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Defensins In Ocular Immunity

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by

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DEDICATION

This work is dedicated to my family,

for their endless love, support and encouragement.

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CHAPTER 1

INTRODUCTION

1.1 Innate immunity

The immune system is a complicated and dynamic network composed of two arms, innate and adaptive immunity, each with distinctive features. Innate immunity is the first line of defense against many common microorganisms. It is activated immediately after infection and rapidly controls replication of the infecting pathogen. There are three types of defense mechanisms in innate immunity: anatomical, cellular and humoral (Janeway CA et al., 2005). Invading microorganisms first encounter the anatomical barrier, which is composed of skin and internal epithelial layers, as well as the chemicals they secrete. Once invaders pass the anatomical barrier, cellular and humoral components come into play. The activation of innate immunity is based on innate immune recognition mediated by germ-line encoded receptors. Each receptor has genetically predetermined specificity. The strategy of the innate immune response is not to recognize every antigen, but to focus on some highly conserved structures existing in large groups of microorganisms (Medzhitov R and Janeway CA, 2000). These structures are referred to as pathogen-associated molecular patterns (PAMPs), and those receptors that recognize them are called pattern-recognition receptors (PRRs) (Medzhitov R and Janeway CA, 1997).

The germ-line encoded receptors in innate immunity are different from those antigen receptors involved in adaptive immunity. PRRs are expressed on many effector cells in the innate immune system, like macrophages (Mϕ), dendritic cells (DC) and B cells, and a certain cell type will display PRRs with identical specificities. The

expression is not clonal, as once the PRRs identify a PAMP, the effector cells are triggered to function immediately rather than after cell proliferation.

Functionally, PRRs can be divided into three classes: secreted, endocytic, and signaling. Secreted PRRs function as opsonins by binding to microbial cell walls and flagging them for recognition by the complement system (e.g., mannan-binding lectin; Fraser IP et al., 1998). Endocytic PRRs occur on the surface of phagocytes and can mediate the uptake and delivery of the pathogen bearing specific PAMPs into lysosomes to be destroyed. Then pathogen-derived proteins can be processed, and the resulting peptide can be presented by major histocompatibility complex (MHC) molecules on the surface of the effector cell. Signaling receptors recognize PAMPs and then activate signal transduction pathways which induce the expression of many genes involved in the immune response, including inflammatory cytokines. The most studied signaling receptors are Toll-like receptors (TLRs), which play an important role in the induction of the immune and inflammatory response (Janeway CA and Medzhitov R, 2002).

1.2 Adaptive immunity

Adaptive immunity provides increased protection to remove microbial invaders and against subsequent reinfection by the same pathogen. Initiated with antigen presentation by antigen presenting cells (APC, including DC, B cells, and sometimes Mϕ), activated individual T and B cells are selected to experience clonal expansion in order to produce sufficient numbers of clones and then differentiate into effector cells, thereby removing pathogens via cell-mediated or humoral [antibody (Ab)-mediated] immune defenses.

For cell-mediated immunity, APCs will display those "non-self" antigens on their surface by coupling them to a "self"-receptor, MHC, which in humans, is also called human leukocyte antigen. Endogenous antigens are typically presented on MHC class I molecules, which activate CD8+ cytotoxic T cells, whereas exogenous antigens are usually bound to MHC class II molecules and then activate CD4+ helper T cells (Das G and Janeway CA, 2003). CD8+ cytotoxic T cells destroy virally-infected cells and tumor cells, and are responsible for transplant rejection, while $CD4^+$ helper T cells further differentiate into Th1 or Th2-type T cells and secrete cytokines that stimulate the activity of other immune cells. Th1-type T cells produce interferon-gamma (IFN-γ) and induce B cells to release immunoglobulins (Ig), which are mainly responsible for defense against intracellular pathogens. Th2 T cells produce interleukins (IL) including IL-4, IL-5 and IL-10 and induce production of IgE antibodies, which mainly function in immune defense against parasites (Jankovic D et al., 2001). Moreover, there is a third type of T lymphocyte called the regulatory T cell, which can mediate tolerance by limiting and suppressing the immune system, as well as controlling aberrant immune responses to self-antigens (Das G and Janeway CA, 2003).

For Ab-mediated immunity, B lymphocytes differentiate into plasma cells that secrete specific Abs. In mammals there are five types of Abs: IgA, IgD, IgE, IgG, and IgM, each with different biological properties and that can recognize different kinds of antigens. Based on those specific Abs, each activated B cell can recognize a unique antigen and neutralize specific pathogens.

1.3 Inflammation

Inflammation is a protective reaction by the host 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. Inflammation is a complex biological response triggered by tissue damage, often in company with redness, pain, heat, swelling, and sometimes loss of function (Stvrtinova V, 1995). Although wounds and infections would never heal without inflammation, improperly regulated inflammation may lead to tissue damage and host disease.

Inflammation can be classified into two basic patterns: acute or chronic, each with distinctive features. Acute inflammation is a response within a relatively short time period (hours to days), which is primarily characterized by exudation of fluid and plasma proteins, as well as a neutrophil (PMN) infiltration (Jaeschke H and Hasegawa T, 2006). Whereas chronic inflammation is a reaction of longer duration (days to years), which is characterized by mononuclear cell infiltration, vascular proliferation and ultimately scarring. During the period of chronic inflammation, an induced progressive shift of cell types (mainly monocytes and lymphocytes) at the site of inflammation will lead to simultaneous destruction and healing of the tissue (Kumar V et al., 2007; Janeway CA et al., 2005).

The inflammatory response must be actively terminated when it is no longer required in order to prevent unnecessary damage to tissues. Several mediators are involved in this active mechanism. For example, platelet-activating factor acetylhydrolase (PAF-AH) can regulate lung inflammation by terminating the signal from

PAF, a pro-inflammatory mediator that plays a central role in acute lung injury (Salluh JI et al., 2007).

Besides production of several endogenous molecules (anti-inflammatory cytokines, lipid mediators and glucocorticoids), there are still several other ways to regulate inflammation. For example, apoptosis is a kind of programmed cell death which acts to remove damaged cells and enhance local homeostasis. Studies indicate that apoptosis of PMN may be involved in controlling acute inflammation. Moreover, it has been revealed that there is a bi-directional communication between the immune and neuroendocrine systems (Weigent DA et al., 1995), suggesting that the inflammatory response also can be regulated by various neuropeptides such as vasoactive intestinal peptide (VIP), which balances pro- and anti-inflammatory cytokines and protects against corneal perforation in the *Pseudomonas aeruginosa* (*P. aeruginosa*)-infected cornea (Szliter EA et al., 2007), as well as Substance P, a potent pro-inflammatory regulator which can enhance immune defenses by overcoming the anti-inflammatory effects of VIP and IL-10 (McClellan SA et al., 2008).

1.4 TLRs

As the most important signaling PRRs, TLRs are expressed in many cell types and recognize a variety of PAMPs. To date, thirteen mammalian TLRs have been identified, 10 in human (lack TLR11, 12, 13) and 12 in mice (lack TLR 10) (Beutler B, 2004; Dowling D, 2008; Takeda K and Akira S, 2004), which differ from each other in ligand specificities, expression patterns, and target genes. Based on their location of expression, TLRs can be divided into two classes.

TLR1, TLR2, TLR4, TLR5 and TLR6 belong to the first TLR class, which is mainly expressed on the plasma membrane and functions at the cell surface. TLR4 is expressed in many cell types involved in the immune system, such as Mϕ and DCs. TLR4 is required in recognition of lipopolysaccharide (LPS) from Gram-negative bacteria, by forming the LPS-recognition complex with several coreceptors like lymphocyte antigen 96 (MD2), and CD14 (Shimazu R et al., 1999; Lien E et al., 2001). TLR2 recognizes the largest number of ligands, including bacterial lipoproteins (both Gram-positive and Gram-negative, lipoteichoic acid (LTA) from Gram-positive bacteria, as well as peptidoglycan (Aliprantis AO et al., 1999; Schwandner R et al., 1999). Usually, TLR2 does not recognize these PAMPs independently, but forms heterodimers with either TLR1 or TLR6 to determine its specificity of ligand binding. For example, TLR2/6 heterodimers recognize LTA (Henneke P et al., 2005; von Aulock S et al., 2003; Takeuchi O et al., 2001), whereas TLR2/1 heterodimers recognize lipoprotein/peptides of bacterial cell walls (Takeda K et al., 2002; Ozinsky A et al., 2000). Besides TLR4 and TLR2, TLR5 also functions at the cell surface by recognizing PAMPs such as flagellin, the protein subunits that compose bacterial flagella (Hayashi F et al., 2001).

On the other hand, TLR3, TLR7, TLR8 and TLR9 compose another class of TLR, which is mainly expressed on endosomal membranes, and bind their ligands in the lumen of intracellular vesicles. TLR3 recognizes double-stranded RNA (dsRNA), a molecular signature of most viruses, and triggers inflammatory responses that prevent viral spread (Liu L et al., 2008). TLR9 is typically expressed intracellularly in immune cells such as DCs and recognizes unmethylated bacterial or viral cytosine-phosphate-guanine DNA (CpG-DNA) (Hemmi H et al., 2000). Murine TLR7 or human TLR8 is

required for the recognition of the single-stranded RNAs found in many viruses *in vivo*, leading to IFN-α production in murine or human Mϕ, respectively (Gantier MP et al., 2008).

As new members of the mammalian TLR family, the ligands for TLR10, 11, 12, and 13 are still unknown (Beutler B, 2004). However, Zhang D et al. (2004) found that TLR11 could respond specifically to some uropathogenic bacteria.

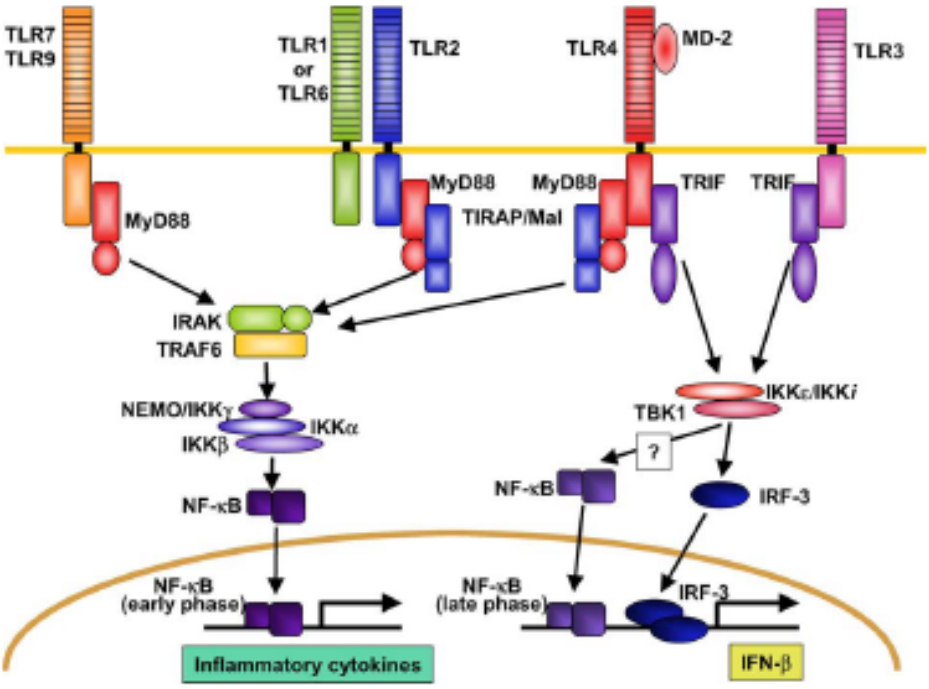

Figure 1. TLR signaling pathways (Takeda K and Akira S, 2004)

The TLR signaling pathways are presented in Figure 1 (Takeda K and Akira S, 2004). There are two TLR signaling pathways: a Myeloid differentiation primary response gene 88 (MyD88)-dependent and a MyD88-independent pathway. MyD88 is essential for inflammatory cytokine production in response to all TLR ligands, except for

dsRNA, the ligand of TLR3. Lipopeptide induces inflammatory cytokine production through a TLR2/MyD88-dependent pathway, but does not induce IFN-β or IFN-inducible genes, and thus lacks an MyD88-independent pathway. LPS induces pro-inflammatory cytokines as well as IFN-β or IFN-inducible genes, via a TLR4/MyD88-dependent pathway and a TLR4/TRIF MyD88-independent pathway, respectively. TLR7, TLR8 and TLR9 lead to a specific pathway in plasmacytoid DCs, which is also through MyD88. However, Wang J et al. (2006) found inhibitory interactions between these three TLRs: TLR8 could inhibit TLR7 and TLR9, and TLR9 could inhibit TLR7, but not *vice versa*.

TLRs also function in the bridging of innate and adaptive immunity. Pathogenic components such as LPS, lipoproteins, and CpG-DNA stimulate TLRs on the surface of DCs, leading to activation of adaptive immunity. The signaling through TLR induces the expression of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor alpha (TNF-α) and co-stimulatory molecules such as CD40, CD80 and CD86 (Dowling D, 2008). These molecules together with presented pathogen antigens promote the development of Th1 cell responses to eliminate those pathogens (Schnare M et al., 2001).

1.5 Antimicrobial peptides

To date, over 800 antimicrobial peptides have been identified from species as widely different as amoeba, plants, penguins and humans. Usually, they are classfied based on secondary structural features: linear α-helical peptides (e.g., cathelicidins), peptides with β-strands linked by disulfide bonds, also called disulfide bridges (e.g., defensins), loop peptides (e.g., bactenecins), and those with a high proportion of specific amino acids (e.g., histatins) (Bals R, 2000; Boman HG, 2003; van't Hof W et al.,

2001). Studies have revealed that the majority of these small peptides (less than 100 amino acids) have a positive charge due to an excess of positively charged amino acids, such as arginine and lysine. Therefore, such peptides are frequently referred to as cationic antimicrobial peptides. Despite significant structural diversity, these peptides play their antimicrobial roles in a similar way. By virtue of their positive charge, these peptides interact electrostatically with negatively charged components of microbial cell membranes (particularly phospholipids), thereby increasing the permeability of the cell membrane and finally resulting in cell death (Hancock RE, 1997; Matsuzaki K, 1999; McDermott AM, 2004). For mammals, there are two main genetic categories for antimicrobial peptides: cathelicidins (Zanetti M, 2004) and defensins (Ganz T, 2003; Lehrer RI, 2004).

1.6 Defensins

Classic mammalian defensins are 29 to 45 amino acids in length and are characterized by the presence of six cysteine residues that interact to form three disulfide bonds and a β-sheet structure. They can be classified into two main classes, α- and β-defensins, based on the location and connectivity of the cysteines. A third novel defensin class has been identified in rhesus macaque leukocytes, referred as θ-defensins (also called minidefensins). Studies show that θ-defensins are only found in non-human primates (Nguyen TX et al., 2003), and are structurally dissimilar to α- and β-defensins. To date, five θ-defensins have been identified: Retrocyclin-1 and -2, as well as rhesus θ-defensin 1 (RTD1), RTD2 and RTD3 (Munk C et al., 2003; Yasin B et al., 2004).

In humans, six α-defensins and four β-defensins have been identified and characterized to date. Their cell sources are listed in Table 1 (Yang D et al., 2004; Selsted ME and Ouellette AJ, 2005) below.

Table 1. Cell source and regulation of human defensins

Name	Cell source	Synthesis	Release
HNP1-4	neutrophil, leukocyte	constitutive, inducible	degranulation
HD5-6	Paneth cell	constitutive	degranulation
HBD1	epithelial cells, keratinocytes	constitutive and inducible	secretion
HBD2-3	epithelial cells, keratinocytes	inducible	secretion
HBD4	epithelial cells (testis, epididymis)	inducible	secretion

*Abbreviations: HNP, human neutrophil peptide; HD, human defensin; HBD, human beta-defensin.

For human α-defensins, HNP 1-4 are found primarily in PMN (highly concentrated in azurophil granules; Greenwald GI and Ganz T, 1987) as well as other leukocytes (e.g., monocytes and lymphocytes), whereas HD5 and HD6 are present in Paneth cells in the small intestine (Mallow EB et al., 1996). It is demonstrated that epithelial cells of the female genital tract also produce HD5 (Quayle AJ et al., 1998). Moreover, HNP 1-3 are detectable in the corneal stroma in cases of rejected transplants and post-infectious keratitis but not in normal cornea (Gottsch JD, 1998). HNP 1-3 also exist in inflamed conjunctiva and the tear film (Haynes RJ et al., 1999; Zhou L et al., 2004). A recent study has demonstrated that pro-inflammatory cytokines can regulate the production of HNP1-3. The role of IL-1β, IL-6 and TNF-α on production of HNP1-3 in immature monocyte-derived DCs (iMMDDCs) has been examined and the results

indicate that each one of those pro-inflammatory cytokines alone, and especially IL-1β, can induce up-regulation of HNP1-3 in iMMDDCs. (Rodriguez-Garcia M et al., 2007).

For human β-defensins, although more than 28 β-defensin genes have been found in the human genome based on a computational search strategy (Schutte BC et al., 2002), only a few have been studied to date. In humans, the most studied β-defensins are HBD1-4. They are expressed chiefly by various epithelial tissues including airway epithelia, urogenital tissues, nasolacrimal duct, and mammary gland (HBD4 is more limited to testes and epididymis), as well as some immune cells such as monocytes, Mϕ and DCs. However, HBD1 is often constitutively expressed, whereas HBD2 and HBD3 are inducible by bacteria (e.g., *P. aeruginosa*) or their products (e.g., LPS), and various pro-inflammatory cytokines (e.g., TNF-α and IL-1β) (Harder J et al., 2000). This difference may be due to their distinct regulation of gene expression. The genomic sequence of HBD1 does not contain transcription factor regulatory elements for nuclear factor-kappa B (NF-κB), leading to constitutive production of the HBD1 gene without transcriptional regulation caused by inflammatory agents (Valore EV et al., 1998). In contrast, it is reported that the 5' region of HBD2 and HBD3 contain a NF-κB binding sequence, and thus their gene expression is inducible by bacteria (or their products) and pro-inflammatory cytokines. For example, in respiratory epithelia, *P. aeruginosa*, LPS, TNF-α and IL-1β can lead to induction of HBD2 via an NF-κB-dependent signaling pathway (Harder J et al., 2000). While HBD3 expression is induced by TNF-α and by heat-inactivated *P. aeruginosa* and *Staphylococcus aureus* (*S. aureus*) in primary keratinocytes and tracheal epithelial cells (Harder J et al., 2001), by IL-1β in fetal lung explants and gingival keratinocytes (Jia HP et al., 2001), and by IFN-γ

in a cultured keratinocyte cell line HaCaT (Garcia JR et al., 2001). Moreover, the expression of HBDs could also be induced through a TLR-MyD88 signaling pathway. It is reported that TLR2 may mediate the induction of HBD3 in keratinocytes stimulated by bacterial lipopeptides (Sumikawa Y et al, 2006). For HBD4, its expression is induced by heat-inactivated *Streptococcus pneumoniae* (*S. pneumoniae*) (Garcia JR et al, 2001), although the signaling pathway remains unknown.

However, in nature, defensins are produced as a functionally inactive preprodefensin form. In order to achieve their biological activities, defensins must undergo posttranslational modification to form a mature peptide by removing a pre- and a pro-sequence (Fig. 2, Selsted ME and Ouellette AJ, 2005). The pre-sequence is usually a highly hydrophobic signal peptide, which is proteolytically cleaved in the Golgi body, however, cleavage of the pro-sequence differs for different defensins (Yang D et al., 2004). After removal of the pro-sequence, the mature peptides of HNP 1-4 are sorted to and stored in the primary PMN granules, whereas the mature forms of HBD 1-4 are secreted onto the surface and immediate surroundings of epithelial cells (Selsted ME and Ouellette AJ, 2005; Yang D et al., 2004). These active defensins have important functions in both innate and adaptive immune response, due to their direct antimicrobial activities against invading pathogens and chemotactic activities on immune cells.

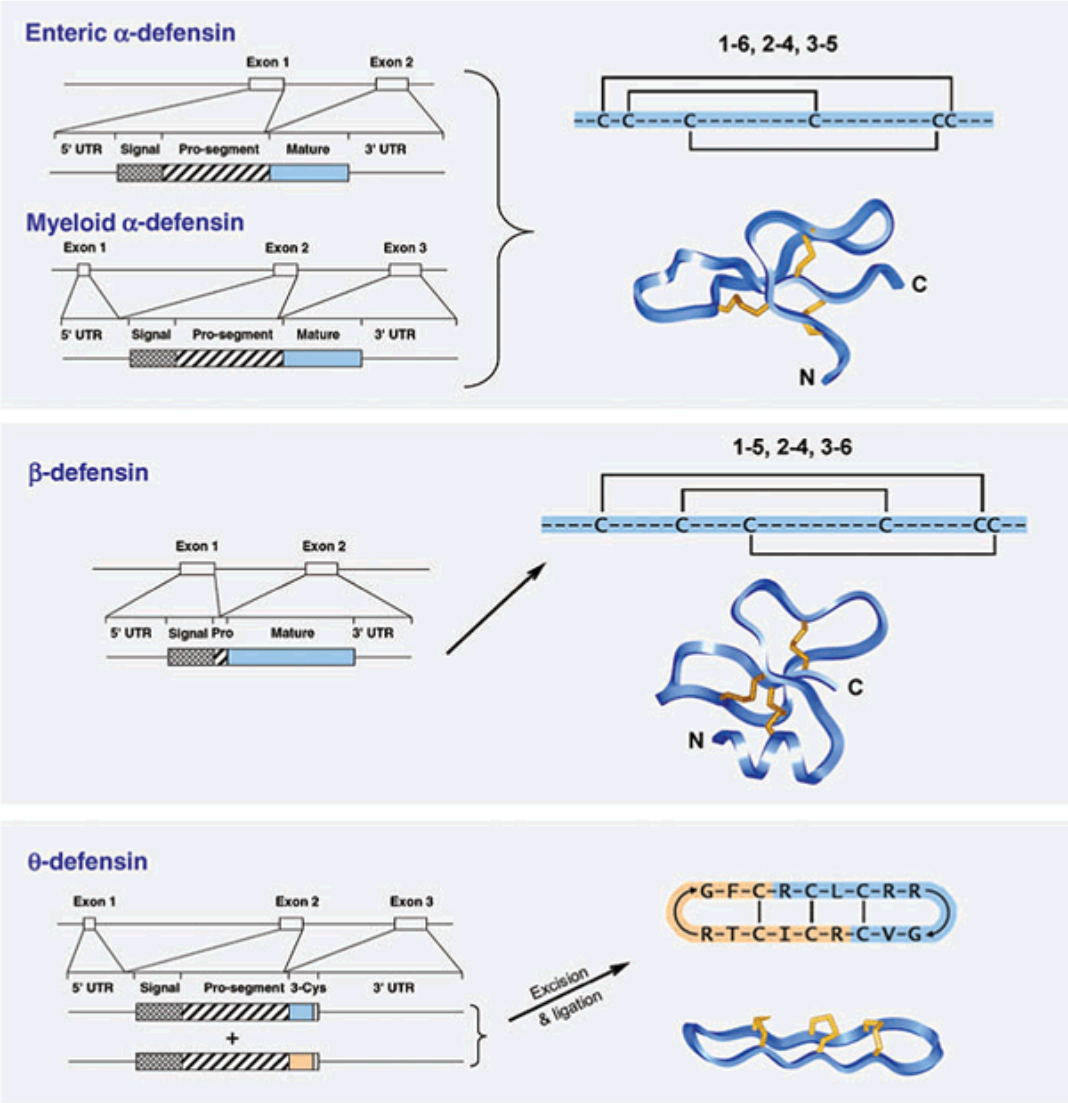

Figure 2. Defensin genes and peptides (Selsted ME and Ouellette AJ, 2005)

1.6.1 Antimicrobial activities of defensins

Much evidence has demonstrated that antimicrobial activities of defensins protect the host against a wide variety of bacteria, fungi and viruses via the innate immune response.

For antibacterial activities, Ericksen B et al. (2004) tested *in vitro* the antibacterial properties of six human α-defensins against Gram-positive bacteria *S. aureus* and

Bacillus cereus and Gram-negative bacteria *Enterobacter aerogenes* (*E. aerogenes*) and *Escherichia coli* (*E. coli*). The results indicated that their potential antibacterial activities against *S. aureus* were HNP2 > HNP1 > HNP3 > HNP4. In contrast, the potential microbicidal effects against *E. coli* and *E. aerogenes* were HNP4 > HNP2 > HNP1 = HNP3. HD5 was as effective as HNP2 against *S. aureus* and as effective as HNP4 against Gram-negative bacteria, whereas HD6 showed little or no antibacterial activity. HBD 1-4 also have microbicidal activities *in vitro* against a variety of bacteria. It is reported that HBD2 preferentially kills Gram-negative bacteria like *P. aeruginosa*, rather than Gram-positive bacteria like *S. aureus* (Schroder JM and Harder J, 1999). Transgenic or knockout models also demonstrate the antibacterial activities of β-defensins. For example, knockout of a single defensin, murine β-defensin-1 (mBD1), results in delayed clearance of *Haemophilus influenzae* from the lung (Moser C et al., 2002) and increased colonization by *S. aureus* in the bladder (Morrison G et al., 2002). Moreover, a recent study has reported that the synthetic peptides Phd1-3 which span the cationic carboxy-terminal region of HBD 1-3 also have antibacterial activities, for gross morphological changes occur in *E. coli* cells treated with these peptides. The peptides differ in their ability to permeabilize the inner membrane of *E. coli*, and Phd3 is less effective than Phd1 and Phd2 (Krishnakumari V and Nagaraj R, 2008).

For antifungal activities, studies have shown that defensins (especially β-defensins) have the capacity to kill or inactivate fungi *in vitro*. For example, HBD1 can inhibit various *Candida* (*C.*) species such as *C. albicans*, *C. krusei* and *C. parapsilosis* and *C. glabrata.* Furthermore, it also has the capacity to inhibit *C. glabrata* adherence to epithelial cells *in vitro* (Feng Z et al., 2005). Whereas HBD2 and HBD3 display similar

antifungal capabilities, they can inhibit *C. albicans, C. krusei* and *C. parapsilosis*, but are inactive against *C. glabrata*. They may also have the capacity to inhibit *C. glabrata* adherence to epithelial cells *in vitro* (Joly S et al., 2005; Feng Z et al., 2005).

For antiviral activities, considerable evidence has indicated that α-defensins can inhibit human immunodeficiency virus (HIV) and herpes simplex virus (HSV) infections *in vitro* (Hazrati E et al., 2006). Due to their lectin-like properties, HNP1-3 can bind with relatively high affinity to HIV viral gp120 and CD4 receptors (Wang W et al., 2004). Meanwhile, as a non-competitive inhibitor of protein kinase C (PKC), HNP1 may have a potential role against HIV-1 via interfering with PKC signaling pathways and blocking nuclear import and transcription of the HIV-1 genome (Chang TL et al., 2005). In addition, Hazrati E et al. (2006) tested the antiviral activities of defensins (HNP1-4, HD6 and HBD3) on CaSki cells against HSV-2 challenge and found that HBD3 inhibited HSV infection in a dose-dependent manner whereas neither HBD1 nor HBD2 blocked HSV-2 infection.

However, the effectiveness and efficiency of defensins to kill pathogens is modulated by many factors. Greenwald GI and Ganz T (1987) purified HNP 1-3 from human PMN granules and tested their microbicidal activities *in vitro*. The results demonstrated that their antibacterial activities are time and dose dependent and could be compromised by low pH or high salt concentration. Furthermore, Vylkova S et al. (2007) found that killing of *C. albicans* cells by HBD2 is salt sensitive and energy dependent. In addition, in a recent study, the antiviral ability of different human defensins to protect against HSV infection was examined, and the results indicated that all α-defensins (HNP1-4, HD5-6) could inhibit HSV infection at a noncytotoxic

concentration between 25 and 50 μg/ml (Hazrati E et al., 2006). Evidence also suggested that β-defensins are salt-sensitive, but with different levels of sensitivity. HBD3 is the least salt-sensitive defensin among HBD1-4.

1.6.2 Chemotactic effects of defensins

In addition to their direct antimicrobial activities, defensins also function as chemoattractants of many immune cells in both the innate and adaptive immune response.

On the one hand, different defensins at nanomolar concentrations could selectively chemoattract different kinds of leukocytes in the innate immune response (Schutte BC and McCray PB, 2002; Territo MC et al., 1989; Garcia JR et al., 2001; Niyonsaba F et al., 2002; Niyonsaba F et al., 2004). HNP1-3 are chemotactic for monocytes, whereas HBD2 and HBD3-4 respectively chemoattract mast cells and Mϕ. Thus, defensins could promote recruitment of leukocytes to combat invading pathogens at infected sites.

On the other hand, defensins could also enhance the adaptive immune response via chemoattracting iDCs, an important class of APCs. At the infected sites, iDCs will take up and present microbial antigens, initiating the adaptive immune response. Then iDCs will undergo a maturational process to become mature DCs, which can migrate to secondary lymphoid organs to stimulate antigen-specific naive T cells (Banchereau J and Steinman RM, 1998; Palucka K and Banchereau J, 2002). It has been demonstrated that β-defensins might function as the the most potentially important chemoattractants of iDCs. For example, studies of mBD2, a homologue of HBD2 in the mouse, have suggested that mBD2 acts directly on iDCs, inducing DC maturation with

up-regulation of costimulatory molecules, such as CD40, CD80, and CD86, MHC class II, and chemokine C-C motif receptor 7 (CCR7). This mBD2-induced DC maturation could occur even in the absence of LPS-binding protein, which is needed for LPS activity (Biragyn A et al., 2002), Functionally, mBD2-activated DCs exhibited Th1 polarized responses such as production of pro-inflammatory cytokines.

1.6.3 Defensins and TLRs

Much evidence has demonstrated a close association between defensins and TLRs, especially between β-defensins and extracellular TLRs. For example, studies have demonstrated that mBD2 and LPS share signaling pathways through the same receptor, namely TLR4 (da Silver Correia J et al., 2001; Hornef MW et al., 2002). mBD2 can activate NF-κB in HEK293 cells transfected with TLR4 and MD2, but not in untransfected HEK293 cells. It also has been reported that mBD2 promotes TLR4/MyD88-dependent and NF-κB-dependent atypical death of APCs via activation of TNF receptor 2 (Biragyn A et al., 2008).

On the other hand, Sumikawa Y et al. (2006) has demonstrated that bacterial lipopeptide stimulation can induce mBD3 expression and TNF-α and IL-1α up-regulation in keratinocytes via a TLR2/MyD88-dependent signaling pathway. It is also reported that activation of professional APCs by HBD3 is mediated by interaction with TLR1 and TLR2. The signal is tranducted via a MyD88-dependent pathway and results in IL-1 receptor-associated kinase-1 phosphorylation and activation of NF-κB (Funderburg N et al., 2007).

In addition, it is demonstrated that pretreatment with a low dose of flagellin, the agonist for TLR5, will increase the mRNA expression of HBD2 in human corneal

epithelial cells after *P. aeruginosa* challenge and elevate the production of pro-inflammatory cytokines like TNF-α and IL-8 (Kumar A et al., 2007).

1.7 Overview and significance

In the United States, microbial keratitis is a disease most frequently associated with contact lens usage. It has considerable medical and economic impact: approximately 25,000 to 30,000 cases are reported annually and the cost of medical treatment as a result of these cases is estimated at between $15 and $30 million (Khatri S et al., 2002). *P. aeruginosa* is a common Gram-negative bacteria associated with induction of microbial keratitis. It is reported that 70% of culture-proven cases of microbial keratitis associated with contact lense wear could be attributed to this pathogen (Schein OD et al., 1989). Studies have demonstrated that *P. aeruginosa*-induced corneal infection usually presents as a rapidly progressing suppurative stromal infiltrate with a marked mucopurulent exudate. Yellowish coagulative necrosis surrounded by inflammatory epithelial edema is distinctive to this bacterial keratitis and can lead to significant stromal tissue damage. A ring infiltrate surrounding the central lesion is often present. In severe cases, descemetocele is also formed, resulting in corneal perforation (Hazlett LD, 2004).

Animal models of *P. aeruginosa*-induced keratitis have been established to elucidate the characteristics of bacterial keratitis by topical application of *P. aeruginosa* after wounding the corneal epithelium, by intrastromal inoculation, or by placement of a contaminated contact lens or suture on the cornea (Hazlett LD et al., 2007). Of particular interest is the defined inbred murine model of *P. aeruginosa*-induced corneal infection. C57BL/6 (B6) mice are Th1 responsive to *P. aeruginosa* challenge, and are

classified as susceptible, as the infected cornea typically perforates by 7 days postinfection (p.i.). By contrast, BALB/c mice, Th2 responders, are classified as resistant, as they can effectively resolve the infection (Hazlett LD et al., 2000; Hazlett LD, 2004). Studies using these susceptible/resistant murine models continue to characterize host defense mechanisms in *P. aeruginosa*-induced corneal infection, including the function of immune cells (e.g., PMN, T cells, Mϕ and DCs) as well as cytokines and chemokines produced by those cells, in regulating inflammation, innate and adaptive immunity, and Th1- vs Th2- responses (Hazlett LD, 2004; Hazlett LD et al., 2000). Nonetheless, little is known regarding the role of defensins in *P. aeruginosa* keratitis.

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

1. Defensins (mBD1 and mBD2) promote resistance against *P. aeruginosa*-induced corneal infection (CHAPTER 2).

2. Defensins (mBD2 and mBD3) act synergistically to promote resistance against *P. aeruginosa*-induced corneal infection (CHAPTER 3).

CHAPTER 2

MBD2 PROMOTES RESISTANCE AGAINST INFECTION WITH *P. AERUGINOSA*

2.1 ABSTRACT

Corneal infection with *P. aeruginosa* results in corneal perforation in susceptible B6 mice, but not in resistant BALB/c mice. To explore the role of two important defensins, mBD1 and mBD2, in the ocular immune defense system, their mRNA and protein expression levels were tested by real-time RT-PCR and Western blot, respectively. mRNA, protein, and immunostaining data demonstrated that both mBD1 and mBD2 were constitutively expressed in normal BALB/c and B6 corneas, and they were disparately up-regulated in BALB/c (more) vs B6 (less) corneas after infection. To determine whether either defensin played a role in host resistance, BALB/c mice were treated with either mBD1 or mBD2 small interfering RNA by subconjunctival injection together with topical application. Increased corneal opacity and worsened disease were displayed after knockdown of mBD2 but not of mBD1. mBD2 silencing also increased bacterial counts and PMN infiltration in BALB/c corneas. Real-time RT-PCR data further demonstrated that mBD2, not mBD1, differentially modulated mRNA expression of proinflammatory cytokines/molecules such as IFN-γ, Mϕ inflammatory protein (MIP)-2, IL-1β, TNF-α, IL-6, and inducible NO synthase (iNOS); TLR signaling molecules, including TLR2, TLR4, TLR9, and MyD88; and the transcription factor NF-κB. Additionally, *in vivo* studies indicated that mBD2 silencing enhanced corneal nitrite levels and NF-κB activation. Collectively, the data provide evidence that mBD2, but not mBD1, is required for host resistance against *P. aeruginosa*-induced corneal infection.

2.2 INTRODUCTION

P. aeruginosa is a common Gram-negative bacteria associated with microbial keratitis, a disease frequently caused by contact lens usage (Wilhelmus KR, 1987). *P. aeruginosa*-induced bacterial infections rapidly progress and result in inflammatory epithelial edema, stromal infiltration, and, oftentimes, corneal ulceration, stromal tissue destruction, and vision loss (Hazlett LD, 2004).

Experimentally, *P. aeruginosa* challenge induces different response outcome in two defined inbred murine models: corneal perforation in susceptible B6 mice (Th1 responders) and corneal healing in resistant BALB/c mice (Th2 responders) (Hazlett LD et al., 2000). Studies using the susceptible/resistant models have provided substantive information of ocular immune defenses against *P. aeruginosa*, including the function of immune cells and cytokines/chemokines in regulating inflammation in innate and adaptive immunity, as well as Th1 vs Th2 responses (Hazlett LD, 2004; Hazlett LD et al., 2000). Nonetheless, little is known regarding the role of defensins in *P. aeruginosa* keratitis.

In this regard, other studies have demonstrated that defensins, especially β-defensins, play an important role in both innate and adaptive immunity due to their antimicrobial, regulatory, and chemotactic effects (Schroder JM and Harder J, 1999; Feng Z et al., 2005; Garcia JR et al., 2001; Niyonsaba F et al., 2002; Biragyn A et al., 2002; McDermott AM, 2004.). In mice, the most studied defensins are mBD1 and mBD2, which are chiefly expressed in a variety of epithelial cells (Bals R et al., 1998; Morrison GM et al., 1999). Both mBD1 and mBD2 can directly kill invading pathogens (Morrison G et al., 2002; Hussain T et al., 2008), while mBD2 also can regulate

production of several inflammatory cytokines and chemokines (Biragyn A et al., 2002; Biragyn A et al., 2008).

Thus, studies described herein investigate the expression and function of mBD1 and mBD2 in susceptible B6 vs resistant BALB/c mice before and after *P. aeruginosa* corneal infection. Our data provide evidence that mBD1 and mBD2 are both disparately expressed in BALB/c (more) vs B6 (less) corneas after *P. aeruginosa* infection. However, only mBD2 is required for host resistance against bacterial infection, and it functions to modulate the production of proinflammatory cytokines, iNOS, TLR signaling molecules, and NF-κB activation.

2.3 MATERIALS AND METHODS

Infection of mice.

Eight-week-old female BALB/c (resistant) and B6 (susceptible) mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with ether and placed beneath a stereoscopic microscope at 40× magnification. The cornea of the left eye was wounded with three 1-mm incisions using a sterile 25 gauge needle. A 5 μl aliquot containing 1 x 10^6 CFU of *P. aeruginosa* (American Type Culture Collection strain 19660), prepared as described before (Kwon B and Hazlett LD, 1997), was topically applied to the ocular surface. Eyes were examined at 1 day p.i. and/or at times described below, to ensure that mice were similarly infected and to monitor disease. Animals were treated humanely and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular response to infection.

Corneal disease was graded using an established scale: (Hazlett LD 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. A clinical score was recorded for each mouse after infection for statistical comparison of disease severity, and slit lamp photography was used to illustrate the disease response.

RNA interference.

In vivo use of small interfering RNA (siRNA) has been described by others (Nakamura H et al., 2004) as well as this laboratory (Huang X et al., 2005) For the studies described herein, mBD1 and mBD2 specific siRNA or appropriate scrambled controls for each (Santa Cruz Biotechnology, Santa Cruz, CA) were injected subconjunctivally (5 µL per mouse at a concentration of 8 µM) into the left eyes of BALB/c mice (n = 5/group/time) 1 day before infection and then topically applied onto the infected corneas (5 µL per mouse per time at a concentration of 4 µM, once on the day of infection, twice on 1 and 3 days p.i.). The efficacy and specificity of silencing of each defensin was tested by RT-PCR. All the siRNAs used in the studies herein were shorter than 21 nucleotides in length to avoid non-specific siRNA suppression effects via cell-surface TLR3 (Kleinman ME et al., 2008).

Real-time RT-PCR.

Total RNA was isolated from individual corneas for analysis (as indicated below) using RNA-Stat 60 (Tel-Test, Friendsville, TX) according to the manufacturer's

recommendations and quantitated by spectrophotometric determination (260 nm). 1 µg 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). Next, cDNA was amplified using SYBR Green Master Mix (Bio-Rad, Hercules, CA) as suggested by the manufacturer. Briefly, the 20-µl reaction system contained: 10 µl of SYBR Green PCR Master Mix, 0.5 µM primers, 2 µl of cDNA (diluted 1:10), and diethyl pyrocarbonate water. Sequences of primer sets for real-time PCR are shown in Table 3. Quantitative real-time RT-PCR reactions were 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 CA et al., 1996; Roux KH, 1995). Relative mRNA levels were calculated after normalization to β-actin.

Immunofluorescent staining.

Normal uninfected and infected eyes were enucleated (n = 3/group/time) at 5 days p.i. from BALB/c and B6 mice, immersed in 1× Dulbecco's PBS (Mediatech, Inc., Herndon, VA), embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) and frozen in liquid nitrogen. 10 µm thick sections were cut, mounted to polylysine-coated glass slides, and incubated at 37 °C overnight. After a 2 min fixation in acetone, slides were blocked with 10 mM sodium phosphate buffer containing 2.5% bovine serum albumin and donkey IgG (1:100) for 30 min at room temperature. After, sections were incubated with primary Abs, goat anti-mouse beta-defensin 2 (M-17, 1:50, Santa Cruz Biotechnology, Inc.) or rabbit anti-mouse beta-defensin 1 (1:50, Santa Cruz

Biotechnology) for 1 h; followed by Alexa Fluor 546 conjugated donkey anti-goat Ab (1:1500, Invitrogen) or Alexa Fluor 594 conjugated donkey anti-rabbit Ab (1:1500, Invitrogen) for another hour. Sections were then incubated for 2 min with SYTOX Green nuclear acid stain (1:20,000, Lonza, Walkersville, MD). Controls were similarly treated, but without the primary Abs. Finally, the sections were visualized and digital images were captured with a Leica TSC SP2 confocal laser scanning microscope (Leica Microsystems, Bannockburn, IL).

Western blot analysis.

Whole corneas (n = 10/group/time) and corneal epithelium (n = 15/group/time) were collected and pooled from normal uninfected and infected BALB/c and B6 mouse eyes at 5 days p.i. Pooled corneas or corneal epithelium were lysed and homogenized using a 1-ml glass tissue homogenizer in 1× sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol). Debris was pelleted by centrifugation for 5 min at 7,500 rpm, and protein concentration of the supernatant was determined by Quick Start Bradford protein assay (Bio-Rad). 10 μg of corneal protein sample or control peptide, (each at 1 μg) for mBD1 (MBD11-P) or mBD2 [HBD21-P, the latter a mixture of 3 different peptides (14aa from human BD-2, 14aa from rat defensin-2 and 17aa from mouse BD-2), Alpha Diagnostic, San Antonio, TX] was added to each respective lane and proteins were separated on 10% acrylamide gels. The electrophoretically separated material was transferred to a supported PVDF membrane (Bio-Rad), and blocked overnight at 4°C in a 5% solution of nonfat dry milk prepared with TTBS (1× TBS containing 0.05% Tween 20, Bio-Rad). Blots were incubated with primary rabbit anti-human beta-defensin 2 IgG Ab (HBD21A, generated from the three different peptides above, 1:500 diluted in TTBS

containing 1% nonfat milk, Alpha Diagnostic) or rabbit anti-mouse beta-defensin 1 (1:500 diluted in TTBS containing 1% nonfat milk, Alpha Diagnostic) for 2 h, washed three times for 15 min each with TTBS, followed by goat anti-rabbit IgG-peroxidase secondary Ab (1:1000 diluted in TTBS containing 1% nonfat milk, Alpha Diagnostic), and developed using the ECL method (ECL Plus, Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol.

ELISA.

Cytokine protein levels were selectively tested using ELISA kits (R&D Systems, Minneapolis, MN). Corneas from mBD2 siRNA and control-treated BALB/c mice were individually collected (n = 5/group/time) at 3 and 5 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 min and an aliquot of each supernatant was assayed in duplicate for IL-1β, TNF-α and IL-6 protein per the manufacturer's instruction. The reported sensitivity of these assays is < 3.0 pg/ml for IL-1β, < 5.1 pg/ml for TNF-α, and 1.3–1.8 pg/ml for IL-6.

Bacterial plate counts.

Corneas from mBD2 siRNA and scrambled control-treated BALB/c mice were collected (n = 5/group/time) at 1, 3 and 5 days p.i. and the number of viable bacteria was quantitated. Individual corneas were homogenized in sterile water containing 0.85% (w/v) NaCl containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on Pseudomonas isolation agar (BD Difco Laboratories, Sparks, MD) in triplicate and plates were incubated overnight at 37°C. Results are reported as $\log_{10}$ number of CFU per cornea ± SEM.

Myeloperoxidase (MPO) assay.

An MPO assay was used to quantitate PMN number in the cornea from both mBD2 siRNA and control-treated BALB/c mice. Infected corneas (n = 5/group/time) were excised at 3 and 5 days p.i. and homogenized in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma, St. Louis, MO). Samples were freeze-thawed four times and centrifuged at 13,000 rpm for 10 min. 0.1 ml of the supernatant was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml, Sigma) and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm was monitored for 5 min at 30-sec intervals, and the results were expressed as units of MPO per cornea. One unit of MPO activity is equivalent to 2 x 10^5 PMN (Williams RN et al., 1982).

Griess reaction.

Nitric oxide (NO) levels were determined by measurement of its stable end product, nitrite, using a Griess reagent (Sigma) for siRNA mBD2 vs control-treated BALB/c mice (n = 5/group/time). First, infected corneas were homogenized in 500 μl of degassed PBS and microcentrifuged at 3500 rpm for 5 min. Next, 100 μl of supernatant was added to an equal volume of Griess reagent in duplicate on a 96-well microtiter plate and incubated at room temperature for 15 min. Absorbance (540 nm) was measured and nitrite concentrations were estimated using a standard curve of sodium nitrite. The results were expressed as the mean micromoles of nitrite per cornea ± SEM.

NF-κB activation.

Infected corneas from mBD2 siRNA and scrambled control-treated BALB/c mice were individually collected (n = 5/group/time) at 3 and 5 days p.i. High-quality nuclear

extract was isolated from corneal samples using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) and protein concentration was determined by Quick Start Bradford protein assay (Bio-Rad). NF-κB activation was determined using a Trans AM NF-κB ELISA (Active Motif) following the manufacturer's protocol. Phosphorylated levels of NF-κB p65 in 5 μg total protein were determined in duplicate following the manufacturer's instruction. The sensitivity of the assay is <0.5 μg.

Statistical analysis.

The difference in clinical score between 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 the significance of viable bacterial counts, MPO, real-time RT-PCR, and protein assays. Data were considered significant at $p < 0.05$.

2.4 RESULTS

Expression of mBD1 and mBD2 in BALB/c vs B6 mice.

To determine whether mBD1 or mBD2 was present in corneas of BALB/c and B6 mice before and after infection with *P. aeruginosa*, mRNA and protein expression levels in normal, uninfected and infected corneas were tested by real-time RT-PCR and western blot, respectively. Representative data are provided in Fig. 3. mRNA levels for mBD1 and mBD2 were both constitutively expressed in normal uninfected corneas of the two mouse strains; however, at 1, 3 and 5 days p.i. (Fig. 3, A and B), mRNA expression levels in BALB/c over B6 mice were significantly up-regulated (mBD1: $p < 0.001$, $p = 0.02$, $p < 0.001$, mBD2: $p = 0.03$, $p < 0.001$, $p < 0.001$, at 1, 3 and 5 days p.i., respectively), and peaked at 5 days p.i. Meanwhile, protein expression of mBD1 and mBD2 in the corneal epithelium of each mouse strain was detected by western blot

before infection and at 5 days p.i. (Fig. 3, C and D). IDV values (Fig. 3, E and F) indicated that both mBD1 and mBD2 protein were constitutively expressed in the two strains, and significantly elevated at 5 days p.i. in BALB/c vs B6 mice ($p < 0.001$). Similar analysis of protein in the whole uninfected and infected cornea at 5 days p.i. (data not shown) exhibited the same pattern as for the epithelium alone.

In addition, we tested normal uninfected and infected corneas of BALB/c and B6 mice at 5 days p.i. using immunofluorescent staining for mBD1 and mBD2 (Fig. 4 and Fig. 5), and the results further confirmed the mRNA and protein data for normal and infected corneas at 5 days p.i. For mBD1, distribution patterns were similar in normal corneas of the two groups (Fig. 4, A–D), while more mBD1 appeared detectable in the infected corneas (most in corneal epithelium, Fig. 5, A–D) of BALB/c over B6 mice at 5 days p.i. For mBD2, similar patterns were displayed and little difference was shown between the two strains before infection (Fig. 4, E–H). However, more mBD2 was detected in the infected corneas (most in corneal epithelium, Fig. 5, E–H) at 5 days p.i. in BALB/c, when compared to B6 mice. Results of SYTOX Green Nuclear staining of normal and infected cornea are shown in Fig. 4 (I and J) and Fig. 5 (I and J), respectively. Controls, in which the primary Ab was omitted, were negative for immunostaining for mBD1 or mBD2, and appeared similar to SYTOX Green Nuclear staining.

Silencing mBD1 and mBD2.

Because the distribution patterns (mRNA and protein) suggested both mBD1 and mBD2 were differentially expressed in infected BALB/c and B6 corneas, the next series of *in vivo* studies were designed to determine their effects in host defense against

corneal infection. First, BALB/c mice were subconjunctivally injected and topically treated with scrambled control or mBD1 or mBD2 specific siRNA to determine whether knock down of either defensin would impair host defense. Since no significant difference was shown between mBD1 siRNA and scrambled control-treated mice at 5 days p.i., we extended observation of this experimental group to 7 days p.i. to determine if any difference between the two groups was detectable at the later time period. By 7 days p.i., clinical score data showed that mBD1 siRNA treatment did not significantly change the host response in BALB/c mice after *P. aeruginosa* infection (Fig. 6A). Representative slit lamp photographs at 7 days p.i. showed similar corneal opacity/disease in control, scrambled (Fig. 6B) vs siRNA-treated (Fig. 6C) mice. RT-PCR also confirmed that silencing was significant and specific for mBD1 and that mBD2 mRNA levels were not changed significantly (Fig. 6, D and E).

On the other hand, by 5 days p.i, the cornea of most mBD2 siRNA-treated mice consistently displayed an enhanced level of disease (grade = +3/+4), whereas all scrambled, control-treated corneas showed less opacity/disease (grade = +1/+2). Clinical score data (Fig. 7A) showed that mBD2 siRNA-treated mice exhibited increased disease at 3 and 5 days p.i. (both $p < 0.001$). Representative slit lamp photographs of control, scrambled (Fig. 7B) vs mBD2 (Fig. 7C) siRNA-treated mice are provided. Treatment with mBD2 siRNA resulted in either perforation (grade = +4, data not shown) or dense opacity covering the entire anterior segment (grade = +3, Fig. 7C) and overall more inflammation than scrambled, control-treated BALB/c corneas at 5 days p.i. RT-PCR also confirmed that silencing was significant and specific for mBD2 and that mBD1 mRNA levels were not changed at all times tested (Fig. 7, D and E).

Effect of silencing mBD2 on plate count, MPO and pro-inflammatory cytokines.

Therefore, we next assessed further the effect of mBD2 siRNA treatment on the bacterial component of disease pathogenesis. Bacterial plate counts were used to detect viable bacteria in the infected cornea of mBD2 siRNA vs scrambled, control-treated mice at 1, 3 and 5 days p.i. Results are shown in Fig. 7F. Elevated bacterial counts were detected in mBD2 siRNA over control-treated corneas ($p = 0.03$, $p < 0.01$, at 3 and 5 days p.i., respectively). In addition, MPO activity was quantitated in infected corneas of the two groups at 3 and 5 days p.i. and results are shown in Fig. 7G. There was no significant difference in the number of PMN between the two groups at 3 days p.i., whereas MPO activity was significantly increased with mBD2 siRNA treatment at 5 days p.i., when compared with controls ($p = 0.03$).

To ascertain whether mBD2 modulated the production of pro-inflammatory cytokines, mRNA expression levels of several were analyzed by real-time RT-PCR in normal uninfected and infected corneas of mBD2 siRNA and control-treated BALB/c mice (Fig. 8). Overall, mBD2 siRNA treatment differentially modulated the expression of pro-inflammatory cytokines in infected corneas. At the mRNA expression level, IFN-γ (Fig. 8A) was increased by mBD2 siRNA treatment at 1, 3 and 5 days p.i. ($p < 0.01$, $p < 0.01$, $p = 0.04$, respectively), peaking at 3 days p.i. Whereas MIP-2 (Fig. 8B) and IL-1β (Fig. 8C) were significantly down-regulated at both 1 and 3 days p.i., and up-regulated at 5 days p.i. (MIP-2: $p < 0.001$, $p < 0.01$, $p < 0.001$; IL-1β: $p < 0.001$, $p = 0.04$, $p < 0.001$, at 1, 3 and 5 days p.i., respectively). Meanwhile, the mRNA expression levels of TNF-α (Fig. 8E) and IL-6 (Fig. 8G) were reduced at 1 day p.i., but significantly enhanced at 3 and 5 days p.i. (peaking at 3 days p.i.) in siRNA mBD2 vs control-treated

corneas (TNF-α: $p < 0.01$, $p = 0.02$, $p < 0.001$, IL-6: $p = 0.24$, $p < 0.01$, $p < 0.001$, at 1, 3 and 5 days p.i., respectively).

In addition, protein expression levels of IL-1β (Fig. 8D), TNF-α (Fig. 8F) and IL-6 (Fig. 8H) were examined by ELISA. Protein expression levels in mBD2 siRNA and control-treated corneas were assessed at 3 and 5 days p.i. After mBD2 siRNA treatment, IL-1β protein expression was slightly decreased at 3 days p.i. ($p = 0.05$), but remained the same at 5 days p.i., whereas TNF-α and IL-6 protein expression levels were similar at 3 days p.i., followed by a significant elevation at 5 days p.i. (both $p = 0.01$), when compared with controls.

Moreover, mRNA expression levels of iNOS (Fig. 9A) were decreased at both 1 and 3 days p.i., followed by an increase at 5 days p.i. in mBD2 siRNA vs control-treated corneas ($p < 0.001$, $p = 0.03$, $p < 0.001$, at 1, 3 and 5 days p.i., respectively). In addition, mBD2 siRNA treatment elevated the amount of nitrite detectable (Fig. 9B) in corneas at 5 days p.i. ($p < 0.001$), whereas no difference was detected at 3 days p.i., when compared with controls.

The role of mBD2 in modulating TLR signaling pathways.

Since mBD2 siRNA treatment differentially regulated the expression of pro-inflammatory cytokines, and increased viable bacteria and disease in BALB/c corneas after *P. aeruginosa* infection, the next series of studies were initiated to investigate early host pathogen immune mechanisms involved. The mRNA expression levels of select TLR signaling molecules (including TLR4, TLR2, TLR9, and MyD88) and transcription factor NF-κB were evaluated by real-time RT-PCR, and results are presented in Fig. 8. TLR4 (Fig. 10A) and TLR2 (Fig. 10B) expression in mBD2 siRNA-treated corneas was

first decreased at 1 and 3 days p.i., and then increased at 5 days p.i. (TLR4: $p = 0.02$, $p < 0.001$, $p < 0.001$; TLR2: $p < 0.01$, $p < 0.001$, $p = 0.001$, at 1, 3 and 5 days p.i., respectively), when compared with controls; whereas no difference in TLR9 mRNA expression was shown between the two groups (Fig. 10C) at any of the times tested. Both MyD88 (Fig. 10D) and NF-κB (Fig. 10E) mRNA expression levels were significantly enhanced at 1, 3 and 5 days p.i. in mBD2 siRNA vs control-treated corneas (MyD88: $p < 0.01$, $p = 0.02$, $p = 0.01$, NF-κB: $p < 0.01$, $p < 0.001$, $p < 0.001$, at 1, 3 and 5 days p.i., respectively). In addition, results from testing NF-κB activation (Fig. 10F) indicated that mBD2 silencing significantly up-regulated phosphorylated levels of p65, indicative of NF-κB activation at both 3 and 5 days p.i. ($p < 0.01$, $p = 0.05$, respectively).

2.5 DISCUSSION

As a family of antimicrobial peptides, β-defensins play an important role in both innate and adaptive immune defense (McDermott AM, 2004; Levy O, 2004; Biragyn A e al., 2001; Yang D et al., 1999). They are chiefly expressed in a variety of epithelial cells (e.g., airway epithelia, urogenital tissues, nasolacrimal duct, and mammary gland) and sometimes in immune cells such as DCs and Mϕ (Garcia JR et al., 2001; Biragyn A et al., 2002; McDermott AM, 2004; Bals R et al., 1998; Morrison G et al., 1999; Morrison G et al., 2002; Hussain T et al., 2008; Biragyn A et al., 2008; Yang D et al., 1999). In mice, the best characterized β-defensins are mBD1 and mBD2. mBD1 is often constitutively expressed (Bals R et al., 1998), whereas mBD2 expression is inducible by Gram-negative bacteria (e.g., *P. aeruginosa*) and their products (e.g., lipopolysaccharide) as

well as various pro-inflammatory cytokines such as TNF-α (Morrison G et al., 1999). In regard to the eye, it has been reported that mBD1 and mBD2 mRNA expression was detected in scraped corneal epithelial cells and whole conjunctival tissues by RT-PCR (Narayanan S et al., 2008). Our studies revealed their distribution patterns (mRNA and protein) in normal, uninfected and infected corneas. RT-PCR and western blot data provided evidence that both mBD1 and mBD2 were constitutively expressed before infection, and disparately up-regulated in BALB/c (more) vs B6 (less) corneas after infection. These data were further supported by immunostaining for each defensin. BALB/c and B6 mice expressed comparable immunostaining patterns in normal cornea (epithelium), whereas BALB/c mice displayed a greater staining intensity in the cornea for both mBD1 and mBD2 at 5 days p.i., when compared with B6 mice. In addition, most mBD1 and mBD2 positive immunostaining was located in the corneal epithelium of both mouse groups which is consistent with previous studies by others (Narayanan S et al., 2008).

The increased levels (mRNA and protein levels) of mBD1 and mBD2 in infected BALB/c vs B6 corneas suggested a potential role for the two defensins in the development of the resistant vs susceptible phenotype. Previous studies reported that mBDs provide an initial block to a variety of pathogens on the epithelial surface (Bals R et al., 1998; Morrison G et al., 1999; Morrison G et al., 2002; Hussain T et al., 2008; Burd RS et al., 2002; Jia HP et al., 2000). Our *in vivo* knock down studies demonstrated that despite the increased level of both defensins in BALB/c mice after infection, mBD2, rather than mBD1, promoted host resistance against *P. aeruginosa*-induced corneal infection. Data to support this tenet included confirming the specificity and selectivity of

each of the the knock downs by RT-PCR, as well as slit lamp photographs and clinical scores which visually showed little difference between mBD1 siRNA vs control-treated corneas. All of these, suggested that mBD1 is not required for ocular immune defense against *P. aeruginosa*. These data were accompanied by similar mRNA distribution patterns (data not shown) of select pro-inflammatory cytokines/molecules and TLR signaling molecules in infected corneas of mBD1 silenced vs control mice. In contrast, mBD2 siRNA-treated BALB/c mice displayed increased corneal opacity and exacerbated ocular disease (at 5 days p.i.). They also showed slightly increased (but significant) bacterial plate counts (at 3 and 5 days p.i.), potentially sufficient to elevate expression of inflammatory mediators and PMN recruitment (at 5 days p.i.), when compared with controls. Together, these results provide direct evidence that mBD2 promotes host resistance against *P. aeruginosa* corneal infection, however, the mechanism of mBD2-dependent protection in the eye remains to be determined.

In this regard, previous studies have revealed that mBD2 not only has the capability to kill a variety of pathogens (especially Gram-negative bacteria) (Schroder JM and Harder J, 1999; Ouhara K et al., 2005), but also can induce immature DC maturation to trigger Th1 responses *in vivo* as well as pro-inflammatory cytokine production (Biragyn A et al., 2002). It has also been demonstrated that mBD2 and lipopolysaccharide share the same receptor, TLR4, and activate the transcription factor NF-κB through a TLR cascade, leading to pro-inflammatory cytokine expression (Biragyn A et al., 2002; Vora P et al., 2004; Wright SD et al., 1990; da Silva Correia J et al., 2001; Hornef MW et al., 2002; Tsutsumi-Ishii Y and Nagaoka I, 2002). Our studies

also revealed that in the eye, mBD2 can regulate pro-inflammatory cytokine and TLR signaling molecule production, contributing to host control of bacterial keratitis.

More specifically, we provide evidence that mBD2 silencing significantly enhanced the mRNA expression levels of IFN-γ, MyD88 and NF-κB at 1, 3 and 5 days p.i., which may be crucial in mBD2-dependent ocular defense against *P. aeruginosa* infection. IFN-γ is an important regulatory cytokine which plays a critical role in inflammation and Th1 responses (McClellan SA et al., 2006; Radhakrishnan S et al., 2007); MyD88 is a key adaptor molecule in all TLR signaling pathways except for TLR3 (Yu FS and Hazlett LD, 2006; Beutler B 2004); and NF-κB is the transcription factor in all TLR/MyD88-dependent signaling pathways whose activation leads to inflammatory cytokine production (Beutler B, 2004; Baldwin AS, 1996). Thus, their up-regulation (peaking at 3 days p.i.) hypothetically may have shifted the normal tight regulation of IFN-γ production and the overall Th2-like response of BALB/c mice (Huang X and Hazlett LD, 2003) to a Th1-like response in mBD2 silenced BALB/c corneas.

On the other hand, for other pro-inflammatory cytokines/molecules (e.g., MIP-2, IL-1β, TNF-α, IL-6 and iNOS) and TLRs (e.g., TLR4 and TLR2), silencing mBD2 led to a shift in mRNA expression: a down-regulation at an earlier period (1-3 days p.i.), followed by an up-regulation at 5 days p.i. To explain this shift as specific to mBD2 silencing, we have provided evidence to confirm both the specificity and effectiveness of silencing using RT-PCR of infected cornea. We also suggest that we can rule out the possibility of silencing being mediated via TLR3 on the cell surface (Kleinman ME et al., 2008), because all the siRNAs used in our studies were shorter than 21 nucleotides, the minimum length required for a sequence- and target-independent suppression.

Therefore, we hypothesize that the shift described above may be caused by a balance between enhanced vs reduced activation of TLR signaling. At an early time period, since there was little difference in bacterial load and PMN recruitment between mBD2 siRNA and control-treated mice, mBD2 silencing overall reduced the expression of pro-inflammatory cytokines and TLRs. In contrast, at a later time period, mBD2 silencing resulted in a slight, but significant increased bacterial load and PMN infiltration, thereby potentially activating TLR signaling cascades to overcome early silencing effects. In addition, our data revealed that mBD2 silencing regulated the mRNA expression of TLR4 and TLR2, but not TLR9. Thus, we propose that mBD2 appears to function via extracellular TLRs (on the plasma membrane surface of cells), rather than intracellularly through TLRs on endosomal cell membranes (Biragyn A et al., 2002; Yu FS and Hazlett LD, 2006; Gariboldi S et al., 2008; Selleri S et al., 2007).

In summary, our studies provide direct evidence that both mBD1 and mBD2 are constitutively expressed similarly in uninfected normal corneas of BALB/c (resistant) and B6 (susceptible) mice, but disparately expressed in resistant (more) vs susceptible (less) mice after *P. aeruginosa* corneal infection; that of the two defensins tested by knock down experiments, only mBD2 is required for host resistance against bacterial infection; and that mechanistically, mBD2 functions to modulate the production of pro-inflammatory cytokines, iNOS, TLR signaling molecules and NF-κB activation in the infected cornea. Based upon these data, mBD2 may provide a promising target for treatment of ocular diseases, such as *P. aeruginosa* keratitis.

Table 2. Nucleotide sequence of the specific primers used in PCR amplification.

Gene	Primer Sequence (5'-3')	
β-actin	GAT TAC TGC TCT GGC TCC TAG C	F
	GAC TCA TCG TAC TCC TGC TTG C	R
mBD1	GGC ATT CTC ACA AGT CTT GGA CGA AG	F
	AGC TCT TAC AAC AGT TGG GCT TAT CTG G	R
mBD2	TCT CTG CTC TCT GCT GCT GAT ATG C	F
	AGG ACA AAT GGC TCT GAC ACA GTA CC	R
TLR2	CTC CTG AAG CTG TTG CGT TAC	F
	TAC TTT ACC CAG CTC GCT CAC TAC	R
TLR4	CGC TTT CAC CTC TGC CTT CAC TAC AG	F
	ACA CTA CCA CAA TAA CCT TCC GGC TC	R
TLR9	AGC TCA ACC TGT CCT TCA ATT ACC GC	F
	ATG CCG TTC ATG TTC AGC TCC TGC	R
IL-6	CAC AAG TCC GGA GAG GAG AC	F
	CAG AAT TGC CAT TGC ACA AC	R
IL-1β	CGC AGC AGC ACA TCA ACA AGA GC	F
	TGT CCT CAT CCT GGA AGG TCC ACG	R
MIP-2	TGT CAA TGC CTG AAG ACC CTG CC	F
	AAC TTT TTG ACC GCC CTT GAG AGT GG	R
IFN-γ	GTT ACT GCC ACG GCA CAG TCA TTG	F
	ACC ATC CTT TTG CCA GTT CCT CCA G	R
TNF-α	ACC CTC ACA CTC AGA TCA TCT T	F
	GGT TGT CTT TGA GAT CCA TGC	R
iNOS	TCC TCA CTG GGA CAG CAC AGA ATG	F
	GTG TCA TGC AAA ATC TCT CCA CTG CC	R
MyD88	AGC AGA ACC AGG AGT CCG AGA AGC	F
	GGG GCA GTA GCA GAT AAA GGC ATC G	R
NF-κB	GCT TTG CAA ACC TGG GAA TA	F
	TCC GCC TTC TGC TTG TAG AT	R

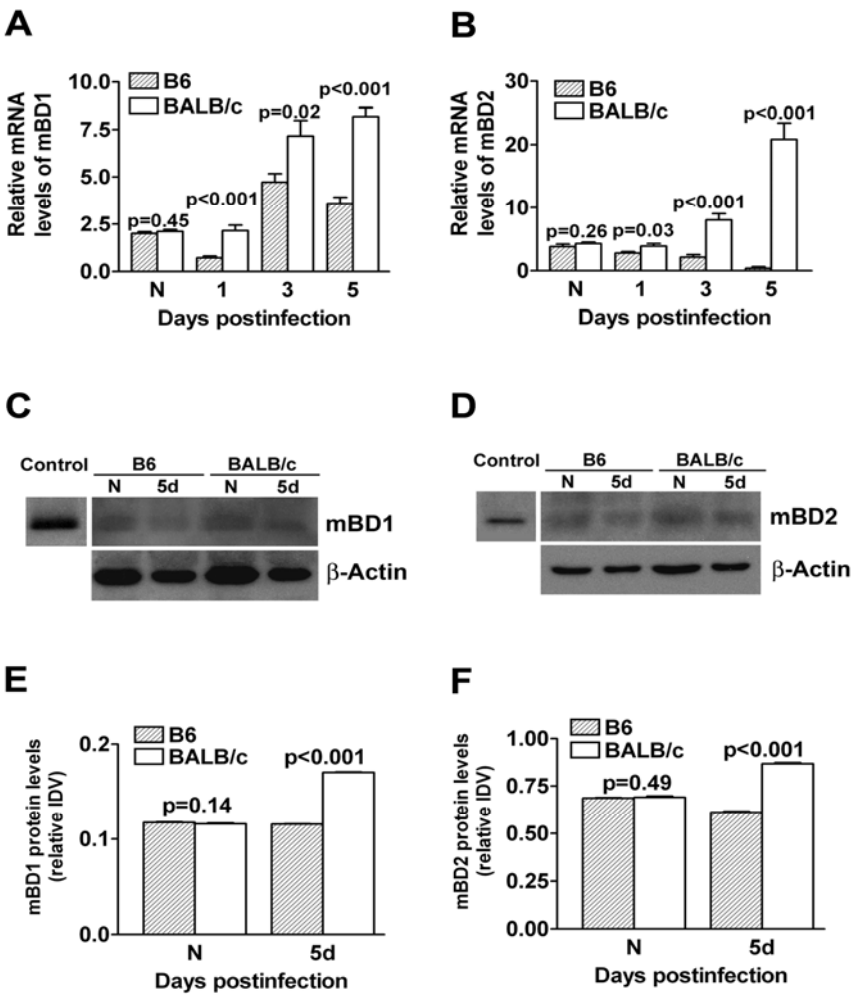

Figure 3. Expression of mBD1 and mBD2 in corneas of B6 and BALB/c mice.
mRNA expression levels of mBD1 (A) and mBD2 (B) were significantly increased in the infected cornea of BALB/c vs B6 mice at 1, 3 and 5 days p.i. (mBD1: $p < 0.001$, $p = 0.02$, $p < 0.001$; mBD2: $p = 0.03$, $p < 0.001$, $p < 0.001$, respectively). No difference was detected between BALB/c and B6 normal corneas for either defensin. Data are the mean ± SEM and represent two individual experiments each with 5 animals/group/time.
Western blot of mBD1 (C) and mBD2 (D) protein levels in the corneal epithelium of B6 and BALB/c mice. Equivalent protein (10 μg) loaded for lanes 2-5 and for lane1 (1 μg control peptide) (Fig. 3C, D). In Fig. 3C, Lanes: 1 (control peptide=1 μg mBD1); 2, normal B6 cornea; 3, B6 5 days p.i.; 4, normal BALB/c cornea; 5, BALB/c 5 days p.i. In Fig. 3D, Lanes: 1 (control peptide=1 μg hBD2); 2, normal B6 cornea; 3, B6 5 days p.i.; 4, normal BALB/c cornea; 5, BALB/c 5 days p.i. Data for the Western blot represent one of three similar experiments each using 15 pooled corneal epithelia/group/time.
Band intensity also was quantitated and normalized to the β-actin control. Protein levels of mBD1 (E) and mBD2 (F) were significantly increased in BALB/c vs B6 cornea at 5 days p.i. (mBD1: $p < 0.001$, mBD2: $p < 0.001$). For each defensin, no difference was detected between the normal cornea of the two groups. Data are the mean ± SEM and represent three individual experiments each using 15 pooled corneal epithelia/group/time.

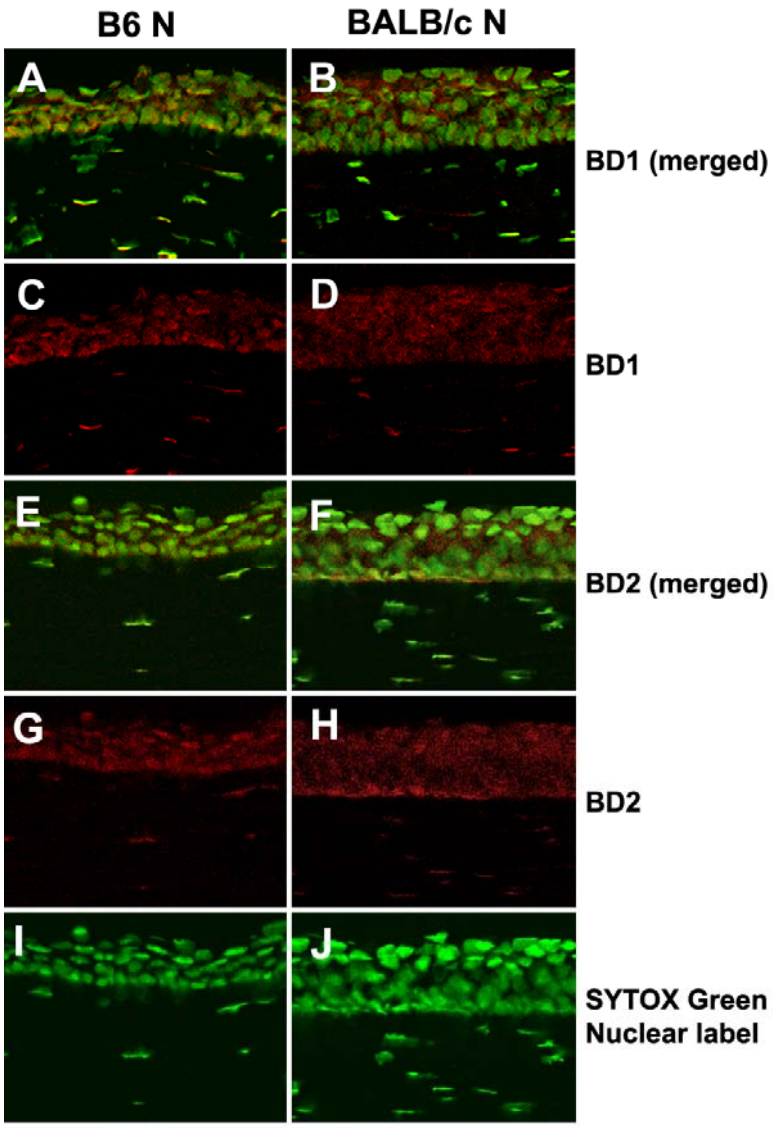

Figure 4. Immunostaining for mBD1 and mBD2 expression in normal corneas of B6 and BALB/c mice. Staining for mBD1 (A–D) and mBD2 (E–H) were similar in normal B6 and BALB/c corneas. Controls, in which the primary Ab was omitted, were negative for immunostaining for mBD1 or mBD2, and appeared similar to SYTOX Green Nuclear staining (I and J). Magnification = × 160. Images shown are representative of two repeat experiments each with 3 mice per group.

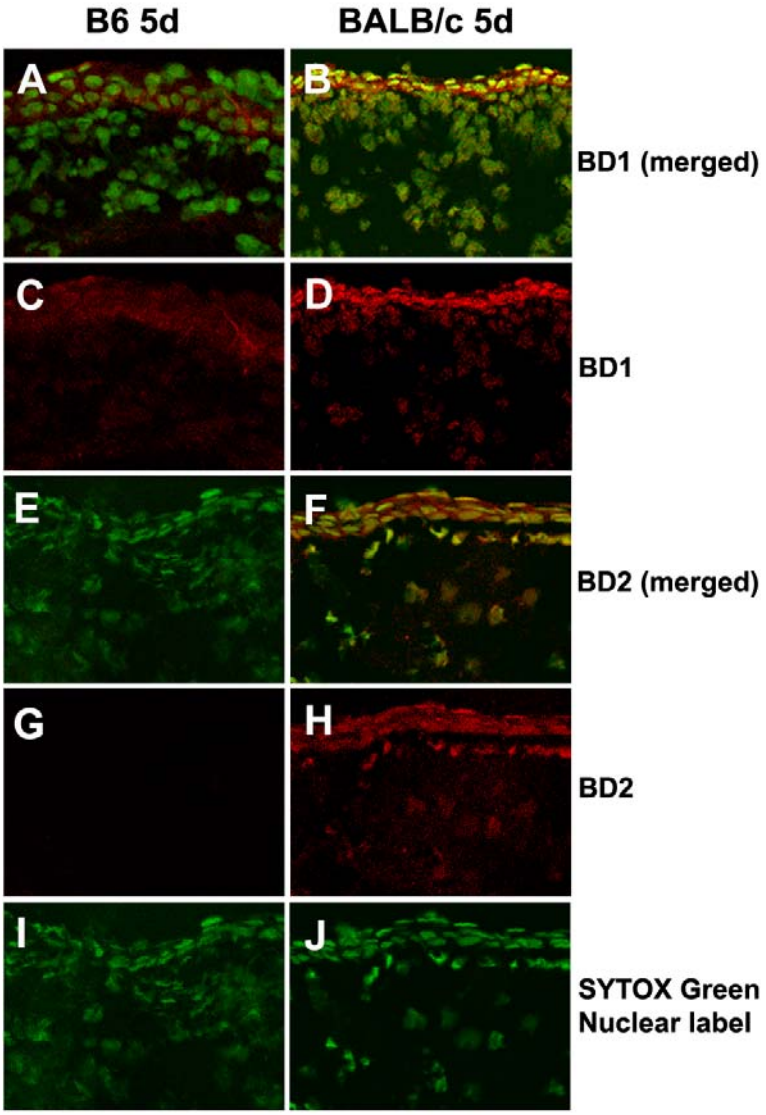

Figure 5. Immunostaining for mBD1 and mBD2 in infected corneas of B6 and BALB/c mice. mBD1 (A–D) and mBD2 (E–H) staining was dissimilar in infected B6 and BALB/c corneas at 5 days p.i. For both proteins, the corneal epithelium of BALB/c mice was stained more intensely than in B6 mice. Controls, in which the primary Ab was omitted, were negative for immunostaining for mBD1 or mBD2, and appeared similar to SYTOX Green Nuclear staining (I and J). Magnification = × 160. Images shown are representative of two repeat experiments each with 3 mice per group.

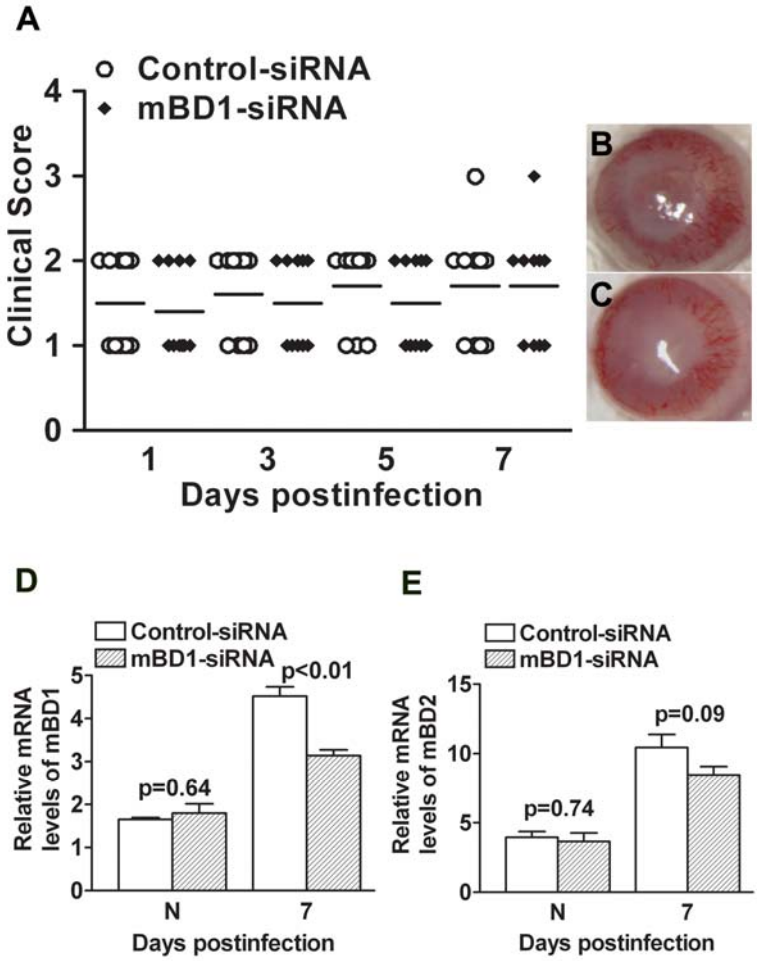

Figure 6. *In vivo* knock down studies of mBD1 in host resistance. Clinical scores (A) indicated no statistically significant differences at 1, 3, 5 and 7 days p.i. between mBD1 siRNA and control-treated BALB/c mice. Representative slit lamp photographs of *P. aeruginosa*-infected eyes were taken for control (B) or mBD1 (C) siRNA-treated mice at 7 days p.i. and showed a similar disease response. (D) RT-PCR confirmed that knock down of mBD1 vs control treatment was effective at 7 days p.i. ($p<0.01$) and that mBD2 mRNA levels were unchanged in mBD1 treated mice at that time (E), with no differences detected in normal, uninfected tissue for either defensin. Data are the mean ± SEM and represent two individual experiments each with 5 animals/group/time/assay.

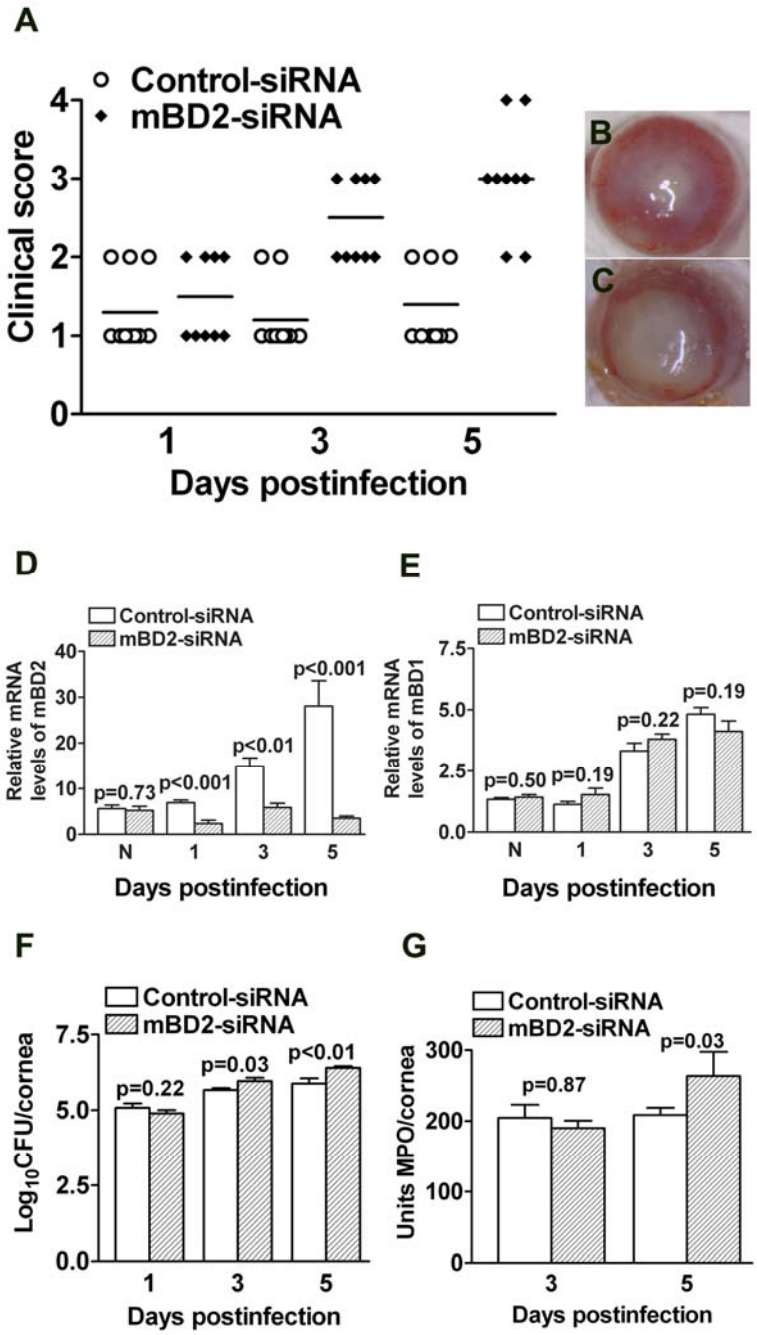

Figure 7. *In vivo* knock down studies of mBD2 in host resistance. For mBD2 siRNA treatment, clinical scores (A) indicated statistically significant differences at 3 and 5 days p.i. (both $p < 0.001$) and no differences at 1 day p.i., when compared with controls. Slit lamp photographs of *P. aeruginosa*-infected eyes at 5 days p.i. displayed more opacity and a worsened disease response when comparing control (B) vs treatment with mBD2 siRNA (C). (D) RT-PCR confirmed that knock down of mBD2 vs control treatment was effective at 1, 3, and 5 days p.i. ($p<0.001$, $p<0.01$ and $p<0.001$) and that mBD1 mRNA levels were unchanged in mBD2 treated mice at those times (E), with no differences detected in normal, uninfected tissue for either defensin. mBD2 silencing also led to increased bacterial counts (F) at 3 and 5 days p.i. ($p = 0.03$, $p < 0.01$, respectively) and enhanced recruitment of PMNs as detected by MPO activity (G) at 5 days p.i. ($p = 0.03$), when compared with controls. Magnification (slit lamp) = × 5. Data are the mean ± SEM and represent two individual experiments each with 5 animals/group/time/assay.

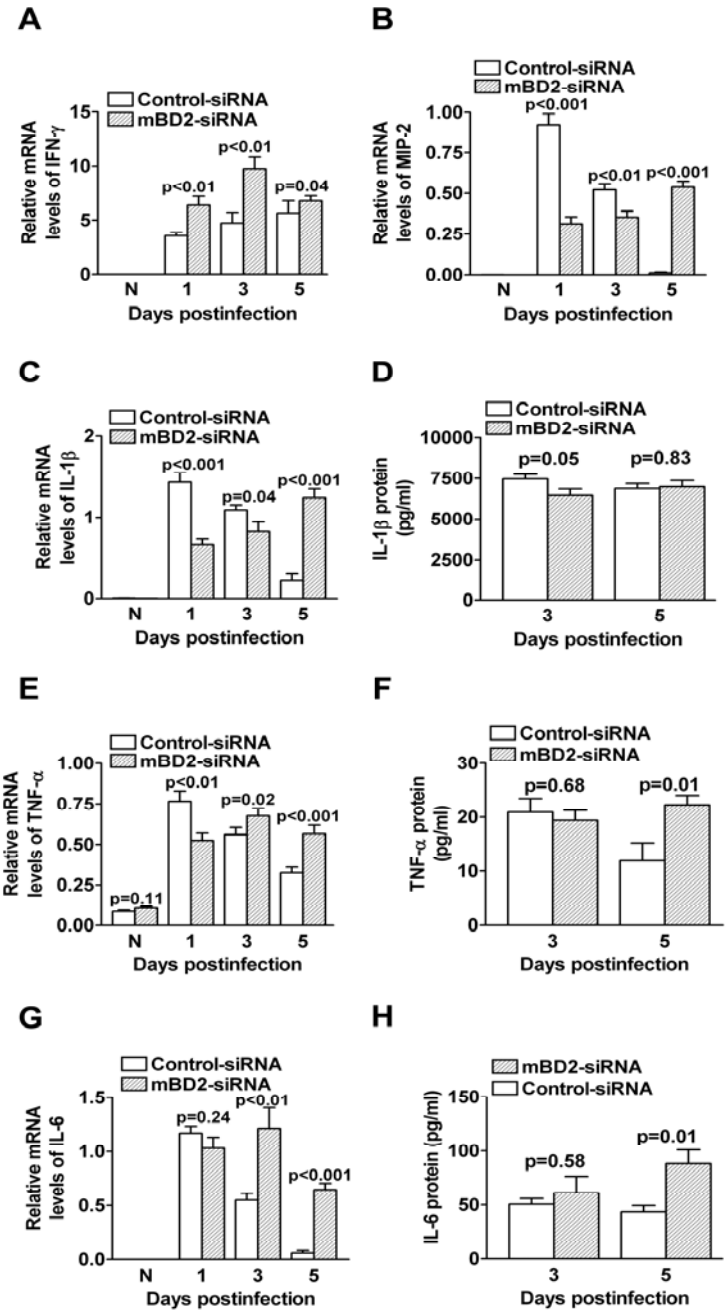

Figure 8. mBD2 silencing differentially regulates the production of pro-inflammatory cytokines. In the mBD2 siRNA-treated group, the mRNA expression level of IFN-γ (A) was significantly increased at 1, 3 and 5 days p.i.; MIP-2 (B) and IL-1β (C) were down-regulated at 1 and 3 days p.i., followed by a significant up-regulation at 5 days p.i.; TNF-α (E) and IL-6 (G) were reduced at 1 day p.i., but significantly enhanced at 3 and 5 days p.i., when compared with controls. Selected protein levels determined by ELISA indicated a down-regulation of IL-1β (D) at 3 days p.i., and up-regulation of TNF-α (F) and IL-6 (H) at 5 days p.i. in siRNA mBD2 vs control-treated mice. Data are the mean ± SEM and represent two individual experiments with 5 mice/group/time.

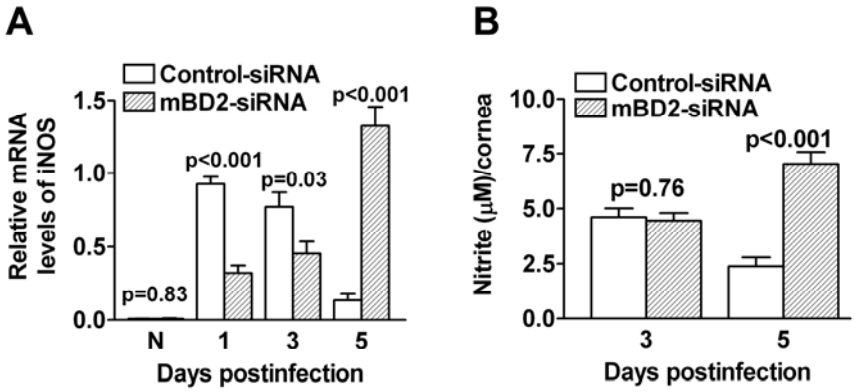

Figure 9. iNOS mRNA expression and nitrite levels as detected in mBD2 siRNA vs control-treated mice after *P. aeruginosa* ocular infection. The mRNA expression levels of iNOS (A) were significantly decreased at 1 and 3 days p.i. and increased at 5 days p.i. Results of Griess reaction (B) indicated that mBD2 siRNA treatment significantly enhanced nitrite levels at 5 days p.i. Data are the mean ± SEM and represent two individual experiments with 5 mice/group/ time.

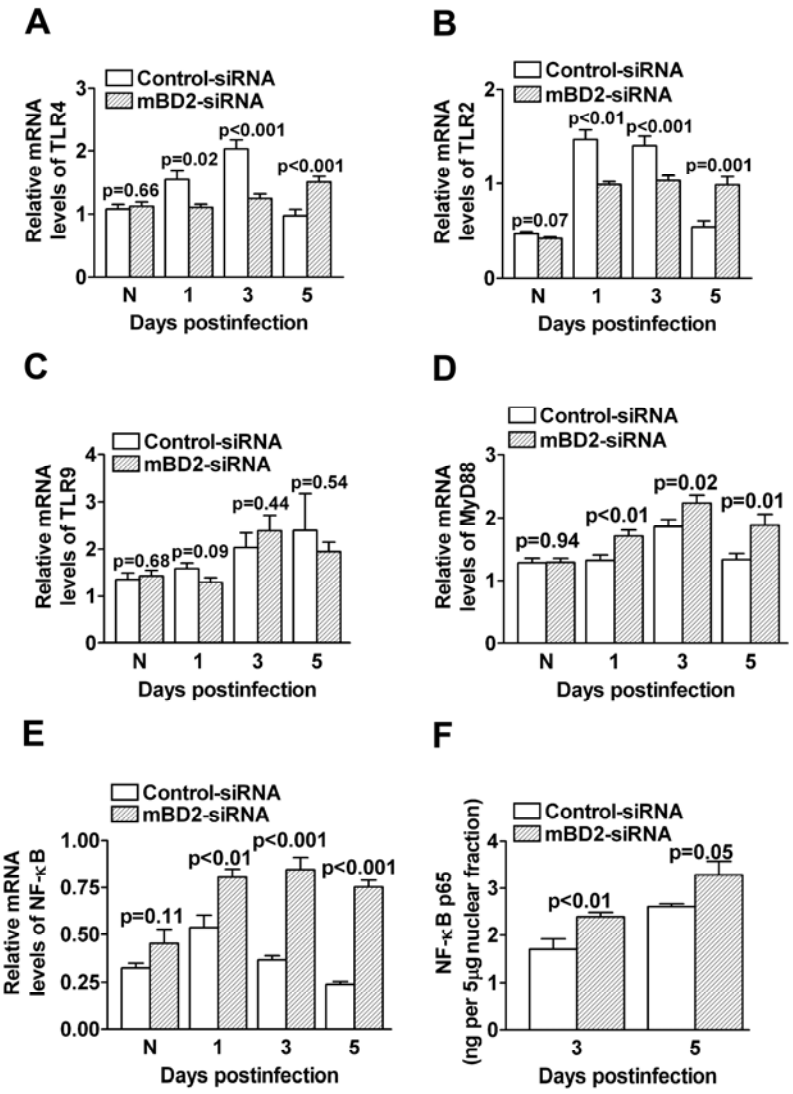

Figure 10. mBD2 siRNA treatment differentially modulates the production of TLR signaling molecules and NF-κB. After knock down of mBD2, mRNA expression levels for TLR4 (A) and TLR2 (B) were significantly decreased at 1 and 3 days p.i., followed by an increase at 5 days p.i.; whereas MyD88 (D) and NF-κB (E) were significantly elevated at 1, 3 and 5 days p.i., when compared with controls. No difference in TLR9 (C) expression was detected between the two groups. Further NF-κB studies (F) indicated that treatment with mBD2 siRNA significantly up-regulated activated levels of NF-κB p65 at both 3 and 5 days p.i. Data are the mean ± SEM and represent two individual experiments with 5 mice/ group/time/assay.

CHAPTER 3

MBD2 AND MBD3 TOGETHER PROMOTE RESISTANCE TO *P. AERUGINOSA* KERATITIS.

3.1 ABSTRACT

Defensins play an important role in both innate and adaptive immunity due to their antimicrobial, regulatory, and chemotactic effects. Nonetheless, the role of mBD3 and mBD4, the murine homologs of HBD2 and HBD3, remains unknown in *P. aeruginosa* keratitis. This study explored their role in corneal infection and potential synergy with mBD2, a defensin associated with better outcome in this disease. Immunostaining and real-time RT-PCR data demonstrated that mBD3 and mBD4 expression was inducible and differentially regulated in the infected cornea of resistant BALB/c vs susceptible B6 mice. Knockdown studies using small interfering RNA treatment indicated that mBD3, but not mBD4, is required in ocular defense. Moreover, *in vivo* studies demonstrated individual and combined effects of mBD2 and mBD3 that modulate bacterial load, PMN infiltration, and production of IFN-γ, MIP-2, IL-1β, TNF-α, iNOS, TLR2, TLR4, MyD88, and NF-κB. Most notably, bacterial load was increased at 5 days postinfection by silencing either mBD2 or mBD3, but it was elevated at both 1 and 5 days postinfection when silencing both defensins. PMN infiltration was increased at 1 day postinfection by silencing both defensins or mBD3, but not mBD2 alone. iNOS expression was elevated by silencing mBD2, but it was reduced after silencing mBD3 or both defensins. Additionally, cell sources of mBD2 (Mϕ, PMN and fibroblasts) and mBD3 (PMN) in corneal stroma were identified by dual label immunostaining after infection.

Collectively, the data provide evidence that mBD2 and mBD3 together promote resistance against corneal infection.

3.2 INTRODUCTION

Clinically, *P. aeruginosa*-induced keratitis is a rapidly progressing corneal disease that results in inflammatory epithelial edema, stromal infiltration, corneal ulceration, stromal tissue destruction, and, frequently, vision loss (Hazlett LD, 2004). Experimentally, *P. aeruginosa* challenge leads to corneal perforation in susceptible C57BL/6 (B6) mice and less severe disease in resistant BALB/c mice (Hazlett LD et al., 2005). In this regard, the antimicrobial properties of the cornea are attributed to several dynamic components, such as defensins, which can kill invading pathogens, regulate inflammation and TLR activation, as well as chemoattract a variety of immune cells (Tomita T and Nagase T, 2001; McDermott AM, 2004; McDermott AM, 2009).

In mice, the most studied defensins are mBD 1-4, which are chiefly expressed in a variety of epithelial cells (Bals R et al., 1998; Morrison GM et al., 1999; Bals R et al., 1999; Chong KT et al., 2008) and promote host resistance against infection in airway tissues (Bals R et al., 1998; Morrison GM et al., 1999; Bals R et al., 1999; Chong KT et al., 2008). mBD2 also can chemoattract and activate immature dendritic cells via TLR4 (Biragyn A et al., 2002). Recently, studies using susceptible/resistant murine models have demonstrated that mBD2, but not mBD1, is required for host resistance against *P. aeruginosa*-induced corneal infection (Wu M et al., 2009a); however, little is known regarding the role of the other two murine defensins in the eye. In this regard, other studies have demonstrated that HBD2 and HBD3, the inducible human homologs of mBD3 and mBD4 (Bals R et al., 1999; Chong KT et al., 2008), play an important role in

the ocular immune defense system. They do so by regulating a variety of immune events, including bacterial killing, cytokine release, mast cell histamine release, dendritic cell activation, immune cell chemotaxis, as well as epithelial cell migration and wound repair (Tomita T and Nagase T, 2001; McDermott AM, 2004; McDermott AM, 2009, Schroder JM and Harder J, 1999; Feng Z et al., 2005; Garcia JR et al., 2001; Niyonsaba F et al., 2002), suggesting that their homologs could be significant in murine ocular immunity. Moreover, *in vitro* studies have demonstrated synergistic activities of HBDs against *S. aureus* and *E. coli* (Chen X et al., 2005), indicating a significant potential for mBDs to act together to protect the ocular surface from invading pathogens.

Thus, studies described herein investigated the expression and function of mBD3 and mBD4 in susceptible B6 vs resistant BALB/c mice and whether either defensin interacts with mBD2, previously shown to be of importance in this disease (Wu M et al., 2009a). Our data provide evidence that mBD3 and mBD4 are inducibly and disparately expressed in BALB/c vs B6 corneal epithelium and stroma after *P. aeruginosa* infection. However, of the two, only mBD3 is required for host resistance and interacts with mBD2 to protect against bacterial infection. Additionally, *in vivo* studies identified the cell sources of mBD2 and mBD3 in the corneal stroma after infection and demonstrated that despite their individual effects on disease outcome (e.g., iNOS expression), these two defensins act together to promote host resistance against corneal infection. This is achieved through modulation of bacterial load and regulation of both PMN infiltration and production of pro-inflammatory cytokines and TLR signaling molecules.

3.3 MATERIALS AND METHODS

Infection of mice.

Eight-week-old female BALB/c (resistant) and B6 (susceptible) mice (The Jackson Laboratory) were anesthetized with ether and placed beneath a stereoscopic microscope at 40x magnification. The cornea of the left eye was wounded with three 1-mm incisions using a sterile 25-gauge needle. A 5-µl aliquot containing 1 x 10^6 CFU of *P. aeruginosa* (American Type Culture Collection, strain 19660), prepared as described before (Kwon B and Hazlett LD, 1997), was topically applied to the corneal surface. Eyes were examined at 1 day p.i. and/or at times described below to ensure that mice were similarly infected and to monitor disease. Animals were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular response to infection.

Corneal disease was graded using an established scale (Hazlett LD et al., 1987). A clinical score was recorded for each mouse after infection for statistical comparison of disease severity, and photography with a slit lamp was used to illustrate the disease response.

RNA interference.

In vivo use of siRNA has been described by others (Nakamura H et al., 2004) as well as by studies from our laboratory (Wu M et al., 2009a; Huang X et al., 2005). For the studies described herein, siRNA for mBD2, mBD3, mBD4, and both mBD2 and mBD3 (composed of an equal mixture of mBD2 and mBD3 siRNA at the same concentration) or appropriate scrambled controls for each (Santa Cruz Biotechnology)

were injected subconjunctivally (5 µl per mouse at a concentration of 8 µM) into the left eye of BALB/c mice (n = 5/group/time) 1 day before infection and then topically applied onto the infected corneas (5 µl/mouse/time at a concentration of 4 µM, once on the day of infection and twice on both 1 and 3 days p.i.). The efficacy and specificity of silencing of each defensin was tested by RT-PCR. All of the siRNAs used in the studies herein were shorter than 21 nucleotides in length to avoid nonspecific siRNA suppression effects via cell-surface TLR3 (Kleinman ME et al., 2008).

Separation of corneal epithelium and stroma.

Corneas from normal, uninfected, or infected eyes of BALB/c and B6 mice were hemisected at 1 and 5 days p.i. and placed into 0.02 M EDTA-PBS buffer (pH 7.2) at 37°C for 15–30 min. Then, the corneal epithelium was gently separated from the underlying stroma using fine forceps and the tissue was processed for real-time RT-PCR.

Real-time RT-PCR.

Total RNA was isolated from an individual whole cornea or separated corneal epithelium/stroma for analysis (as indicated below) using RNA-Stat 60 (Tel-Test), according to the manufacturer's recommendations, and quantitated by spectrophotometric determination (260 nm). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase. The 20-µl reaction mixture contained 200 U of Moloney murine leukemia virus-reverse transcriptase, 10 U of RNasin, 500 ng of oligo(dT) primers, 10 mM dNTPs, 100 mM DTT, and Moloney murine leukemia virus reaction buffer (Invitrogen). Next, cDNA was amplified using SYBR Green Master Mix (Bio-Rad), as suggested by the manufacturer.

Briefly, the 20-µl reaction system contained 10 µl of SYBR Green PCR Master Mix, 0.5 µM primers, 2 µl of cDNA (diluted 1:10), and diethyl pyrocarbonate water. All primers for the PCR, except for mBD3 and mBD4, were designed using PrimerQuest (Integrated DNA Technologies) and reported previously (Wu M et al., 2009). Primers for mBD3 and mBD4 were purchased from SABiosciences. Quantitative real-time RT-PCR reactions were 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 CA et al., 1996; Roux KH, 1995). mRNA transcription levels were normalized to the β-actin gene and/or calculated as the n-fold difference of transcription in siRNA for mBD2, mBD3, or both defensins compared with scrambled control treated mice. Data are shown as means ± SEM for relative mRNA levels and means ± SD for n-fold difference, respectively, and represent two individual experiments each with five mice per group per time.

Immunofluorescent staining.

Normal uninfected and infected eyes were enucleated (n = 3/group/time) at 5 days p.i. from BALB/c and B6 mice, immersed in 1x Dulbecco's PBS (Mediatech), embedded in Tissue-Tek OCT compound (Miles), and frozen in liquid nitrogen. Ten-micrometer-thick sections were cut, mounted to polylysine-coated glass slides, and incubated 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, for single label immunostaining, sections were incubated with primary Abs, rabbit anti-mouse β-defensin 3 (1:50; Santa Cruz Biotechnology) or rabbit anti-mouse β-defensin 4 (1:50; Santa Cruz Biotechnology) for 1

h, followed by Alexa Fluor 594-conjugated donkey anti-rabbit Ab (1:1500; Invitrogen) for another hour. For dual label immunostaining, sections were incubated for 1 h with goat anti-mouse β-defensin 2 (1:50; Santa Cruz Biotechnology) or rabbit anti-mouse β-defensin 3 (1:50; Santa Cruz Biotechnology), together with either rat anti-mouse Mϕ marker F4/80 (1:100; Santa Cruz Biotechnology), rat anti-mouse fibroblast marker (ER-TR7, 1:50; Santa Cruz Biotechnology), or rat anti-mouse PMN marker Gr-1/Ly6G (3 μg/ml; R&D Systems). This was followed by a secondary Ab, Cy5-conjugated donkey anti-rat IgG (H+L) (1:500, 1:1000, and 1:750 for Mϕ, fibroblast, and PMN, respectively; Jackson ImmunoResearch Laboratories) and Alexa Fluor 594-conjugated donkey anti-goat or donkey anti-rabbit Ab (for mBD2 and mBD3, respectively, 1:1500; Invitrogen) for another hour. Sections were then incubated for 2 min with SYTOX Green nuclear acid stain (1:20,000 and 1:30,000, for single and dual label immunostaining, respectively; Lonza). Controls were similarly treated, but the primary Abs were replaced with the same host IgG, ChromPure goat, rabbit, or rat IgG (Jackson ImmunoResearch Laboratories). Finally, sections were visualized and digital images captured with a Leica TSC SP2 confocal laser scanning microscope (Leica Microsystems).

ELISA.

Cytokine protein levels were selectively tested using ELISA kits (R&D Systems). Corneas from siRNA for mBD2, mBD3, or both defensins, and scrambled control-treated BALB/c mice were individually collected (n = 5/group/time) at 1 and 5 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 min and an aliquot of each supernatant was assayed in duplicate for MIP-2, IL-1β, and TNF-α protein per the manufacturer's instruction. The reported

sensitivity of these assays is <1.5 pg/ml for MIP-2, <3.0 pg/ml for IL-1β, and <5.1 pg/ml for TNF-α.

Bacterial plate counts.

Corneas from siRNA mBD2, mBD3, or both defensins, and scrambled control-treated BALB/c mice were collected (n = 5/group/time) at 1 and 5 days p.i. and the numbers of viable bacteria were quantitated. Individual corneas were homogenized in sterile water containing 0.85% (w/v) NaCl containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on Pseudomonas isolation agar (Difco Laboratories) in triplicate and plates were incubated overnight at 37°C. Results are reported as number of 10^5 CFU per cornea ± SEM.

MPO assay.

A MPO assay was used to quantitate PMN number in the cornea from siRNA mBD2, mBD3, or both defensins, and scrambled control-treated BALB/c mice. Infected corneas (n = 5/group/time) were excised at 1 and 5 days p.i. and homogenized in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% HTAB (Sigma-Aldrich). Samples were freeze-thawed four times and centrifuged at 13,000 rpm for 10 min, and 0.1 ml of the supernatant was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml; Sigma-Aldrich) and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm was monitored for 5 min at 30-s intervals, and the results were expressed as units of MPO per cornea. One unit of MPO activity is equivalent to 2 x 10^5 PMN (Williams RN et al., 1982).

Statistical analysis.

The difference in clinical score between 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 the significance of viable bacterial counts, MPO, real-time RT-PCR, and protein assays. Data were considered significant at $p < 0.05$.

3.4 RESULTS

Expression of mBD3 and mBD4 in BALB/c vs B6 mice.

The distribution of mBD3 and mBD4 in corneal epithelium and stroma of BALB/c and B6 mice before and after infection with *P. aeruginosa* was tested in BALB/c and B6 mice at 1 and 5 days p.i. using immunofluorescent staining (Figs. 11 and 12). No staining for mBD3 or mBD4 was detected in normal, uninfected BALB/c and B6 corneas (data not shown). At 1 day p.i., mBD3 staining was similar in infected B6 and BALB/c corneas (Fig. 11, A and B), while qualitatively more mBD4-positive cells were detectable in both corneal epithelium and stroma in B6 vs BALB/c mice (Fig. 11, C and D). At 5 days p.i., mBD3 staining of increased intensity was detected in BALB/c vs B6 corneal epithelium, but no difference was seen in stromal staining between the two groups (Fig. 12, A and B). In contrast, qualitatively more mBD4-positive cells were detectable in both epithelium and stroma in B6 vs BALB/c infected corneas (Fig. 12, C and D). Controls (primary Ab replaced with donkey IgG) were similar to SYTOX Green Nuclear staining (Figs. 11, E and F, and 12, E and F). Additionally, mRNA transcription levels of mBD3 and mBD4 in separated epithelium and stroma of normal uninfected and infected BALB/c and B6 corneas were tested at 5 days p.i. using real-time RT-PCR (data not shown) and confirmed the 5 days p.i. immunostaining data.

Silencing mBD3 and mBD4.

Because the distribution patterns (mRNA and immunostaining) suggested that mBD3 and mBD4 were differentially expressed in infected BALB/c and B6 corneal epithelium and stroma, the next series of *in vivo* studies were designed to determine their effects in host defense against corneal infection. First, BALB/c mice were subconjunctivally injected and topically treated with scrambled control or mBD3- or mBD4-specific siRNA to determine whether knock down of either defensin would impair host defense. The results indicated that by 5 days p.i., the cornea of most mBD3 siRNA-treated mice displayed an enhanced level of disease (grade = +3/+4), whereas almost all scrambled control-treated corneas showed less opacity/disease (grade = +1/+2). Clinical score data (Fig. 13A) showed that mBD3 siRNA-treated mice exhibited increased disease at 3 and 5 days p.i. (both $p < 0.001$). Representative photographs taken with a slit lamp of control, scrambled (Fig. 13B) vs mBD3 (Fig. 13C) siRNA-treated mice at 5 days p.i. are provided. Treatment with mBD3 siRNA resulted in either perforation (grade = +4, data not shown) or dense opacity covering the entire anterior segment (grade = +3, Fig. 13C) and overall more inflammation than scrambled, control treatment (Fig. 13B) at 5 days p.i.

On the other hand, no significant difference was shown between mBD4 siRNA and scrambled control-treated mice at 1, 3, and 5 days p.i. By 5 days p.i., clinical score data showed that mBD4 siRNA treatment did not significantly change the host response in BALB/c mice after *P. aeruginosa* infection (Fig. 13D). Representative photographs taken with a slit lamp at 5 days p.i. showed similar corneal opacity/disease in scrambled control (Fig. 13E) vs mBD4 siRNA-treated (Fig. 13F) mice. RT-PCR data confirmed that

silencing was significant and specific for mBD3 and mBD4 (Fig. 13, G and I), and that mRNA levels for mBD4 expression in mBD3 siRNA-treated mice as well as mBD3 expression in mBD4 siRNA-treated mice were not changed significantly (Fig. 13, H and J).

In vivo studies of silencing mBD2, mBD3, or both defensins.

Our previous studies demonstrated that mBD2 promotes resistance against *P. aeruginosa*-induced corneal infection. Therefore, we next used siRNA treatment to determine whether mBD2 and mBD3 function together in ocular immune defense. Results indicated that after infection, the cornea of mice treated with siRNA for both defensins displayed an enhanced level of disease when compared with scrambled control or either mBD2 or mBD3 siRNA-treated mice. Clinical scores (Fig. 14A) indicated statistically significant differences at 1, 3, and 5 days p.i. (all $p < 0.001$) after silencing both defensins. For either mBD2 or mBD3 siRNA treatment, clinical score differences were shown at 3 and 5 days p.i., but not at 1 day p.i., when compared with scrambled controls. Representative photographs taken with a slit lamp of infected eyes at 5 days p.i. are provided for mice treated with scrambled control (Fig. 14B, grade = +1/+2), siRNA for mBD2 (Fig. 14C, grade = +3), mBD3 (Fig. 14D, grade = +3), or both defensins (Fig. 14E, grade = +4). Meanwhile, bacterial plate counts (Fig. 14F) and MPO activity (Fig. 14G) were used to detect viable bacteria and PMN infiltration in the infected cornea of mice treated with siRNA for mBD2, mBD3, or both defensins vs scrambled controls at 1 and 5 days p.i. When compared with controls, silencing both defensins led to enhanced bacterial counts and PMN recruitment at 1 and 5 days p.i. (bacteria: $p < 0.01$, $p < 0.001$; PMN: $p < 0.001$, $p < 0.001$); silencing mBD2 elevated

both at 5 days p.i. only (both $p < 0.001$); silencing mBD3 increased bacterial counts at 5 days p.i. ($p < 0.01$) and PMN infiltration at 1 and 5 days p.i. ($p = 0.02$, $p < 0.001$). Furthermore, when comparing knockdown of both defensins with either mBD2 or mBD3 alone, bacterial counts were elevated at 5 days p.i. ($p = 0.02$, $p < 0.01$) and at 1 and 5 days p.i. (all $p < 0.001$), PMN were increased. RT-PCR data also demonstrated that siRNA treatment for mBD2, mBD3, or both defensins was significant and specific at 5 days p.i. (Fig. 15).

Effects of silencing on proinflammatory cytokines and TLR signaling.

Since silencing both mBD2 and mBD3 elevated bacterial load and PMN infiltration in BALB/c corneas earlier after *P. aeruginosa* infection than did silencing either defensin alone, the next series of studies were initiated to investigate whether the two defensins acted together to regulate host pathogen immune mechanisms. mRNA transcription levels of proinflammatory cytokines and TLR signaling molecules were evaluated at 5 days p.i. by real-time RT-PCR (Table 4, A-C). At 5 days p.i., the mRNA transcription levels of IFN-γ, MIP-2, TNF-α, IL-1β, TLR4, TLR2, MyD88, and NF-κB were significantly up-regulated by siRNA treatment for mBD2, mBD3, or both defensins, while iNOS levels were significantly up-regulated after silencing mBD2 alone, but down-regulated after silencing mBD3 or both defensins, when compared with controls. No changes in TLR5 and TLR9 transcription were shown between silenced and control groups. Moreover, mRNA transcription levels of selected cytokines/molecules at 5 days p.i. were calculated as the n-fold differences of transcription in mice treated with siRNA for mBD2, mBD3, or both defensins vs scrambled control. The results indicated that silencing both defensins significantly elevated the relative fold increase of IFN-γ, TLR2,

MyD88, and NF-κB transcription, when compared with either mBD2 or mBD3 siRNA treatment (data not shown). Additionally, to determine whether mBD2 and mBD3 function together to modulate inflammatory process, protein expression levels of MIP-2 (Fig. 16A), TNF-α (Fig. 16B), and IL-1β (Fig. 16C), key regulators and mediators of inflammatory responses, such as PMN activation, proliferation and chemotaxis, were examined by ELISA at 1 and 5 days p.i. Results suggested that protein levels of these cytokines were significantly up-regulated at 5 days p.i. in siRNA mBD2, mBD3, and both defensins vs scrambled control-treated mice (MIP-2: $p = 0.01$, $p = 0.03$, $p < 0.001$; TNF-α: $p = 0.03$, $p = 0.03$, $p < 0.01$; IL-1β: $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively), as well as when comparing knockdown of both defensins with either mBD2 or mBD3 silencing (MIP-2: $p = 0.04$, $p = 0.03$; TNF-α: $p = 0.03$, $p = 0.02$; IL-1β: $p = 0.02$, $p < 0.01$, respectively).

Cell sources of mBD2 and mBD3 in stroma.

To identify the sources of defensins 2 and 3 in the infected corneal stroma of BALB/c mice, dual label immunostaining was used. mBD2 staining was detected in fibroblasts (Fig. 17A), Mϕ (Fig. 17C), and PMN (Fig. 17E), while mBD3-specific staining was detected only in PMN (Fig. 17G) but not in the other cell types (data not shown). Controls (Fig. 17, B, D, F, and H), in which the primary Abs were replaced with the same host IgG, were similar to SYTOX Green Nuclear staining (not shown).

3.5 DISCUSSION

In humans, the most important defensins are HBD2 and HBD3. They are mainly expressed in a variety of epithelial cells and play an important role in both innate and adaptive immunity (Tomita T and Nagase T, 2001; Schroder JM and Harder J, 1999;

Feng Z et al., 2005; Garcia JR et al., 2001; Niyonsaba F et al., 2002; Yang D et al., 1999). However, little is known regarding the functions of their murine homologs (Bals R et al., 1999; Chong KT et al., 2008), mBD3 and mBD4, especially in the ocular immune defense system.

In this regard, our studies revealed the distribution patterns of these defensins in normal uninfected and infected corneas. Immunostaining and RT-PCR data (not shown) provided evidence that both mBD3 and mBD4 were inducibly expressed and disparately regulated in the corneal epithelium and stroma of BALB/c vs B6 mice after infection, suggesting a potential role for the two defensins in the development of the resistant vs susceptible phenotype. *In vivo* knockdown studies demonstrated that mBD3, rather than mBD4, promoted host resistance against *P. aeruginosa*-induced corneal infection. Data to support this tenet included confirming the specificity and selectivity of each knockdown by RT-PCR, as well as photographs taken with a slit lamp and clinical scores that visually showed little difference between mBD4 siRNA vs scrambled control-treated corneas. All of these suggested that mBD4, similar to mBD1 (Wu M et al., 2009a), is not required for ocular immune defense against *P. aeruginosa*. These data were accompanied by similar mRNA distribution patterns (data not shown) of selected proinflammatory cytokines and TLR signaling molecules in infected corneas of mBD4 silenced vs control-treated mice. In contrast, at 5 days p.i., mBD3 siRNA-treated BALB/c mice displayed increased corneal opacity and exacerbated ocular disease, increased bacterial plate counts and PMN recruitment, as well as elevated mRNA levels of selected proinflammatory cytokines and TLR signaling molecules, when compared with controls. Taken together, these results provide direct evidence that mBD3 promoted

resistance to *P. aeruginosa* keratitis by modulating bacterial load, PMN infiltration, inflammation, and TLR activation.

Nonetheless, *in vitro* studies have reported that defensins may act synergistically in immune defense (McDermott AM, 2009; Chen X et al., 2005). As a matter of fact, previous studies have revealed that mBD2 is required in ocular defense against *P. aeruginosa*-induced corneal infection (Wu M et al., 2009a). Thus, we tested the interaction of the two defensins by silencing both. This led to a significantly increased disease and enhanced bacterial counts earlier (1 day p.i.) than when silencing either defensin alone, indicating their antimicrobial synergy. Additionally, compared with knockdown of either mBD2 or mBD3 alone, silencing both defensins elevated mRNA levels of TLR2, MyD88, NF-κB, and IFN-γ (calculated as *n*-fold difference, data not shown), as well as protein expression of MIP-2, IL-1β, and TNF-α at 5 days p.i. Regarding these molecules, IFN-γ is a hallmark regulatory cytokine in inflammation and Th1 responses, which can also induce PMN survival due to its antiapoptotic effect (McClellan SA et al., 2006; Radhakrishnan S et al., 2007; Sekiya M et al., 1997). TLRs are critical in innate immune response, and their activation can induce the production of inflammatory cytokines, including MIP-2, TNF-α, and IL-1β, which can modulate PMN activation, proliferation, and chemotaxis (Yu FS and Hazlett LD, 2006; Beutler B, 2004; Esen N and Kielian T, 2006; Matsumoto K et al., 2005; Hazlett LD, 2005). Thus, it is logical to predict that mBD2 and mBD3 function together to modulate ocular inflammatory responses through IFN-γ and TLR signaling pathways and their interactions (Zhao J et al., 2006).

Although both mBD2 and mBD3 contribute to host resistance against bacterial keratitis, the mechanisms by which they do so appear to differ. First, both have antimicrobial activities; however, mBD2 is salt sensitive and preferentially kills Gram-negative bacteria (Morrison GM et al., 1999), while mBD3 can kill both Gram-negative and -positive bacteria, with much less salt sensitivity (Burd RS et al., 2002). Second, in our study, silencing of mBD3, not mBD2, resulted in earlier PMN infiltration (at 1 day p.i.), compared with controls, indicating that in mice, mBD3 may be more important than mBD2 in regulating PMN recruitment. Third, studies described herein provide evidence that iNOS expression was up-regulated by mBD2 silencing, but down-regulated by silencing mBD3 or both defensins. These differences are puzzling, but they may be explained, in part, in the light of other studies suggesting that LPS and IFN-γ regulate iNOS expression through differential activation of TLR4 and JAK/STAT pathways (Yu FS and Hazlett LD, 2006; Marrero MB et al., 1998). More specifically, regarding TLR4, both defensins have potential LPS neutralization activity (Lai Y and Gallo RL, 2009), but only mBD2 can activate TLR4 (Biragyn A et al., 2002; da Silva Correia J et al., 2001). This difference may contribute to the disparate outcome in iNOS transcription levels observed after silencing mBD2 vs mBD3 or both defensins.

Additionally, regarding IFN-γ and iNOS expression, other scenarios require consideration. In this regard, iNOS is expressed maximally after an inflammatory stimulus and induces the production of large micromolar quantities of NO, a free radical gas with both signaling and antimicrobial functions (Bogdan C et al., 2000; Hazlett LD et al., 2005). NO is an essential mediator of immune defense against bacterial infection, whereas excessive production of NO often results in tissue destruction and pathology

(McClellan SA et al., 2006; Hazlett LD et al., 2005). Moreover, it has been reported that the synergistic interaction between IFN-γ and NO levels regulates ocular disease outcome in resistant BALB/c mice (McClellan SA et al., 2006). Thus, because silencing mBD3 or both defensins down-regulates NO, mBD3 may be more important than mBD2 in this outcome.

Additionally, studies have demonstrated that defensins are produced as a functionally inactive preprodefensin form in nature and must undergo posttranslational modification to form a biologically active mature peptide, which is sequentially secreted onto the surface such as an epithelium or immediate surroundings to execute their function (Yang D et al., 2004). Thus, we hypothesize that mBDs at the ocular epithelial surface might be functionally more important early in infection than those in the stroma. Our studies demonstrated that both mBD2 and mBD3 are mainly expressed in the corneal epithelium, which may contribute to their synergy in bacterial killing early after infection. In the stroma, whereas PMN produce both defensins, Mϕ and fibroblasts produce mBD2 but not mBD3, which may contribute to their individual and combined effects later in the disease process.

In summary, our studies provide direct evidence that both mBD3 and mBD4 are inducibly expressed and disparately regulated in infected corneal epithelium and stroma of resistant and susceptible mice; that of the two defensins tested by knockdown experiments, only mBD3 is required for host resistance against bacterial infection; and that mBD2 and mBD3 function together to modulate bacterial load, PMN infiltration, production of proinflammatory cytokines, iNOS, TLR signaling molecules as well as

transcription factor NF-κB in the infected cornea. These data suggest that the defensins may provide a novel target for treatment of ocular infections induced by *P. aeruginosa*.

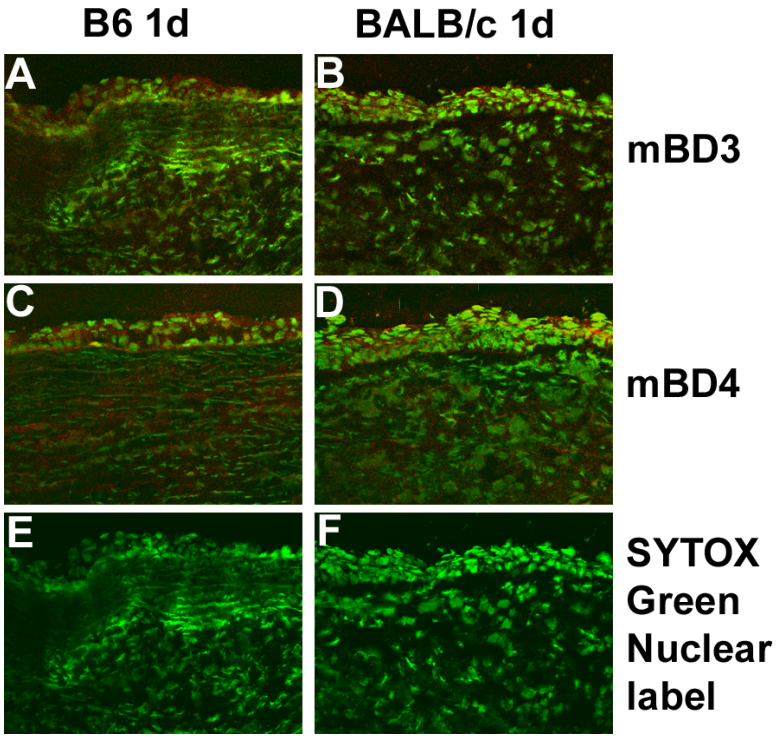

Figure 11. Immunostaining for mBD3 and mBD4 in infected cornea at 1 day p.i. For mBD3 (A and B), staining was similar in infected cornea of B6 and BALB/c mice. For mBD4 (C and D), the corneal stroma of B6 mice was stained more intensely than in BALB/c mice, while no difference was seen in the corneal epithelium between the two groups. Controls, in which the primary Ab was replaced by donkey IgG, were negative for immunostaining for mBD3 or mBD4 and appeared similar to SYTOX Green Nuclear staining (E and F). Magnification = x 100. Images shown are representative of two repeated experiments each with three mice per group.

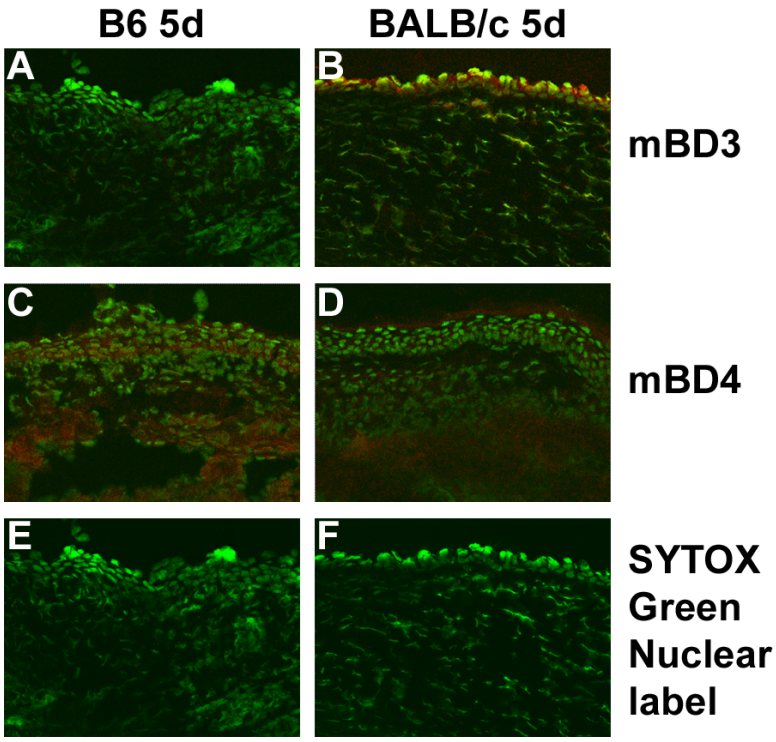

Figure 12. Immunostaining for mBD3 and mBD4 in infected cornea at 5 days p.i. For mBD3 (A and B), the corneal epithelium of BALB/c mice was stained more intensely than in B6 mice, while no difference was shown in the corneal stroma of the two groups. For mBD4 (C and D), both corneal epithelium and stroma of B6 mice was stained more intensely than in BALB/c mice. Controls, in which the primary Ab was replaced by donkey IgG, were negative for immunostaining for mBD3 or mBD4 and appeared similar to SYTOX Green Nuclear staining (E and F). Magnification = x 100. Images shown are representative of two repeated experiments each with three mice per group.

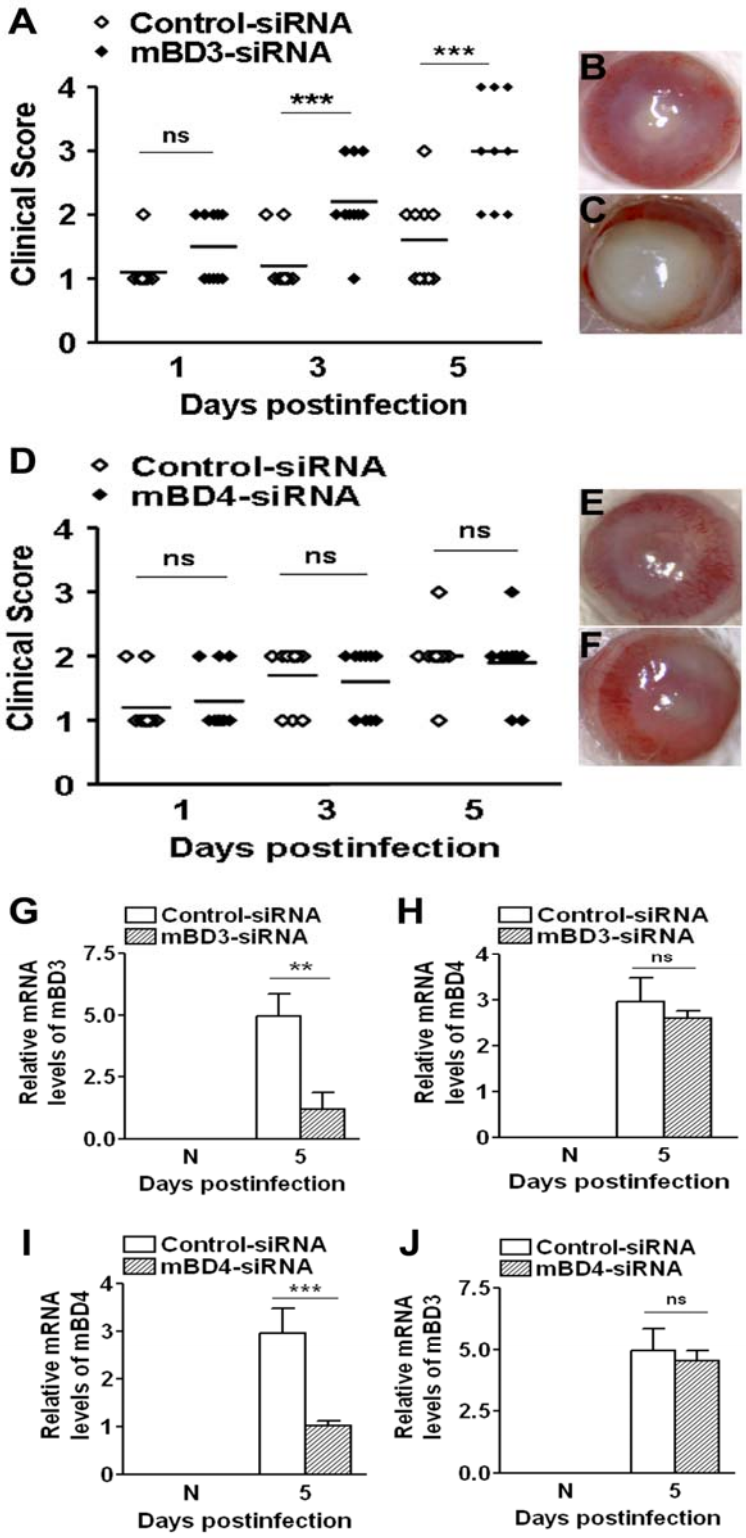

Figure 13. *In vivo* knockdown studies of mBD3 and mBD4. For mBD3 siRNA treatment, clinical scores (A) indicated statistically significant differences at 3 and 5 days p.i., compared with controls. Photographs taken with a slit lamp of infected eyes at 5 days p.i. displayed more opacity and a worsened disease response when comparing control (B) vs mBD3 siRNA (C) treatment. For mBD4 siRNA treatment, clinical scores (D) and similar photographs indicated no differences between control (E) and mBD4 silenced (F) mice after infection. RT-PCR (G–J) confirmed the efficacy and specificity of each silencing. Magnification = x 6. Data are the means ± SEM and represent two individual experiments each with five animals/group/time/assay. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by Mann-Whitney U or Student's t test; ns, not significant

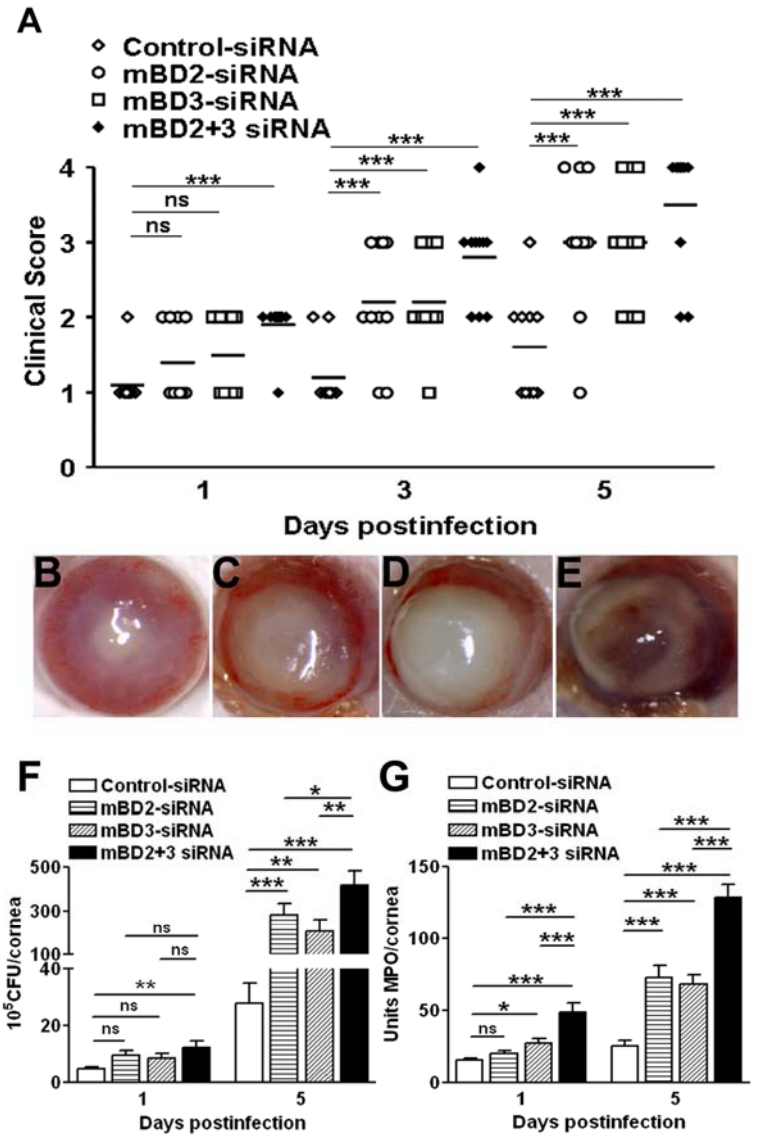

Figure 14. *In vivo* silencing of mBD2, mBD3, and both defensins. Clinical scores (A) and photographs taken with a slit lamp of *P. aeruginosa*-infected eyes at 5 days p.i. displayed an enhanced disease level and more opacity when comparing control (B) vs siRNA treatment for mBD2 (C), mBD3 (D), or both defensins (E). Bacterial plate counts (F) and PMN recruitment as detected by MPO activity (G) are shown in each group. Magnification = x 6. Data are the means ± SEM and represent two individual experiments each with five animals/group/time/assay. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by Mann-Whitney U or Student's t test; ns, not significant.

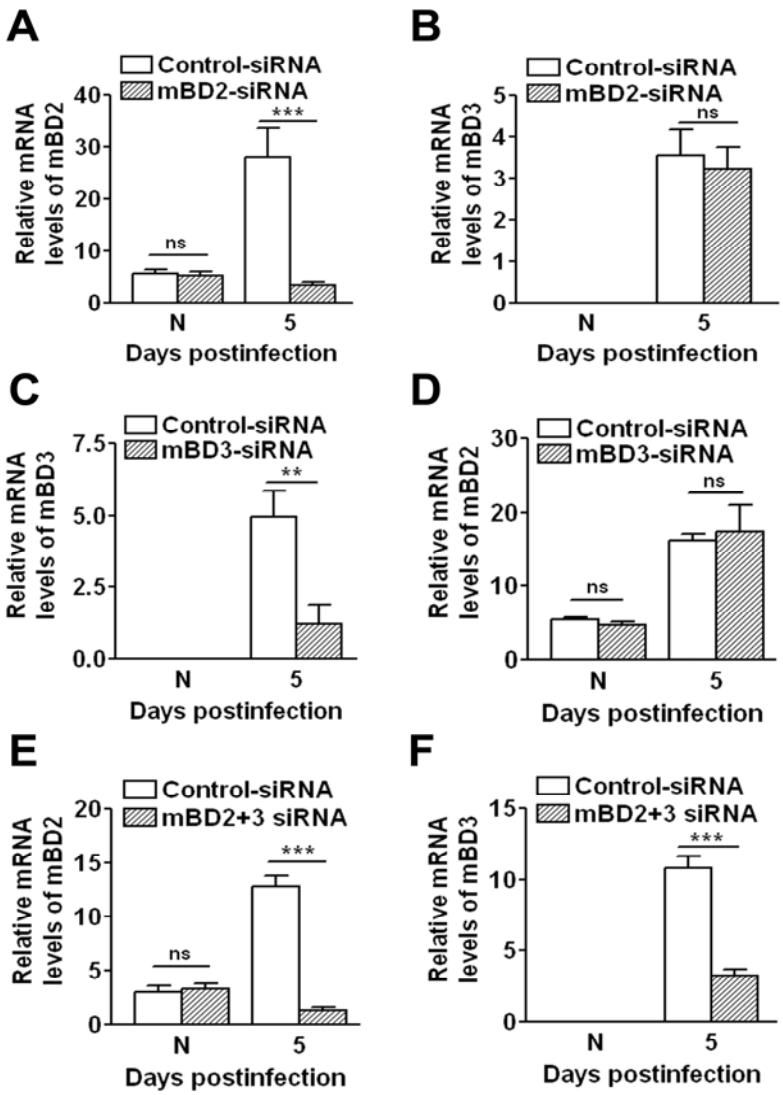

Figure 15. Efficacy and specificity of *in vivo* siRNA treatment. RT-PCR confirmed that knockdown of mBD2 (A), mBD3 (C), and both defensins (E and F) vs control treatment was effective at 5 days p.i., and that mBD3 mRNA levels in mBD2 siRNA-treated mice (B) as well as mBD2 mRNA levels in mBD3 siRNA-treated mice (D) were unchanged at that time, with no differences detected in normal, uninfected tissue for either defensin. Data are the means ± SEM and represent two individual experiments each with five animals/group/time/assay. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by Student's t test; ns, not significant.

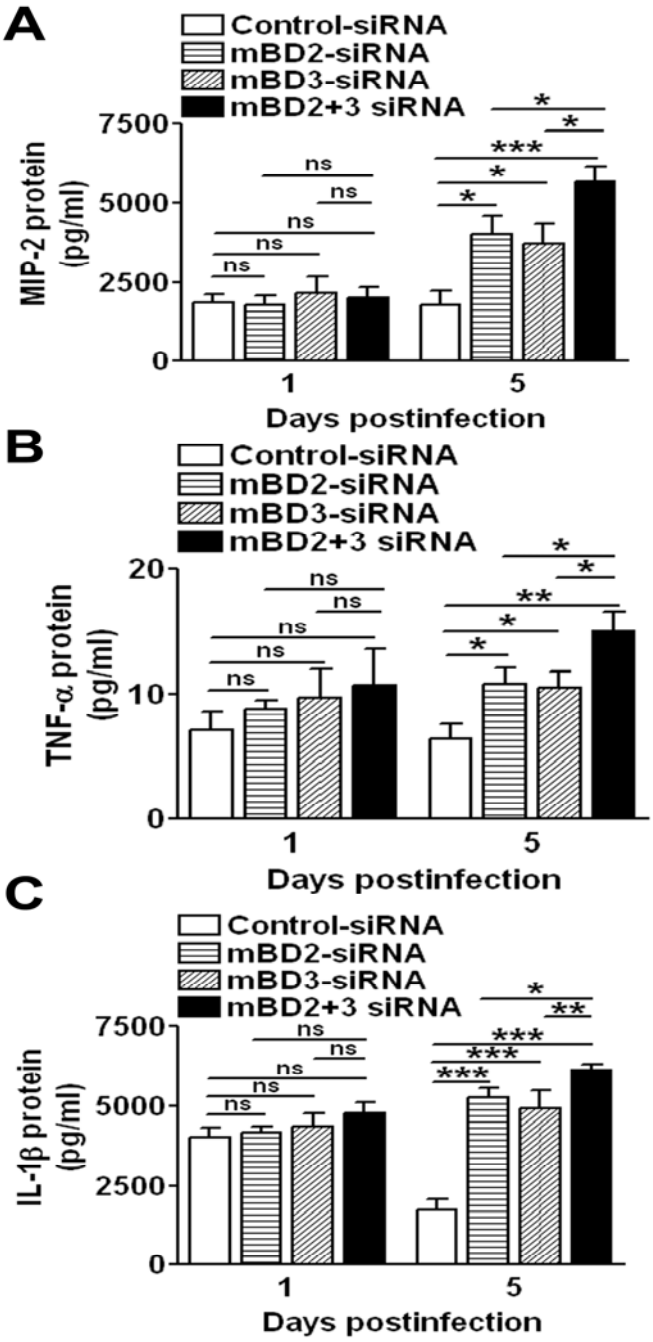

Figure 16. Protein expression levels after *P. aeruginosa* infection. Protein levels of MIP-2 (A), TNF-α (B), and IL-1β (C) were significantly up-regulated in siRNA mBD2, mBD3, and both defensins vs scrambled control-treated mice at 5 days p.i. Silencing both defensins also elevated protein levels of MIP-2, TNF-α, and IL-1β at 5 days p.i., when compared with silencing either defensin alone. Data are the means ± SEM and represent two individual experiments each with 5 animals/group/time. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by Student's t test; ns, not significant.

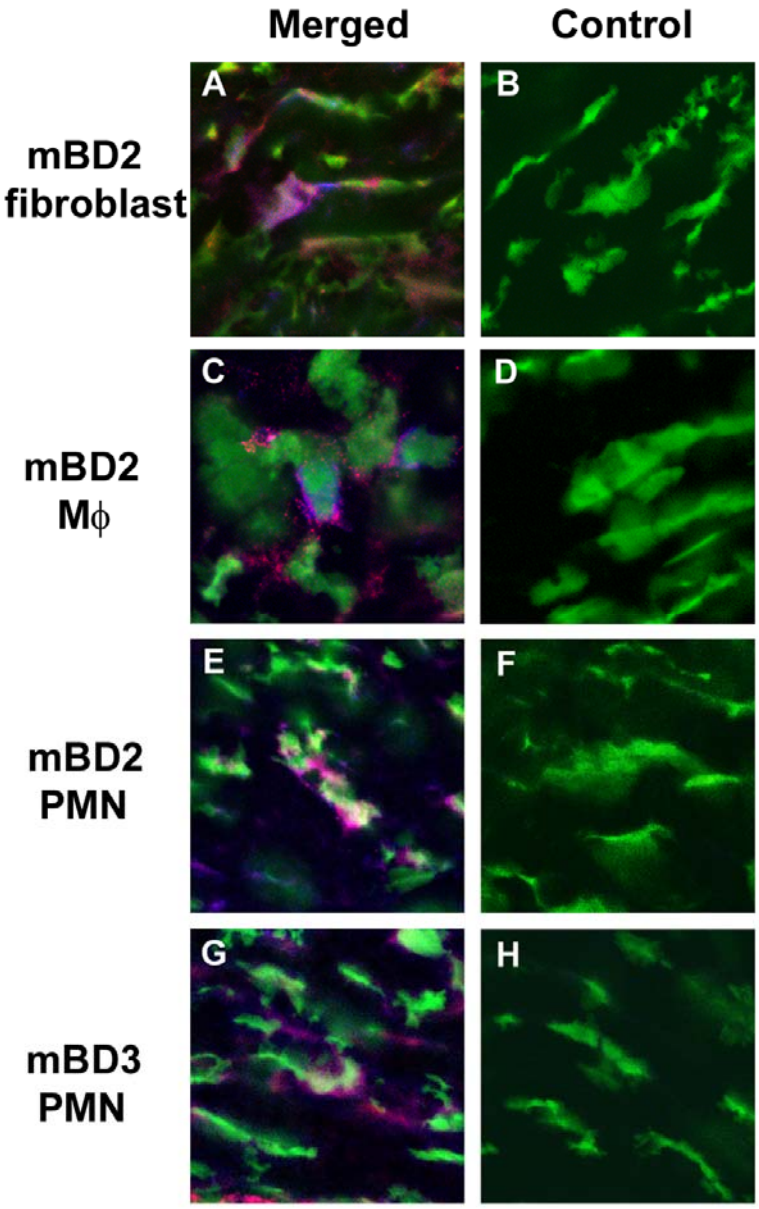

Figure 17. mBD2 and mBD3 staining in BALB/c infected stroma. mBD2-positive staining was found in fibroblasts (A), Mϕ (C), and PMN (E). PMN also stained positively for mBD3 (G). Controls (B, D, F, H), in which the primary Abs were replaced by the same host IgG, were negative for staining and appeared similar to SYTOX Green Nuclear staining (not shown). Magnification = x 500. Images shown are representative of two repeated experiments each with three mice per group.

Table 3. mRNA transcription of cytokines/molecules after silencing mBD2 or mBD3 or both at 5 days p.i.

(A) silencing mBD2

	Control siRNA	mBD2 siRNA	p-value
IFN-γ	0.75±0.29	2.22±0.42	0.01
MIP-2	0.35±0.07	0.75±0.11	0.01
TNF-α	0.39±0.11	1.04±0.12	<0.01
IL-1β	0.62±0.10	0.97±0.05	0.01
iNOS	0.25±0.05	1.09±0.09	<0.001
TLR4	1.03±0.16	1.63±0.14	0.01
TLR2	0.65±0.12	1.18±0.12	0.01
MyD88	1.24±0.12	1.96±0.15	<0.001
NF-κB	0.65±0.07	1.00±0.06	0.001
TLR5	2.73±0.28	2.21±0.27	0.2
TLR9	1.33±0.22	1.66±0.12	0.32

(B) silencing mBD3

	Control siRNA	mBD3 siRNA	p-value
IFN-γ	3.05±0.27	6.27±1.29	0.02
MIP-2	0.31±0.06	0.55±0.07	0.03
TNF-α	0.43±0.05	0.74±0.10	0.01
IL-1β	0.79±0.04	1.16±0.11	0.01
iNOS	1.08±0.08	0.64±0.04	<0.001
TLR4	1.02±0.02	1.23±0.09	0.04
TLR2	0.84±0.04	1.16±0.14	0.04
MyD88	1.78±0.07	2.06±0.02	<0.01
NF-κB	0.65±0.03	0.77±0.04	0.03
TLR5	2.28±0.12	2.08±0.16	0.34
TLR9	1.83±0.13	1.89±0.19	0.76

(C) silencing both defensins

	Control siRNA	mBD2+3 siRNA	p-value
IFN-γ	1.05±0.14	5.38±1.27	0.01
MIP-2	0.29±0.04	0.58±0.09	0.01
TNF-α	0.26±0.04	0.72±0.17	0.01
IL-1β	0.63±0.10	1.24±0.03	<0.001
iNOS	1.25±0.28	0.52±0.07	0.02
TLR4	0.73±0.11	1.43±0.12	0.001
TLR2	0.75±0.16	1.54±0.16	0.01
MyD88	1.23±0.17	2.68±0.15	<0.001
NF-κB	0.73±0.09	1.41±0.07	<0.001
TLR5	1.99±0.27	2.4±0.29	0.32
TLR9	1.24±0.11	1.44±0.12	0.33

CHAPTER 4

CONCLUSIONS

P. aeruginosa is a common Gram-negative bacteria associated with microbial keratitis, a disease frequently caused by contact lens usage. Significant evidence has been proved that the outcome of *P. aeruginosa*-induced keratitis is largely determined by the host immune response (Hazlett LD et al., 2004). Among many regulators, defensins play an important role in both innate and adaptive immunity by killing invading pathogens, chemoattracting immune cells as well as regulating production of pro-inflammatory cytokines. However, their role in the ocular immune defense system remains unknown. In this regard, our studies have provided important insights into their mechanisms to promote resistance against bacterial infection by using an experimental model of corneal infection with *P. aeruginosa* in susceptible B6 vs resistant BALB/c mice.

First, in Chapter 2 of this dissertation (This part of the work has been published: Wu M et al., 2009a), the role of mBD1 and mBD2 at the ocular surface was investigated. After infection, the expression levels of these defensins were both increased in the two murine strains, with a disparate pattern in BALB/c (more) vs B6 (less) corneas, indicating their potential role in resistance against bacterial infection. However, *in vivo* studies provided evidence that only mBD2 is required for host resistance against bacterial infection, and it functions to modulate bacterial load, PMN infiltration, as well as production of pro-inflammatory cytokines, iNOS, TLR signaling molecules, and NF-κB activation. The mechanisms leading to differences in host protection between the two defensins need to be further addressed, but it is highly likely

that the ability of mBD2 to inhibit an LPS-induced inflammatory response by competing for the same receptor, TLR4, may be of importance.

Furthermore, in Chapter 3 of this dissertation (This part of the work also has been published: Wu M et al., 2009b), we investigated the expression and function of mBD3 and mBD4, the murine homologs of HBD2 and HBD3, in susceptible B6 vs resistant BALB/c mice and whether either defensin interacted with mBD2, previously shown to be of importance in this disease (Wu M et al., 2009a). Our data provide evidence that mBD3 and mBD4 are inducibly and disparately expressed in BALB/c vs B6 corneal epithelium and stroma after *P. aeruginosa* infection. However, of the two, only mBD3 is required for host resistance and interacts with mBD2 to protect against bacterial infection. Additionally, *in vivo* studies have demonstrated the individual and combined effects of mBD2 and mBD3 on modulating bacterial load, PMN infiltration, and production of pro-inflammatory cytokines, TLR signaling molecules as well as NF-κB. Despite some distinct individual effects (e.g., iNOS induction), these two defensins act together to promote host resistance against corneal infection. To further understand the mechanisms involved, the cell sources of mBD2 and mBD3 were identified in the corneal stroma after infection. Among three major corneal stromal cell types, mBD2 was expressed in PMN, Mϕ and fibroblasts, while mBD3 was expressed in PMN only, which may contribute to the individual and combined effects of mBD2 and mBD3.

In summary, this dissertation has focused upon the role of mBDs in the *P. aeruginosa* infected mouse cornea. Based upon these data, among the most studied mBDs, only mBD2 and mBD3 are required for host resistance against *P. aeruginosa*-induced keratitis, and they act together to promote this resistance. The findings

presented herein indicate strong clinical relevance, which may suggest development and testing comparable targets for treatment of human diseases.

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ABSTRACT

DEFENSINS IN OCULAR IMMUNITY

by

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Corneal infection with *P. aeruginosa* results in corneal perforation in susceptible B6, but not resistant BALB/c mice. This study explored their role mBD 1-4 in corneal infection, and their potential synergy. Immunostaining and real-time RT-PCR data demonstrated that their expression was either constitutive (mBD1 and mBD2) or inducible (mBD3 and mBD4) in normal BALB/c and B6 corneas, and disparately regulated in BALB/c vs B6 corneas after infection. Knock down studies using siRNA treatment indicated that mBD2 and mBD3, but neither mBD1 nor mBD4, is required in ocular defense. Moreover, *in vivo* studies demonstrated individual and combined effects of mBD2 and mBD3 that modulate bacterial load, PMN infiltration, and production of pro-inflammatory molecules (e.g., IFN-γ, MIP-2, IL-1β, TNF-α), iNOS, as well as TLR signaling molecules (e.g., TLR2, TLR4, MyD88) and transcription factor NF-κB. Most notably, bacterial load was increased at 5 days p.i. by silencing either mBD2 or mBD3, but was elevated at both 1 and 5 days p.i. when silencing both defensins. PMN infiltration was increased at 1 day p.i. by silencing both defensins or mBD3, but not mBD2 alone. iNOS expression was elevated by silencing mBD2, but reduced after

silencing mBD3 or both defensins. Additionally, cell sources of mBD2 and mBD3 in corneal stroma were identified by dual label immunostaining after infection: PMN produce both defensins, whereas Mϕ and fibroblasts produce mBD2 but not mBD3. Collectively, the data provide evidence that mBD2 and mBD3 together promote resistance against corneal infection. The conclusions may be relevant to potential treatment of other ocular diseases, in addition to *P. aeruginosa* keratitis.

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