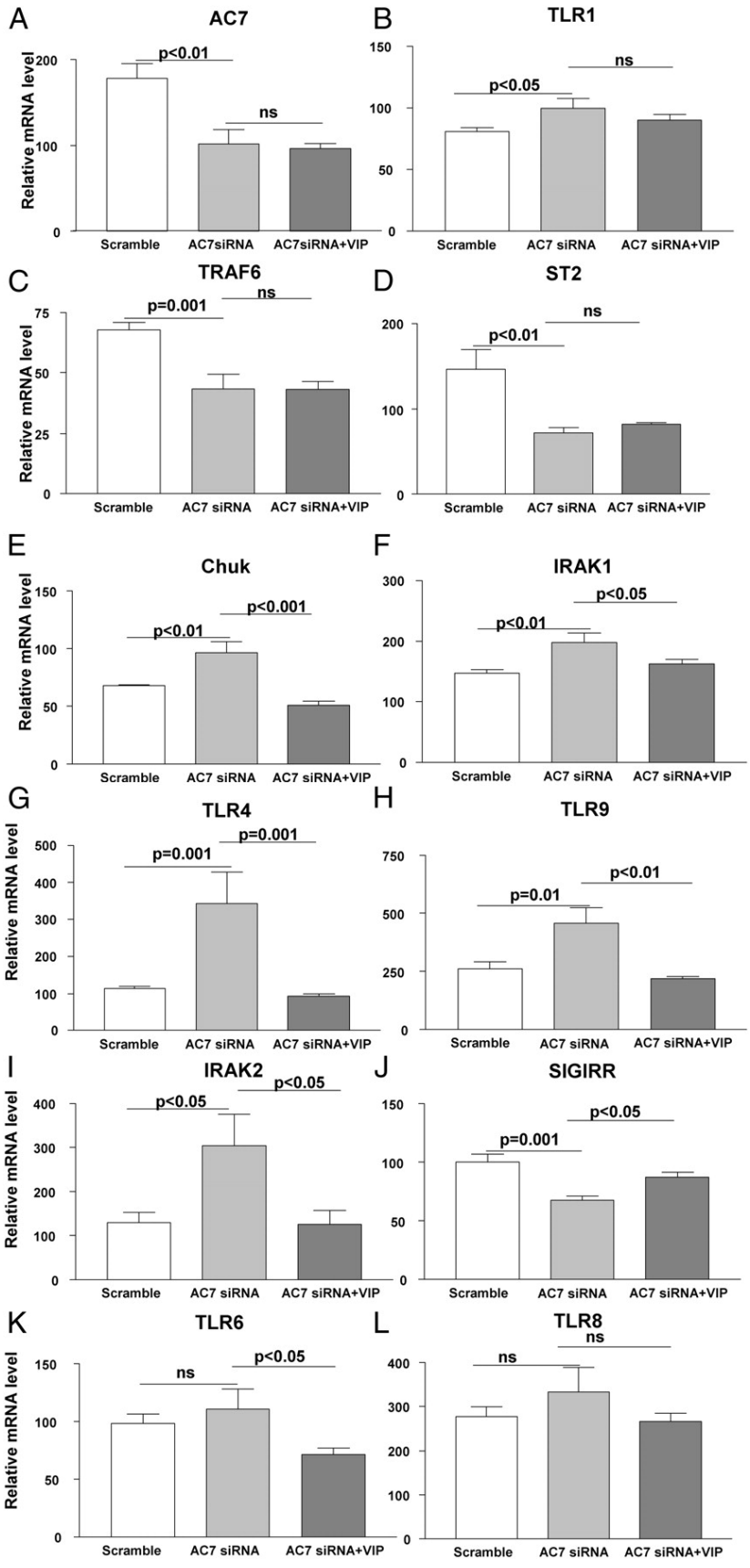

FIGURE 14. (A) When tested at 5 d p.i., RT-PCR confirmed that, compared with scrambled control treatment, silencing AC7 significantly decreased AC7 mRNA levels; silencing plus VIP treatment did not reverse the effect. When tested at 5 d p.i., silencing AC7 increased mRNA levels of TLR1 (B), whereas TRAF6 (C) and ST2 (D) were decreased. Silencing plus VIP injection did not change the effect of silencing for any of the above-mentioned molecules. In contrast, compared with scrambled control treatment, Chuk (E), IRAK1 (F), TLR4 (G), TLR9 (H), and IRAK2 (I) mRNA levels were increased after silencing AC7. Silencing plus VIP injection decreased mRNA expression of each of these molecules significantly. (J) SIGIRR mRNA levels were decreased after silencing AC7; however, silencing plus VIP treatment resulted in increased mRNA levels over silencing alone. No significant changes in mRNA levels of TLR6 (K) or TLR8 (L) were seen with AC7 siRNA treatment; however, silencing plus VIP treatment resulted in reduced mRNA levels of TLR6.

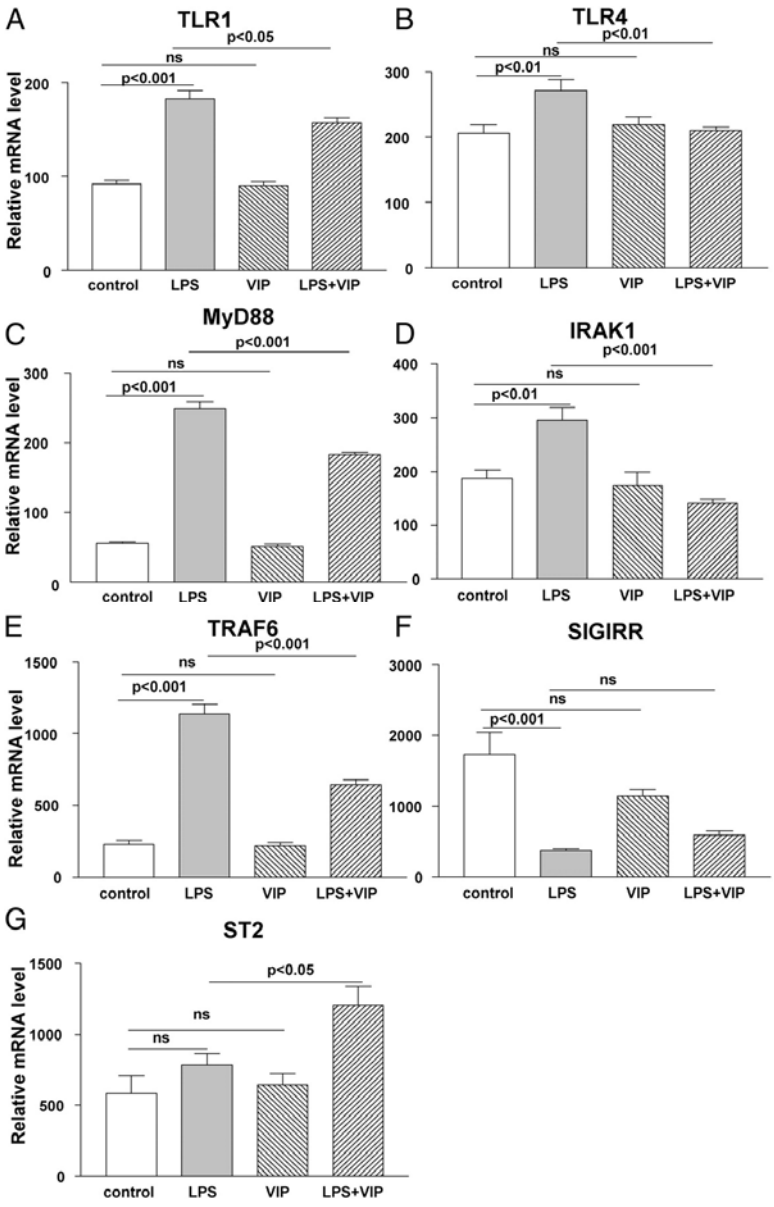

FIGURE 15. In vitro studies using macrophages. Compared with stimulation of macrophages with media control, ultrapure LPS significantly increased mRNA levels of TLR1 (A), TLR4 (B), MyD88 (C), IRAK1 (D), and TRAF6 (E), whereas it decreased mRNA levels of SIGIRR (F) and had no effect on ST2 (G). Compared with LPS alone, LPS plus VIP treatment significantly decreased mRNA expression of TLR1 (A), TLR4 (B), MyD88 (C), IRAK1 (D), and TRAF6 (E), whereas it increased ST2 (G) and had no effect on SIGIRR (F).

Compared with media control, VIP alone had no significant effect on any of the molecules tested.

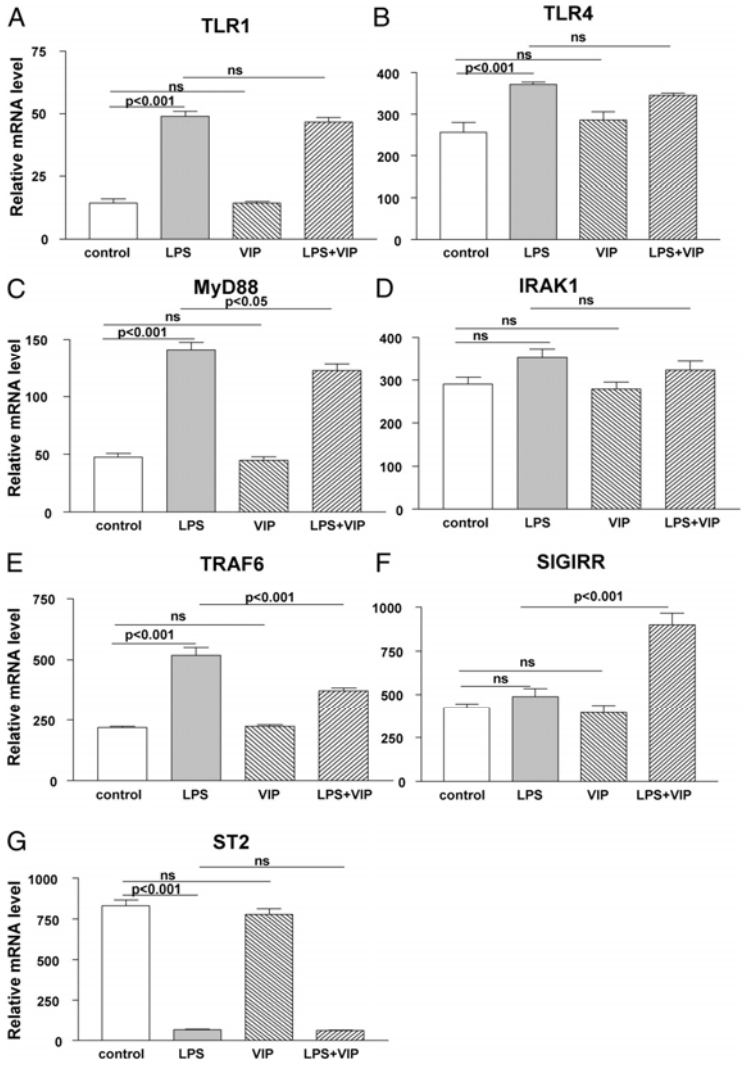

FIGURE 16. In vitro studies using XS52 (Langerhans) cells. Compared with media control, stimulation of these cells with ultrapure LPS significantly increased mRNA levels of TLR1 (A), TLR4 (B), MyD88 (C), and TRAF6 (E), whereas it decreased mRNA levels of ST2 (G) and had no effect on IRAK1 (D) or SIGIRR (F). Compared with LPS alone, VIP plus LPS treatment significantly decreased MyD88 (C) and TRAF6 (E), increased SIGIRR (F), and had no significant effect on TLR1 (A), TLR4 (B), IRAK1 (D), or ST2 (G). Cultured cells treated with VIP alone did not differ from media-treated controls for any of the molecules tested.

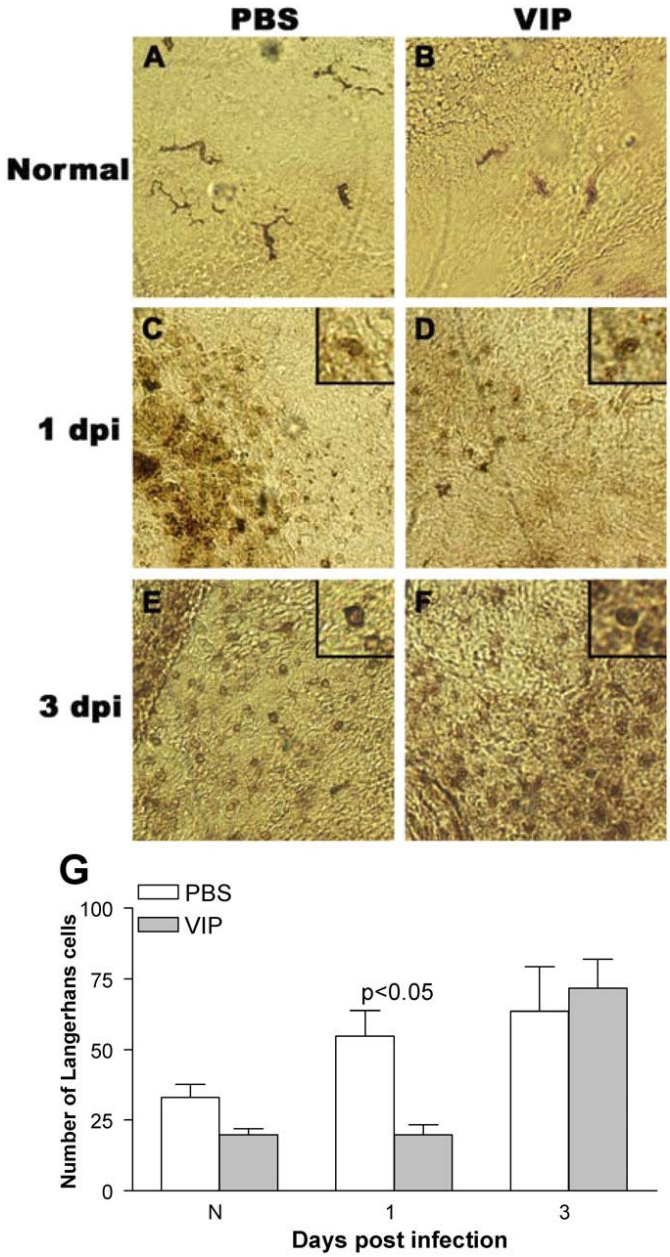

FIGURE 17. Staining and quantitation of Langerhans cells. In the normal, uninfected eye (conjunctiva/peripheral cornea), dendritic-shaped ADPase$^+$ Langerhans cells appeared similar in number after VIP (B) or PBS (A) treatment. At 1 d p.i., rounded ADPase$^+$ cells in the peripheral cornea appeared to be decreased in number after VIP treatment (D) compared with PBS treatment (C). At 3 d p.i., cells appeared qualitatively similar after PBS (E) or VIP (F) treatment.

Insets show examples of cells. (G) Quantitation confirmed these findings. (A–F) Original magnification ×375; field size, 270 μm^2.

Chapter 4

THE ROLE OF VIP IN CORNEA

4.1 Abstract

Purpose: Exogenous VIP down-regulates pro- but upregulates anti-inflammatory cytokines, growth factors (GFs) and TLRs promoting healing in experimental *P. aeruginosa* keratitis. Whether VIP is required for GF or GF receptor (R) expression in normal and infected cornea is unknown and the purpose of this study. Methods: VIP knockout ($^{-/-}$) and wild type (WT) C57BL/6 (B6) mice were infected and tested using PCR array, real-time RT-PCR, ELISA and immunostaining. VIP antagonist treatment studies also were done using B6 and BALB/c mice. Results: Infected corneas of VIP$^{-/-}$ vs WT B6 mice perforated earlier [2 vs 5 days postinfection (p.i.)] and array data showed that GFs were differentially changed between groups. RT-PCR revealed that the infected cornea of VIP$^{-/-}$ vs WT mice expressed higher mRNA levels of EGF and HGF, reduced FGF, EGFR and HGFR, with no difference in FGFR; differences between groups were not seen in normal cornea. Immunostaining for GF and GFR in the normal cornea of VIP$^{-/-}$ vs WT mice were similar for both. However, at 1 day p.i., VIP$^{-/-}$ vs WT mice had more intense EGF and HGF, similar FGFR, and reduced FGF, EGFR, and HGFR staining. VIP antagonist treatment decreased protein levels for GFR at 5 days p.i. in both B6 and BALB/c mice, with no significant changes in normal cornea. Conclusions: The data show that endogenous VIP is not requisite for GF or GFR expression in the normal cornea, but after infection, its absence or reduction is critical for their regulation.

4.2 Introduction

P. aeruginosa, an opportunistic, Gram negative pathogen, is one of the most virulent organisms associated with microbial keratitis and is often associated with contact lens use (Wilhelmus, 1987). Infections progress rapidly and lead to inflammatory epithelial edema, stromal infiltration, corneal ulceration, and oftentimes vision loss (Hazlett, 2004). Experimental murine models of the disease have been established: Th1 responder mouse strains (e.g., C57BL/6, B6) are susceptible (cornea perforates), whereas Th2 responder strains (e.g., BALB/c) are resistant (cornea heals) (Hazlett et al., 2000).

In previous studies using these murine models, we provided evidence that an anti-inflammatory neuropeptide, VIP promotes resistance against *P. aeruginosa* corneal infection by regulation of cytokine production and subsequent alteration of the host inflammatory cell response (Szliter et al., 2007). In addition, recent studies from this laboratory have provided mechanistic information that VIP treatment modulates keratitis through regulation of growth factors, angiogenic molecules, beta defensins (Jiang et al., 2011) and Toll-like receptors (TLR) (Jiang el al., 2012) in the infected cornea, contributing to healing. However, whether endogenous VIP is required for production of growth factors (GF) or growth factor receptors (GFR) in the normal, uninfected cornea and after infection remains untested. This is of importance to determine, since it has been demonstrated in other experimental models (Ware and Matthay, 2002; Woodworth et al., 2005) that one of the ways that inflammation can be regulated is through growth factor binding to their receptors. Thus, the current study

investigated the expression of growth factors and their receptors in the normal and infected cornea of VIP knockout ($VIP^{-/-}$) vs wild-type (WT) mice. Data from the studies provide evidence that the normal cornea of both groups of mice is similar morphologically and have a similar pattern of GF and GFR expression, suggesting that endogenous VIP is not requisite for their expression. However, after infection, corneal perforation occurred more rapidly (2 days p.i.) in knockout vs WT mice and real time RT-PCR and immunostaining studies showed disparate expression of GF and their receptors in the two groups. As the GFR appeared to be most consistently affected, B6 and BALB/c mice were treated with a VIP antagonist, infected and tested for GFR expression. In this experiment, GFR proteins were reduced for both murine strains, confirming the trend in the knockout data. Overall, the current study provides evidence that endogenous VIP is not required for normal corneal expression of GF or GFR. However, if it is absent or reduced, GFs and their receptors are disregulated and the infected cornea perforates rapidly.

4.3 Materials and Methods

Infection

Eight-week-old female C57BL/6, $VIP^{-/-}$ (B6.129S4-*Vip*^{tm1Clw}/J) and BALB/c mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were anesthetized with ethyl ether and placed beneath a stereoscopic microscope at x40 magnification. The cornea of the left eye was wounded (Rudner et al., 2000) and a 5-µl aliquot containing 1.0 x 10^6 CFU of *P. aeruginosa* (American Type Culture Collection, strain 19660, Manassas, VA) was topically delivered. Animals

were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

Ocular response to bacterial infection

Corneal disease was graded: (Hazlett et al., 1987) 0 = clear or slight opacity, partially or fully covering the pupil; +1 = slight opacity, fully covering the anterior segment; +2 = dense opacity, partially or fully covering the pupil; +3 = dense opacity, covering the entire anterior segment; and +4 = corneal perforation or phthisis. After infection, a clinical score was recorded (days 1 and 2) for each group of mice (n = 5/group/treatment).

VIP antagonist treatment

BALB/c and B6 mice (n = 5/group/time/assay) were injected intraperitoneally with 100 µL PBS containing 10 µg VIP antagonist (McClellan et al., 2008) (Bachem, San Carlos, CA) on days −1, 0 (day of infection), and daily through 5 days p.i. Control mice were similarly injected with PBS. Normal, uninfected and infected corneas were collected at 3 and 5 days p.i. for real-time RT-PCR mRNA detection and 5 days p.i. for ELISA assay.

Real-time RT-PCR

Total RNA was isolated from an individual cornea using RNA-Stat 60 (Invitrogen, Carlsbad, CA), per the manufacturer's recommendations and quantitated spectrophotometrically (260 nm). One microgram of total RNA was reverse transcribed using MMLV reverse transcriptase. The 20 µl reaction mixture containing 200 U of MMLV-reverse transcriptase, 10 U of RNase Inhibitor,

500 ng of oligo (dT) primers, 10 mM dNTP, 100 mM DTT, and MMLV reaction buffer (Invitrogen, Carlsbad, CA). After, cDNA was amplified using SYBR Green Master Mix (Bio-Rad), per the manufacture's recommendation. Briefly, the 20 µl reaction system contained: 10 µl of SYBR Green PCR Master Mix, 0.5 µM primers, 2 µl of cDNA (diluted 1/25), and diethyl pyrocarbonate water. All primer sets for the PCR array were purchased as a 96-well plate (RT^2 Profiler Mouse Wound Healing PCR Array; SABiosciences Corporation, Frederick, MD). The individual primer sets were designed using PrimerQuest (Integrated DNA Technologies, Cambridge, MA). Sequences of primers for β-actin, EGF, EGFR, FGF, FGFR, HGF and HGFR are shown (Table 4).

Quantitative real-time RT-PCR was performed using the MyiQ single color real-time RT-PCR detection system (Bio-Rad). Optimal conditions for PCR amplification of cDNA were established using routine methods (Heid et al., 1996). Relative mRNA levels were calculated after normalization to β-actin.

Immunofluorescent staining

Normal, uninfected and infected eyes were enucleated (n = 3/group/time) at 1 day p.i. from WT and $VIP^{-/-}$ mice, immersed in 1X Dulbecco's PBS (Mediatech, Inc., Herndon, VA), embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Ten-micrometer sections were cut, mounted to polylysine-coated glass slides, and stored at 37°C overnight. After a 2 min fixation in acetone, slides were blocked with 10 mM sodium phosphate buffer containing 2.5% BSA and donkey IgG (1/100) for 30 min at room temperature. Then, sections were incubated for 1 h with goat anti-mouse EGF

(15 µg/ml; R&D Systems, Minneapolis, MN) or goat anti-mouse HGF (15 µg/ml; R&D Systems) antibody. Antibodies for EGF and HGF were incubated simultaneously with antibodies for their receptors, goat anti-mouse EGFR (15 µg/ml; R&D Systems) or goat anti-mouse HGFR (15 µg/ml; R&D Systems). This was followed by secondary antibodies, Alexa Fluor 594-conjugated donkey anti-goat antibody (for EGF and HGF, respectively, 1/1500; Invitrogen), and Alexa Fluor 633-conjugated donkey anti-goat antibody (for EGFR and HGFR, respectively, 1/1500; Invitrogen) for another hour.

Staining for FGF and its receptor, FGFR was done sequentially. After incubating the sections with goat anti-mouse FGF antibody (1/100, Santa Cruz Biotechnology) for 1 h followed by the secondary Alexa Fluor 633-conjugated donkey anti-goat (1/2000; Invitrogen) for another hour, sections were incubated with rabbit anti-mouse FGFR AB (1/100, Santa Cruz), 1 h, and Alexa Fluor 546-conjugated donkey anti-rabbit secondary (1/1500; Invitrogen) for another hour. Controls were similarly treated, but the primary Abs were replaced with the same host IgG, ChromPure goat or rabbit IgG (Jackson ImmunoResearch Laboratories). Finally, sections were visualized and digital images captured with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems).

ELISA

Protein levels for GFR were tested using ELISA kits. Corneas from PBS and VIP antagonist treated mice were individually collected (n = 5/group/time) from normal uninfected and infected corneas at 5 days p.i. For EGFR (EIAab Science Co. Ltd, Wuhan, China), for HGFR (R&D Systems) and for FGFR

(MyBioSource, San Diego, CA), corneas were homogenized in 0.5 ml of PBS with 0.1% Tween 20 and protease inhibitor (1 tablet/10 ml, Roche). All samples were centrifuged at 13,000 rpm for 5 min and an aliquot of each supernatant was assayed in duplicate per the manufacturer's instructions. Sensitivities of the ELISA assays were <0.08 ng/ml for EGFR, <10.0 g/ml for HGFR, and <0.1 ng/ml for FGFR.

Statistics

The difference in clinical score between two groups was tested by the Mann-Whitney U test. For all other experiments, an unpaired, two-tailed Student's *t* test (for comparisons between two groups) was used. P<0.05 was considered to be statistically significant.

4.4 Results

Ocular response in VIP$^{-/-}$ mice

VIP$^{-/-}$ vs WT mice showed similar disease scores at 1 day after infection. At 2 days p.i., a significantly higher mean clinical score (Fig. 18, p<0.05), indicative of worsened disease and/or perforation was recorded for the knockout mice. Typical responses for each of the two groups was documented by photography using a slit lamp and showed that WT (B) mice had opacity over the anterior segment, while VIP$^{-/-}$ (C) mice exhibited corneal perforation, with the remainder of the eye heavily opaque due to the presence of an inflammatory infiltrate.

PCR array and real-time RT-PCR

To determine whether endogenous VIP is required for GF production,

mRNA levels of 84 Wound Healing related genes were profiled by PCR array at 1 day p.i. (Table 5). Eighteen of these GF and related molecules (e.g., EGF, EGFR, FGF, and HGF) were differentially changed in $VIP^{-/-}$ vs WT B6 mice and are listed in Table 5. RT-PCR was used to selectively confirm the array data and to test for FGFR and HGFR which are receptors for FGF and HGF but not included on the array. In the normal, uninfected cornea, we found no significant difference at the mRNA level between the two mouse groups for GF or GFR expression (Fig. 19 A-F). However, at 2 days p.i., $VIP^{-/-}$ vs WT mice showed significantly increased mRNA levels for EGF (Fig. 19A, $p<0.01$) and HGF (Fig. 19E, $p=0.01$) but lower levels of FGF (Fig. 19C, $p<0.01$). For GFR, EGFR (Fig. 19B, $p<0.01$) and HGFR (Fig. 19F, $p<0.01$) were significantly decreased in $VIP^{-/-}$ vs WT mice, with no significant changes to FGFR (Fig. 19D).

Immunostaining

Immunostaining confirmed the mRNA data above and showed no differences between the two groups of mice in the normal cornea for either the GFs (Fig. 20) or for the GFRs (Fig. 21). When comparing the two groups at 1 day p.i. (Fig. 22), immunostaining of the cornea of $VIP^{-/-}$ vs WT mice revealed increased EGF and HGF, while FGF staining was decreased. For the GFRs, comparison in immunostaining between the two groups at 1 day p.i. (Fig. 23) revealed that $VIP^{-/-}$ mice had decreased EGFR and HGFR staining, while staining for FGFR was similar. All controls (Figs. 20-23) in which the primary antibody was replaced with same species IgG, were negative for immuunostaining.

Real-time RT-PCR

Next, to further test the effects of VIP on GF and GFR expression, real-time RT-PCR was done using B6 mice treated with a VIP antagonist vs PBS. Similar to the $VIP^{-/-}$ experiments, there was no significant difference between the two groups of mice at the mRNA level in either GFs or GFRs in the normal, uninfected cornea (Fig. 24 A-F). However, at 5 days p.i., VIP antagonist vs PBS treated mice showed significantly increased mRNA levels for EGF (Fig. 24A, $p=0.01$) and HGF (Fig. 24E, $p<0.01$), with decreased levels for FGF (Fig. 24C, $p=0.05$). For GFRs, EGFR (Fig. 24B, $p=0.01$) and HGFR (Fig. 24F, $p=0.05$) were significantly decreased in VIP antagonist vs PBS treated mice, while there was no significant change for FGFR (Fig. 24D).

ELISA

GFRs were tested at 5 days p.i. in B6 (Fig. 25 A,C,E) and BALB/c (Fig. 25 B,D,F) mice after treatment with VIP antagonist. For B6 mice, treatment with the VIP antagonist resulted in reduced levels of EGFR (A), although not significant, while reduction in both FGFR (C, $p=0.01$) and HGFR (E, $p<0.01$) were significant. For BALB/c mice, VIP antagonist treatment significantly reduced all three GFR, EGFR (B, $p<0.001$), FGFR (D, $p<0.01$) and HGFR (F, $p<0.01$). No difference in GFRs between groups was detected in normal cornea.

4.5 Discussion

The neuropeptide VIP is a multifunctional molecule and regulates immune reactions and also participates in the maintenance and restoration of immune homeostasis (Souza-Moreira et al., 2011). In addition, VIP has been considered in itself to be a growth factor (Gressens et al., 1997), as well as a type 2 cytokine

(Pozo and Delgado, 2004).

In prior work, our laboratory has provided evidence that treatment with synthetic VIP regulates expression of pro- and anti-inflammatory cytokines (Szliter et al., 2007), growth factors (Jiang et al., 2011), and Toll-like receptors (Jiang et al., 2012) in *P. aeruginosa* induced keratitis. This leads to decreased stromal destruction in a B6 mouse model in which the untreated, infected cornea perforates within 5-7 days after experimental infection (Szliter et al., 2007). The present study, using $VIP^{-/-}$ mice tested whether the endogenous absence of VIP or its reduction using an inhibitor, contributed to modulation of growth factors and their receptors. No detectable differences in either GFs or GFRs were detected in the normal cornea by either RT-PCR or by immunostaining. Neither was there detectable differences in corneal morphology between the two groups. In contrast, in other models, the absence of VIP has been shown to lead to overall altered intestinal morphology, as well as thickening of smooth muscle and increased villi length in the bowel (Lelievre et al., 2007). In whole cultured embryonic 9.5 mouse embryos, VIP regulated insulin growth factor-I and embryonic development and when pregnant mice were treated with a VIP antagonist, inhibition of embryonic growth occurred (Servoss et al., 2001).

After infection, in the absence of the neuropeptide, GFs and GFRs were dysregluated when tested by PCR array, RT-PCR and immunostaining and the cornea perforated more rapidly (within 2 days after infection) for the majority of the knockout vs WT mice. Furthermore, either the endogenous absence of VIP or antagonist treatment of B6 mice led to this dysregulation. Specifically, VIP

antagonist treatment of B6 mice reduced all three GFRs at the mRNA level (all significantly except for FGFR). And when either B6 or BALB/c mice were treated with a VIP antagonist, GFR protein levels were decreased in both strains of mice when compared with controls. In this regard, others have shown interaction of VIP with the EGFR previously. *In vitro* studies have shown that G_q protein-coupled receptor agonists such as VIP can directly activate (phosphorylate) the EGFR in T_{84} colonic epithelial cells by signaling pathways involving cAMP and protein kinase A and regulate Cl^- secretion (Bertelsen et al., 2004). In another study, it was reported that during infection of human alveolar epithelial cells with *Pseudomonas fluorescens,* a gram-negative rod, blocking EGFR increased epithelial susceptibility to pathogen induced epithelial cell death (Choi et al., 2011), consistent with, although not tested in our keratitis model. Another growth factor, KGF/FGF-7 (FGF) and its receptor (FGFR), were both found to reduce *Pseudomonas* infection in a lung model, through reducing bacterial load, since intratracheal FGF was found to increase clearance of *P. aeruginosa* (Viget et al., 2000). Another study using an experimental burn model in human keratinocytes, also found that when FGF was added to *P. aeruginosa* in the presence of keratinocytes, bacterial growth was inhibited and the same was observed when genetically modified keratinocytes were used (Sobral et al., 2007). In addition, *P. aeruginosa* infection in a bioengineered skin model, in which FGF7 was expressed in diploid human keratinocytes, led to increased antimicrobial β-defensin-2 and cathelicidin (LL-37) production and reduced bacterial load when compared with controls (Erdag et al., 2004). Previous studies from our laboratory

also found that treatment using a GF mixture, composed of EGF, FGF and HGF, increased murine beta-defensin-2 (mBD-2) and mBD3 expression, as well as decreasing bacterial plate counts, resulting in less disease severity in the infected cornea (Jiang et al., 2011). FGF also has been shown to promote wound healing in skin epithelium (Marchese et al., 1995) and participates in lung epithelial repair as well (Ware and Matthay, 2002). Since many cases of bacterial keratitis result either directly or indirectly from disruption of the corneal epithelium (O'Brien, 2003), it also is possible that earlier perforation observed in $VIP^{-/-}$ mice may be due to reduced FGF levels with subsequent epithelial defect. HGFR mRNA and protein (immunostaining) are also decreased in the $VIP^{-/-}$ mice. HGFR recognizes HGF which has anti-inflammatory properties, in that it can target vascular endothelial cells and disrupt nuclear factor-kappa B (NF-κB) signaling in these cells (Gong et al., 2006),critical to regulating inflammatory cytokines.

Overall, this study provides evidence that although VIP is not required for GF and GFR expression in normal cornea, either its absence or reduction results in dysregualted GF and GFR expression in the cornea and enhanced disease.

Table 4. Primer sequence of the growth factors and their receptors primers for Real-Time PCR amplification.

Gene	Primer Sequence (5' - 3')	Size (bp)
β-actin	F – GAT TAC TGC TCT GGC TCC TAG C R – GAC TCA TCG TAC TCC TGC TTG C	147
EGF	F – ACG GTT TGC CTC TTT TCC TT R – GTT CCA AGC GTT CCT GAG AG	130
EFGR	F – GTG GAG GGA CAT CGT CCA AA R – ATT GGG ACA GCT TGG ATC ACA T	100
FGF	F – AAC AGC TAC AAC ATC ATG GAA ATC AG R – AAT CAG TTC TTT GAA GTT GCA ATC CT	153
FGFR	F – GTG CTT ATT GGG GAG TAT CTC CA R – GAT CCA AGT TTC ACT GTC TAC CG	102
HGF	F – ACT TCT GCC GGT CCT GTT G R – GGG ATG GCG ACA TGA AGC A	66
HGFR	F – GTG AAC ATG AAG TAT CAG CTC CC R – TGT AGT TTG TGG CTC CGA GAT	100

Table 5. Selected GFs from The Mouse Wound Healing RT^2 Profiler^{TM} PCR array

Genes	Fold Differences
	VIP$^{-/-}$ / WT
Angpt1	-6.45
Csf2	-1.79
Csf3	1.03
Ctgf	-2.06
Egf	1.47
Egfr	-1.83
Fgf10	4.69
Fgf2	-1.41
Fgf7	-5.03
Hbegf	-2.11
Hgf	1.25
Igf1	-3.63
Mif	1.21
Pdgfa	1.16
Tgfa	-2.03
Tgfb1	1.45
Tnf	1.92
Vegfa	1.27

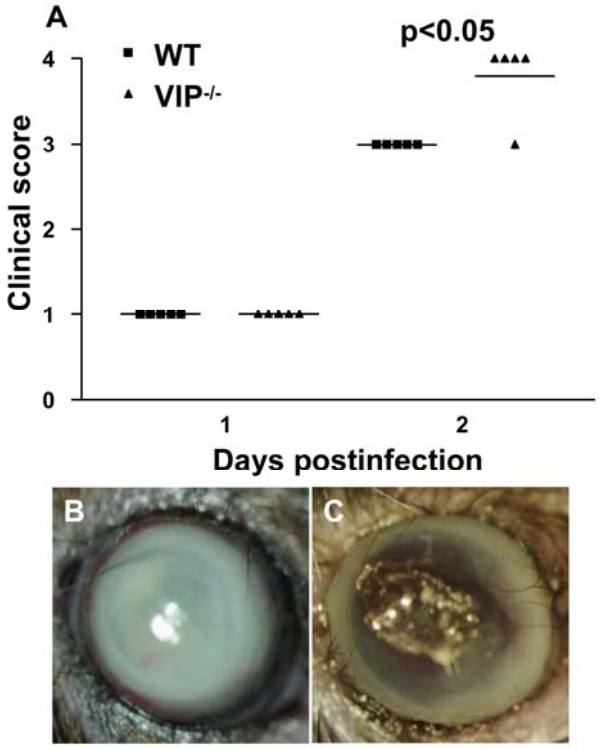

Figure 18. VIP$^{-/-}$ and WT B6 mice. Clinical scores (A) indicated statistically significant differences at 2 days p.i. between groups. Photographs taken with a slit lamp at 2 days p.i. showed an early perforation in VIP$^{-/-}$ (C) vs WT (B) mice. Magnification = ×7.

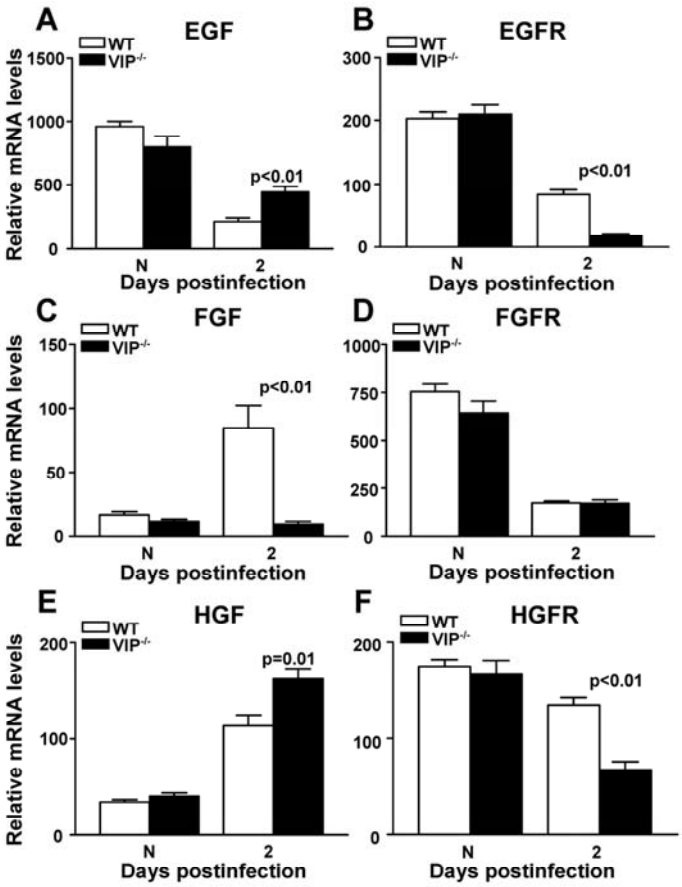

Figure 19. GF and GFR mRNA expression. At 2 days p.i., EGF (A) and HGF (E) were significantly increased, while FGF (C) mRNA levels were significantly decreased in VIP$^{-/-}$ vs WT mice. EGFR (B) and HGFR (F) were decreased significantly, while FGFR (D) mRNA was similar in the two groups. No significant changes between the two groups were seen in normal cornea.

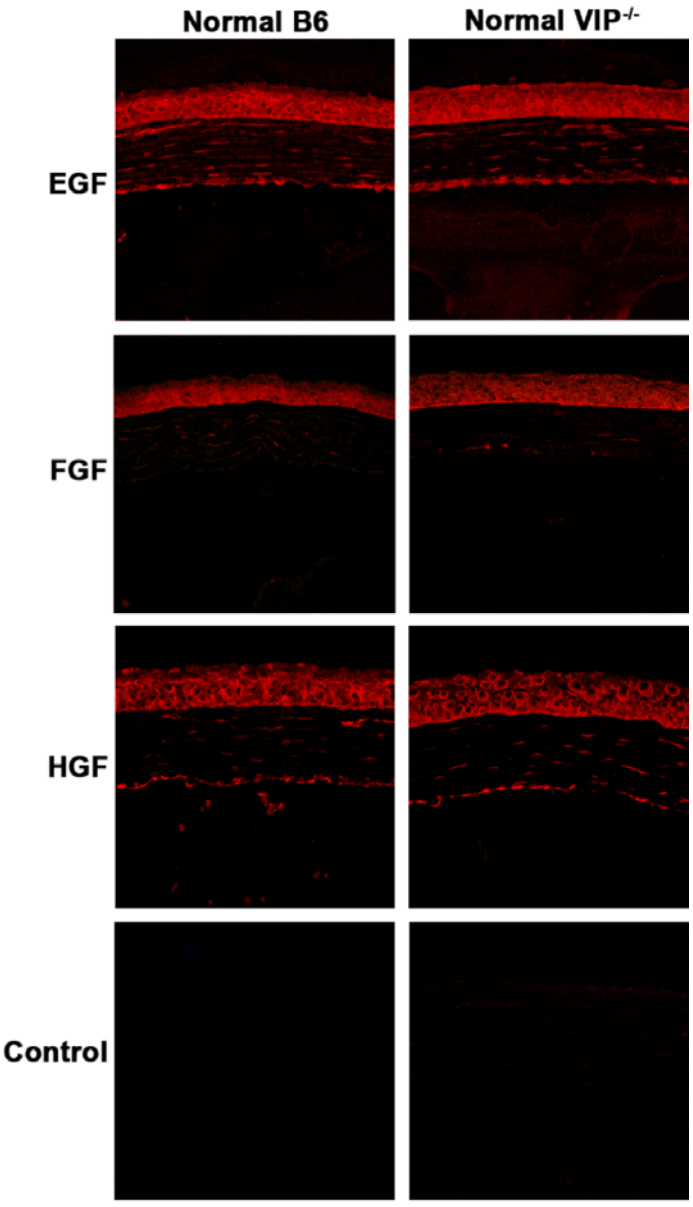

Figure 20. GF immunostaining in normal cornea. GF staining was similar in VIP$^{-/-}$ and WT B6 mice. Negative controls, in which the primary antibody was replaced with a species-specific IgG, showed no positive staining. Magnification = 170X

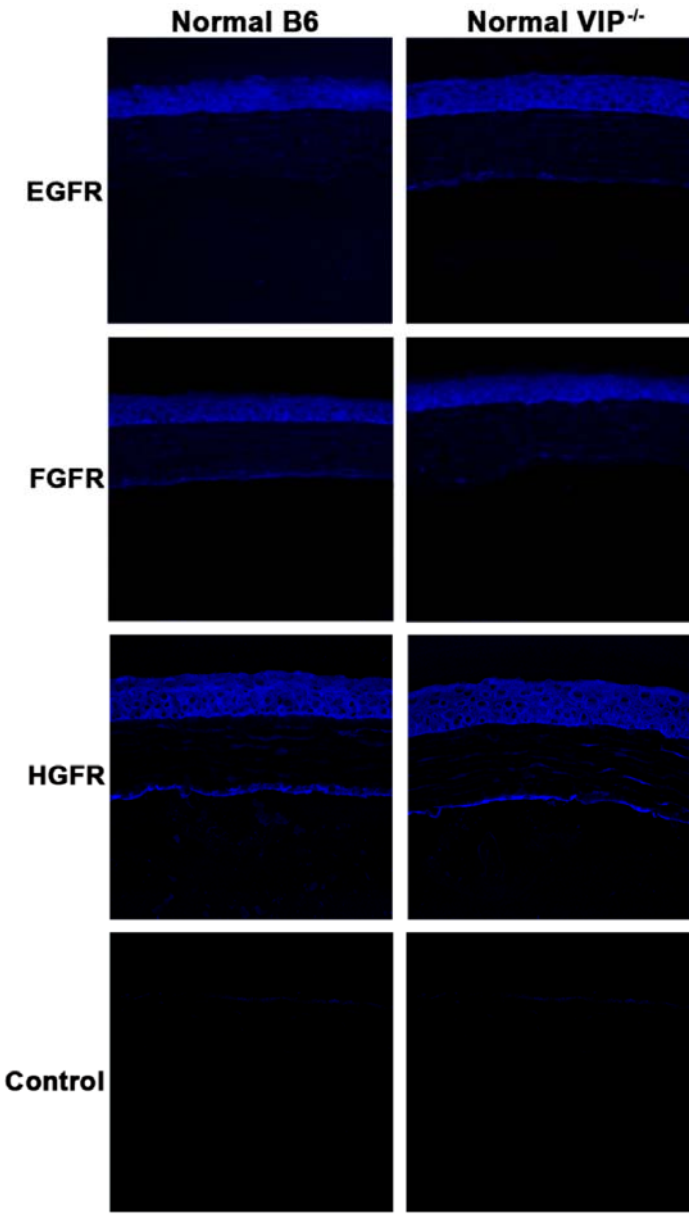

Figure 21. GFR immunostaining in normal cornea. GFR staining did not difference between $VIP^{-/-}$ and WT B6 mice. Negative controls, in which the primary antibody was replaced with a species-specific IgG, showed no positive staining. Magnification = 170X

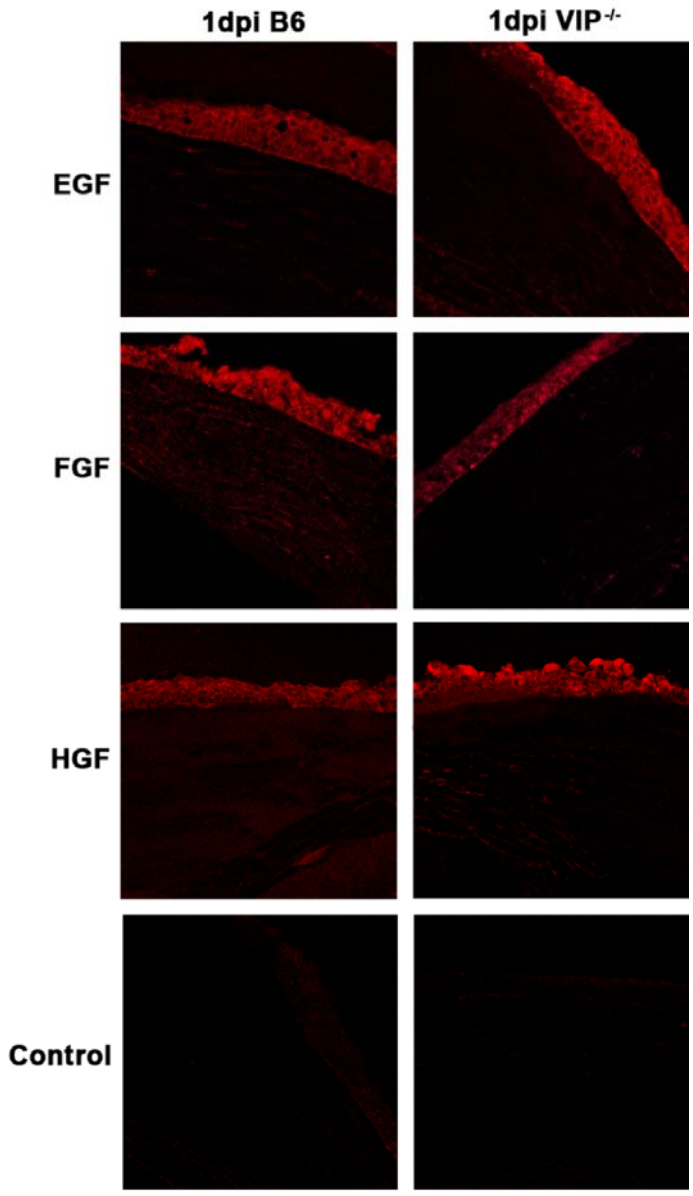

Figure 22. GF immunostaining at 1 day p.i. Greater staining intensity for EGF and HGF, with reduced intensity for FGF was seen in $VIP^{-/-}$ compared to WT B6 mice. Negative controls, in which the primary antibody was replaced with a species-specific IgG, showed no positive staining. Magnification = 170X.

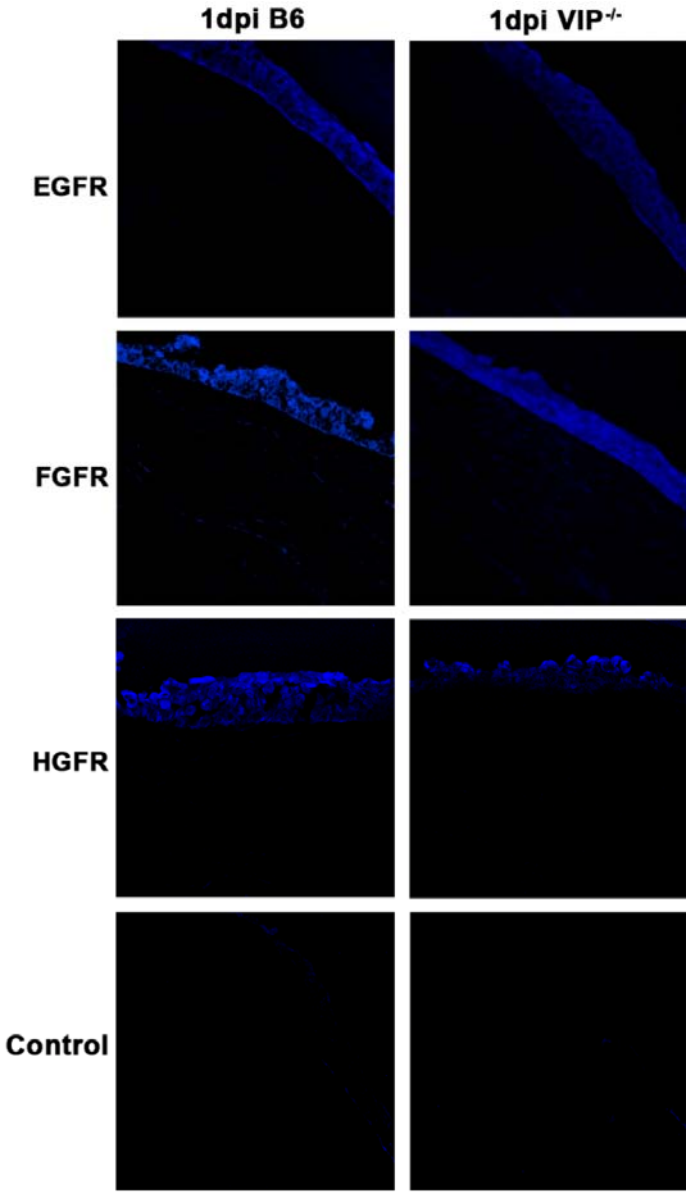

Figure 23. GFR immunostaining at 1 day p.i. Staining for GFRs was less intense for EGFR and HGFR in $VIP^{-/-}$ compared to WT B6 mice. No difference for FGFR was noted between groups. Negative controls, in which the primary antibody was replaced with a species-specific IgG, showed no positive staining. Magnification = 170X.

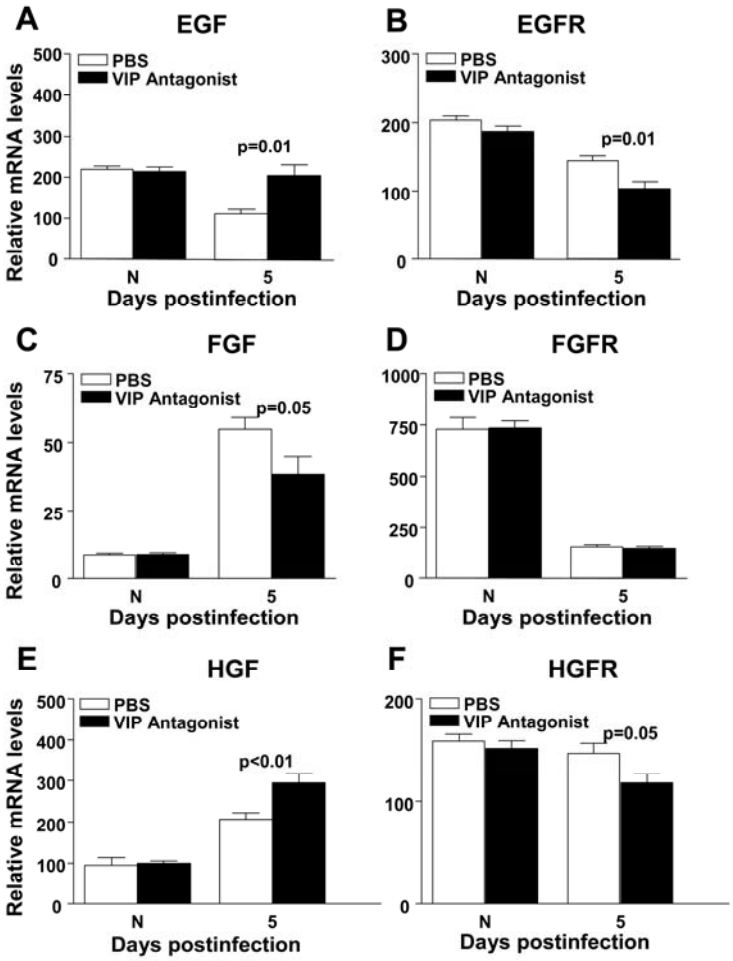

Figure 24. GF and GFR mRNA expression. EGF (A) and HGF (E) were significantly increased, while FGF (C) was decreased in VIP antagonist treated B6 mice at 5 days p.i. EGFR (B) and HGFR (F) were significantly decreased with no difference in FGFR (D) after VIP antagonist treatment. No difference was detected between groups in normal, uninfected cornea.

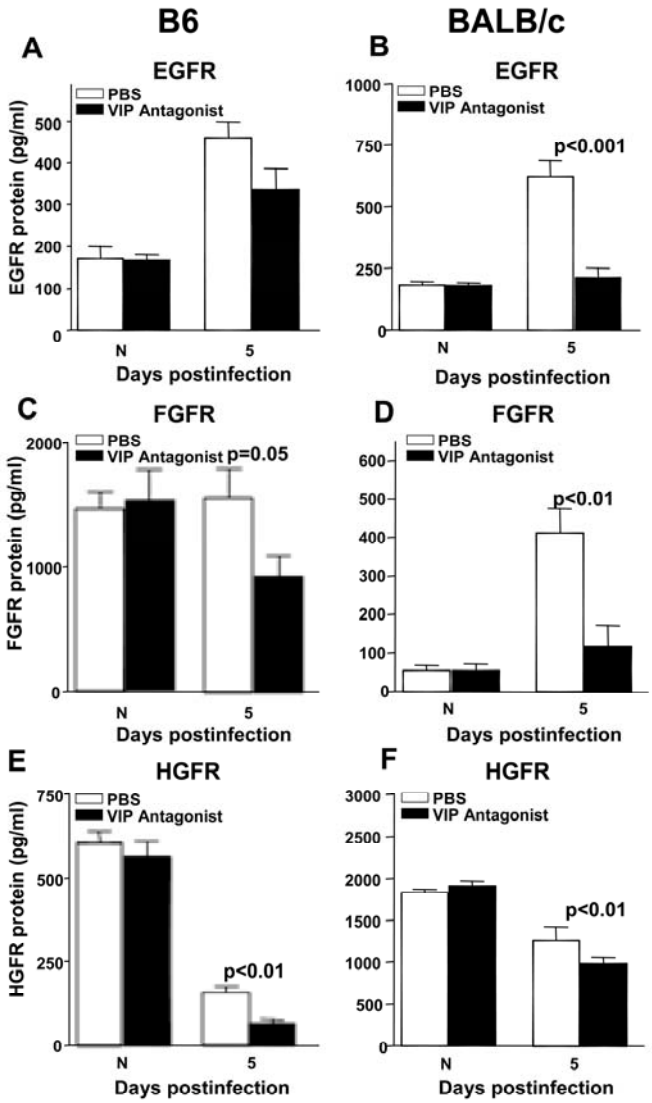

Figure 25. GFR ELISA analysis. VIP antagonist vs PBS treatment decreased GFR (A-F) in both B6 and BALB/c mice at 5 days p.i. All were significant except for EGFR (A). No significant changes were seen in the normal cornea between two groups.

Chapter 5

CONCLUSIONS

Host immunity has been considered as an important factor in determining the prognosis of microbial keratitis (Hazlett LD et al., 2004). Previous study has found that VIP promoted resistance against *P. aeruginosa* induced corneal infection by regulating cytokine production and subsequently altering the host inflammatory response (Szliter et al., 2007). However, the role of VIP in regulation of growth factors and TLRs during *P. aeruginosa* keratitis remained untested. In this regard, current studies provide important insights showing that VIP modulates growth factors and TLR signaling in the infected cornea and these in turn, contribute to better disease outcome.

In chapter 2 (This part of the work has been published: Jiang et al., 2011), we found that VIP vs PBS treatment increased growth factor and angiogenic molecules mRNA and protein levels in infected B6 mouse cornea. In order to assess whether the anti-inflammatory effect of VIP was through regulating growth factors, a mixture of EGF, FGF and HGF was topically applied after infection. Treatment with a mixture of growth factors prevents corneal perforation, down-regulated pro-inflammatory cytokines but up-regulated anti-inflammatory cytokines and β defensins, as well as delaying bacterial growth, indicating the anti-inflammatory effects of VIP are partially achieved through regulating growth factor production.

In chapter 3 (This part of work has been published: Jiang et al., 2012), our work provided evidence that VIP vs PBS treatment decreased pro-inflammatory

TLRs but increased anti-inflammatory TLRs in infected B6 mouse cornea and also in XS52 (Langerhans) cells and peritoneal macrophages. To determine whether VIP effects were cAMP dependent, mice were injected with AC7 siRNA with or without VIP treatment and tested for TLR and related molecules mRNA expression. The results indicated that VIP regulates TLR1, TRAF6 and ST2 in a cAMP dependent manner, but Chuk, IRAK1, 2, TLR4, 9 and SIGIRR were found to be cAMP independent. Moreover, we found that exogenous VIP treatment, compared to PBS control, reduced Langerhans cell number in early (1 day p.i.) infected cornea.

In chapter 4 (This part of work has been submitted for publication and is in revision, 2012), we investigated whether VIP is required for expression of growth factors and their receptors in normal and infected cornea. Our data provide solid evidence of the importance of VIP in host resistance against corneal *P. aeruginosa* infection. We observed that $VIP^{-/-}$ vs WT B6 mouse corneas showed more severe infection and earlier perforation. PCR array and real time RT-PCR data indicated that growth factors and their receptors were differentially changed in infected $VIP^{-/-}$ vs WT mouse cornea; differences between groups were not seen in normal cornea. Immunostaining for growth factors and their receptors in normal and infected cornea of both groups of mice showed similar results when compared with the mRNA data. Additionally, VIP antagonist treatment decreased protein levels for growth factor receptors at 5 days p.i. in both B6 and BALB/c mice, with no significant changes in normal cornea. These results indicate that endogenous VIP is not required for the expression of growth factors and their

receptors in normal cornea, but critical for their regulation after infection.

In summary, this dissertation has focused upon how VIP regulates host immunity to promote resistance against corneal infection. Our data suggest that VIP modulates growth factors and their receptors as well as TLR expression, contributing to corneal healing. The findings presented herein provide further evidence to justify testing VIP clinically either alone, or with antibiotics, to treat microbial keratitis.

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ABSTRACT

VIP AND HOST IMMUNITY

by

XIAOYU JIANG

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Advisor: Dr. Linda D. Hazlett

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The purpose of the current dissertation was to examine how VIP regulates host immunity and corneal healing, specifically, its control of growth factors and TLR expression in the *P. aeruginosa* infected cornea. Firstly, VIP treatment increased growth factor expression (EGF, HGF, FGF and VEGF) in infected cornea. Notably, treatment with a mixture of EGF, FGF and HGF prevented corneal perforation, reduced pro-inflammatory cytokines and bacterial plate count, while increasing anti-inflammatory cytokines and antimicrobials such as murine β-defensin2 and 3.

We also investigated the expression of TLR-signaling pathways in *P. aeruginosa*-infected corneas with or without VIP treatment. PCR array and real-time RT-PCR data demonstrated that VIP treatment decreased pro-inflammatory, but increased anti-inflammatory TLRs. Immunohistochemistry, ELISA and western blot results further confirmed the PCR data. AC7 siRNA experiments indicated that VIP regulated TLR1, TRAF6 and ST2 in a cAMP dependent manner, but Chuk, IRAK1, 2, TLR4, 9 and SIGIRR were cAMP independent. In

vitro, VIP down-regulated pro-inflammatory, but increased anti-inflamamtory TLRs in macrophages and Langerhans (XS52) cells. Exogenous VIP also decreased Langerhans cell number in the infected cornea.

Furthermore, we used $VIP^{-/-}$ mice to asses whether VIP is required for expression of growth factors and their receptors in normal and infected cornea. $VIP^{-/-}$ vs WT B6 mice showed earlier corneal perforation. PCR array showed growth factors are differentially changed between groups. Real time RT-PCR and immunostaining studies revealed that the infected cornea of $VIP^{-/-}$ vs WT mice expressed higher EGF and HGF, reduced FGF, EGFR and HGFR and similar FGFR; no significant difference between the two groups of mice were seen in normal cornea. VIP antagonist treatment decreased protein levels for growth factor receptors at 5 days p.i. in both B6 and BALB/c mice, with no significant changes in normal cornea.

In summary, these data provide evidence that VIP modulate growth factors, angiogenic molecules and beta defensins in the infected cornea; that it reduced pro-inflammatory, but increased anti-inflammatory TLRs after corneal infection; and that it is not required for growth factor production in the normal cornea but required in the infected cornea to delay perforation.

AUTOBIOGRAPHICAL STATMENT

XIAOYU JIANG

7341 Scott Hall, 540 E. Canfield Ave.

Detroit, MI 48201

xyjiang@med.wayne.edu

EDUCATION

2008-2012
Wayne State University School of Medicine
Detroit, MI
Ph.D., December 2012

2003-2008
Hebei Medical University
Shijiazhuang, Hebei, China
M.D., Jun 2008

PUBLICATIONS

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