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β-Defensin-2 Promotes Resistance against Infection with P. aeruginosa

Minhao Wu, Sharon A. McClellan, Ronald P. Barrett, and Linda D. Hazlett

Corneal infection with Pseudomonas aeruginosa results in corneal perforation in susceptible C57BL/6 (B6) mice, but not in resistant BALB/c mice. To explore the role of two important defensins, murine β-defensin-1 (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 polymorphonuclear neutrophil 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, MIP-2, IL-1β, TNF-α, IL-6, and inducible NO synthase; 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.

Pseudomonas aeruginosa is a common Gram-negative bacteria associated with microbial keratitis, a disease frequently caused by contact lens usage (1). 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 (2).

Experimentally, P. aeruginosa challenge induces different response outcome in two defined inbred murine models: corneal perforation in susceptible C57BL/6 (B6) mice (Th1 responders) and corneal healing in resistant BALB/c mice (Th2 responders) (3). 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 (2, 3). 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 (4–9). In mice, the most studied defensins are murine β-defensin (mBD)1–1 and -2, which are chiefly expressed in a variety of epithelial cells (10, 11). Both mBD1 and mBD2 can directly kill invading pathogens (12, 13), while mBD2 also can regulate production of several inflammatory cytokines and chemokines (8, 14).

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, inducible NO synthase (iNOS), TLR signaling molecules, and NF-κB activation.

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 ×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 × 106 CFU of P. aeruginosa (American Type Culture Collection strain 19600), prepared as described before (15), was topically applied to the ocular surface. Eyes were examined at 1 day postinfection (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 on the Use of Animals in Ophthalmic and Vision Research.

Ocular response to infection

Corneal disease was graded using an established scale: (16) 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 (17) as well as studies in our laboratory (18). For the studies described herein, mBD1- and mBD2-specific siRNA or appropriate scrambled controls for each (Santa Cruz Biotechnology) were injected subconjunctivally (5 μl/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/mouse/time at a concentration of 4 μM), on the day of infection and twice on 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 (19).

Real-time RT-PCR

Total RNA was isolated from individual corneas 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 (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). Next, cDNA was amplified using SYBR Green reaction buffer (Invitrogen). Next, cDNA was amplified using SYBR Green PCR Master mix, 0.5 μl of cDNA (diluted 1/10), and diethyl pyrocarbonate water. Optimal conditions for PCR amplification of cDNA were established using routine methods (20, 21). 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), 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. Afterward, sections were incubated with primary Abs, goat anti-mouse mBD2 (M-17, 1/50; Santa Cruz Biotechnology), or rabbit anti-mouse mBD1 (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). 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).

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 epithelia were lysed and homogenized using a 1-ml glass tissue homogenizer in 1× sample buffer (2% SDS, 10% glycerol, 5% 2-ME). Debris was pelleted by centrifugation for 5 min at 7500 rpm, and protein concentration of the supernatant was determined by Quick Start Bradford protein assay (Bio-Rad). Ten micrograms of corneal protein sample or control peptide (each at 1 μg) for mBD1 (MBD11-P) or mBD2 (HBD21-P, being a mixture of three different peptides (14 aa from human BD2, 14 aa from rat defensin-2, and 17 aa from mouse BD2; Alpha Diagnostic International) was added to each respective lane, and proteins were separated on 10% acrylamide gels. The electrophotorethetically separated material was transferred to a supported polyvinyliden difluoride 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 mBD2 IgG Ab (HBD21A, generated from the three different peptides above, 1/500 diluted in TTBS containing 1% nonfat milk; Alpha Diagnostic International) or rabbit anti-mouse mBD1 (1/500 diluted in TTBS containing 1% nonfat milk; Alpha Diagnostic International) 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 International), and developed using the ECL method (ECL Plus; Amersham Biosciences) following the manufacturer’s protocol.

ELISA

Cytokine protein levels were selectively tested using ELISA kits (R&D Systems). 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

| Table 1. Nucleotide sequence of the specific primers used in PCR amplification |
|------------------|------------------|------------------|
| **Gene**        | **Primer Sequence (5′-3′)** | **Product size** |
| β-actin         | GAT TAC TGC TCT GCC TCC TAG C | F               |
| mBD1            | GAC TCA TCG TAC ACC TGC TGG T | R               |
| mBD2            | TCT CTC TCT GCC GCT GAT ATG C | F               |
| TLR2            | TCT CTC TAC AGC TCT GAC ACC C | R               |
| TLR4            | GCC TCT CAT CTT GGA TCC TAC C | F               |
| TLR9            | AGC TCA ACC TGT CTC TGA ACC GC | F               |
| IL-1β           | GGC AGC AGC ACA TCA ACA AGA GC | F               |
| MIP-2           | TGG CAA TGC CGA ACC TGG CGC | F               |
| IFN-γ           | ACC ACT CTT TCA GCA GAC TCA TGT C | F               |
| TNF-α           | GCA AGC TTC GCT CTA TCA TCT CAG | R               |
| iNOS            | GCC TCA GTG CTA CAG CAG CAG AGC | R               |
| MyD88           | GCC ACA ACT CTT GCA TGC TCT ACT GAA CTT CGG | F               |
| NF-κB           | GCT TAC TGC TCC TAG AT | R               |
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 instructions. 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 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 instructions. 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.

**Myeloperoxidase (MPO) assay**

An MPO assay was used to quantitate PMN numbers 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-Aldrich). Samples were freeze-thawed four times and centrifuged at 13,000 rpm for 10 min. The supernatant (0.1 ml) 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 × 10³ PMN (22).

**Griess reaction**

NO levels were determined by measurement of its stable endproduct, nitrite, using a Griess reagent (Sigma-Aldrich) 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), and protein concentration was determined by the 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 of total protein were determined in duplicate following the manufacturer’s instructions. 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.

**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. 1. 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. 1, 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, and mBD2: p = 0.03, p < 0.001, p < 0.001, at 1, 3, and 5 days p.i., respectively).

**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. 1. 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. 1, 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, and 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. 2, A–D). Relative integrated density values (Fig. 1, E and F) indicated that both mBD1 and mBD2 proteins were constitutively expressed in the two strains and were 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.

Additionally, 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. 2 and Fig. 3), 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. 2A–D), while more mBD1 appeared detectable in the infected corneas (most in corneal epithelium, Fig. 3A–D) of BALB/c over B6 mice ($p < 0.001$). Scrambled control-treated mice 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 three mice per group.

Silencing mBD2 and mBD1

Because the distribution patterns (mRNA and protein) suggested that 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 knockdown 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 whether 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. 4A). Representative slit lamp photographs at 7 days p.i. showed similar corneal opacity/disease in control, scrambled (Fig. 4B) vs siRNA-treated (Fig. 4C) mice. RT-PCR also confirmed that silencing was significant and specific for mBD1 and that mBD2 mRNA levels were not changed significantly (Fig. 4, 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 of 3/4), whereas all scrambled, control-treated corneas showed less opacity/disease (grade of 1/2). Clinical score data (Fig. 5A) 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. 5B) vs mBD2 (Fig. 5C) siRNA-treated mice are provided. Treatment with mBD2 siRNA resulted in either perforation (grade of 4, data not shown) or dense opacity covering the entire anterior segment.

**FIGURE 2.** 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 three mice per group.

**FIGURE 3.** 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 three mice per group.


that knockdown of mBD1 vs control treatment was effective at 7 days p.i. and showed a similar disease response. RT-PCR confirmed that knockdown 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 means ± SEM and represent two individual experiments each with five animals per group per time per assay.

(grade of 3, Fig. 5C) 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. 5, D and E).

**Effect of silencing mBD2 on plate count, MPO, and proinflammatory 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. 5F. Elevated bacterial counts were detected in mBD2 siRNA over control-treated corneas (p = 0.03 and p < 0.01 at 3 and 5 days p.i., respectively). Additionally, MPO activity was quantitated in infected corneas of the two groups at 3 and 5 days p.i. and results are shown in Fig. 5G. 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 proinflammatory cytokines, mRNA expression levels of several cytokines were analyzed by real-time RT-PCR in normal uninfected and infected corneas of mBD2 siRNA and control-treated BALB/c mice (Fig. 6). Overall, mBD2 siRNA treatment differentially modulated the expression of proinflammatory cytokines in infected corneas. At the mRNA expression level, IFN-γ (Fig. 6A) 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. 6B) and IL-1β (Fig. 6C) 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 and 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. 6E) and IL-6 (Fig. 6G) 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 = 0.03 and 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 means ± SEM and represent two individual experiments each with five animals per group per time per assay.

**FIGURE 4.** In vivo knockdown studies of mBD1 and mBD2 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. A, RT-PCR confirmed that knockdown 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 means ± SEM and represent two individual experiments each with five animals per group per time per assay.

**FIGURE 5.** 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 knockdown 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 and 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 means ± SEM and represent two individual experiments each with five animals per group per time per assay.
Moreover, mRNA expression levels of iNOS (Fig. 7A) 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 = 0.02, p < 0.001 at 1, 3, and 5 days p.i., respectively). Additionally, mBD2 siRNA treatment elevated the amount of nitrite detectable (Fig. 7B) 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 proinflammatory 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 selected TLR signaling molecules (including TLR4, TLR2, TLR9, and MyD88) and transcription factor NF-κB were evaluated by real-time RT-PCR, and the results are presented in Fig. 8. TLR4 (Fig. 8A) and TLR2 (Fig. 8B) 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 and TLR2: p < 0.01, 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. 8C) at any of the times tested. Both MyD88 (Fig. 8D) and NF-κB (Fig. 8E) mRNA expression levels were significantly increased at 1 and 3 days p.i., followed by an increase at 5 days p.i. when compared with controls. No difference was detected at 3 days p.i. when compared with controls.
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 and NF-κB: p < 0.01, p < 0.001, p < 0.001 at 1, 3, and 5 days p.i., respectively). Additionally, results from testing NF-κB activation (Fig. 8F) 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 and p = 0.05, respectively).

Discussion
As a family of antimicrobial peptides, β-defensins play an important role in both innate and adaptive immune defense (9, 23–25). They are chiefly expressed in a variety of epithelial cells (e.g., airway epithelia, urogenital tissues, nasolacrimal duct, and mammary gland) and sometimes immune cells (e.g., dendritic cells (DCs) and macrophages) (6, 8–14, 25). In mice, the best characterized β-defensins are mBD1 and mBD2. mBD1 is often constitutively expressed (10), whereas mBD2 expression is inducible by Gram-negative bacteria (e.g., P. aeruginosa) and their products (e.g., LPS) as well as various proinflammatory cytokines (e.g., TNF-α) (11). 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 (26). 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 were 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. Additionally, most mBD1- and mBD2-positive immunostaining was located in the corneal epithelium of both mouse groups, which is consistent with previous studies by others (26).

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 (10–13, 27, 28). Our in vivo knockdown 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 knockdowns by RT-PCR, as well as slit lamp and clinical scores, which visually showed little difference between mBD1 siRNA vs control-treated corneas. All of these data 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 selected proinflammatory cytokines/molecules and TLRs 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. Taken together, these results provided direct evidence that mBD2 promotes host resistance against P. aeruginosa corneal infection; however, the mechanism of mBD2-dependent protection in the eye remained to be determined.

In this regard, previous studies have revealed that BD2 not only has the capability to kill a variety of pathogens (especially Gram-negative bacteria) (4, 29) but also can induce immature DC maturation to trigger Th1 responses in vivo as well as proinflammatory cytokine production (8). It has also been demonstrated that mBD2 and LPS share the same receptor, TLR4, and activate the transcription factor NF-κB through a TLR cascade, leading to proinflammatory cytokine expression (8, 30–34). Our studies also revealed that in the eye, mBD2 can regulate proinflammatory 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 that plays a critical role in inflammation and Th1 responses (35, 36). MyD88 is a key adaptor molecule in all TLR signaling pathways except for TLR3 (37, 38), and NF-κB is the transcription factor in all TLR/MyD88-dependent signaling pathways whose activation leads to inflammatory cytokine production (38, 39). 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 (40) of BALB/c mice to a Th1-like response in mBD2-silenced BALB/c corneas.

On the other hand, for other proinflammatory 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 (19), because all of 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 proinflammatory 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. Additionally, 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 endothelial cell membranes (8, 37, 41, 42).

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 they are disparately expressed in resistant (more) vs susceptible (less) mice after P. aeruginosa corneal infection; that of the two defensins tested by knockdown experiments, only mBD2 is required for host resistance against bacterial infection; and that mechanistically, mBD2 functions to modulate the production of proinflammatory cytokines, iNOS, TLR signaling molecules, and NF-κB activation in the infected cornea. Based on these data, mBD2 may provide a promising target for treatment of ocular diseases, such as P. aeruginosa keratitis.

Disclosures
The authors have no financial conflicts of interest.
References


