# TLR4 Is Required for Host Resistance in *Pseudomonas aeruginosa* Keratitis

*Xi Huang, Wenjin Du, Sharon A. McClellan, Ronald P. Barrett, and Linda D. Hazlett*

**Purpose.** To determine the role of Toll-like receptor 4 (TLR4) in *Pseudomonas aeruginosa* (*P. aeruginosa*) keratitis in resistant (cornea-healing) BALB/c mice.

**Methods.** Corneal TLR4 mRNA levels were tested by real-time PCR in BALB/c mice before and after infection. Clinical score, slit lamp, histopathology, bacterial counts, and polymorphonuclear neutrophil (PMN) quantitation were performed in the infected cornea of TLR4-deficient ($TLR4^{lps\text{-}d}$) and wild-type BALB/c mice. mRNA for IL-1$\beta$, MIP-2, IFN-$\gamma$, IL-18, inducible nitric oxide synthase (iNOS), and $\beta$-defensin-2 levels were measured by real-time PCR. Protein levels for IL-1$\beta$, MIP-2, and IFN-$\gamma$ were tested by ELISA.

**Results.** In resistant BALB/c mice, TLR4 mRNA expression was significantly upregulated in the cornea after *P. aeruginosa* infection. In contrast, TLR4-deficient mice were susceptible to infection with *P. aeruginosa* and showed increased corneal opacity, PMN infiltration, bacterial counts, and perforated infected corneas. After infection, TLR4-deficient mice also showed increased mRNA expression of proinflammatory cytokines (IL-1$\beta$ and MIP-2) and type-1–associated cytokines (IFN-$\gamma$ and IL-18) when compared with wild-type BALB/c mice. ELISA analyses showed that IL-1$\beta$, MIP-2, and IFN-$\gamma$ protein levels also were significantly upregulated in the cornea of TLR4-deficient versus wild-type mice. In contrast, levels of iNOs and $\beta$-defensin-2 were significantly decreased in TLR4-deficient compared with wild-type mice.

**Conclusions.** TLR4 is critical in host resistance to *P. aeruginosa*, as its deficiency results in increased PMN infiltration and proinflammatory cytokine production, decreased iNOs and $\beta$-defensin-2 production, impaired bacterial killing, and a susceptible phenotype. (*Invest Ophthalmol Vis Sci.* 2006;47:4910–4916) DOI:10.1167/iovs.06-0537

Keratitis induced by *Pseudomonas aeruginosa* is a rapidly progressive and devastating corneal disease that leads to epithelial defects, stromal ulceration, scarring, and visual impairment.[1] Compelling evidence suggests that the innate immune response plays a critical role in disease,[2,3] and both bacterial (e.g., lipopolysaccharide [LPS]) and host factors released from infiltrating cells during infection contribute to a rapidly progressing liquefactive stromal necrosis.[4–6] Recent studies also have shown that epithelial cells actively participate in the host response to infection of both Gram-negative and -positive bacteria. This first line of defense is effected through recognition of pathogens by Toll-like receptors (TLRs) and subsequent expression and secretion of proinflammatory cytokines that recruit inflammatory cells in response to bacterial virulence factors.[7–9]

TLRs, a family of pathogen-recognition receptors (PRR), play an essential role in the innate immune response by detecting microbial products and triggering antimicrobial host responses.[10,11] TLR4, a classic member of the TLR superfamily, has been studied extensively in pathogen-mediated host responses and functions as a primary sensor to detect LPS, a component of Gram-negative bacteria such as *P. aeruginosa*. TLR4 activation induces secretion of proinflammatory molecules,[12,13] mainly chemokines and cytokines which amplify the response to infection. These include but are not limited to interleukin (IL)-1$\beta$, macrophage inflammatory protein (MIP)-2, IL-6, IL-12, IL-18, and interferon (IFN)-$\gamma$.[14]

In the eye, several groups have reported detecting the expression of TLRs, such as TLR2,[15] TLR3,[16] TLR4,[17] TLR5,[18] and TLR9,[19] in mouse cornea and cultured human corneal epithelial cells. LPS was used for challenge of cultured human corneal epithelial cells[2,20,21] and in an in vivo sterile keratitis mouse model.[15,17] In addition, in a recent report of a study in which gene array was used, expression of TLR4 and coreceptors including but not limited to CD14, IL-1R antagonist, and TLR6 were detected in *P. aeruginosa* keratitis in the mouse.[22] All the molecules were significantly elevated at the mRNA level in resistant BALB/c mice after ocular bacterial infection.[22] These data suggested an important immunomodulatory role for these molecules that could influence early as well as later events that occur in the disease response, resulting in the resistance phenotype. Despite the plethora of information that microbial infection activates the TLR4 signaling cascade and leads to expression of various proinflammatory cytokines and chemokines,[10] essential in the host defense against invading pathogens,[23] the direct role of TLR4 in *P. aeruginosa* keratitis remains unexplored.

In the present study, we began by investigating the expression and function of TLR4 in the normal BALB/c mouse cornea and after induction of bacterial keratitis. Our data show that mRNA expression of TLR4 was significantly upregulated in the cornea of BALB/c mice after bacterial infection and that TLR4 deficiency rendered a normally resistant mouse susceptible to *P. aeruginosa.* We provide evidence that susceptibility is brought about by several mechanisms, including: impaired bacterial killing and stasis, increased polymorphonuclear neutrophil (PMN) infiltration, enhanced proinflammatory cytokine and chemokine expression, and inhibition of the production of inducible (i)NOs and $\beta$-defensin-2 in the cornea.

## Methods

### Infection of the Corneas

Eight-week-old female BALB/c and TLR4-deficient ($TLR4^{lps\text{-}d}$) mice on a BALB/c background (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with ether and placed beneath a stereoscopic microscope at 40× magnification, and the cornea of the left eye was wounded with three 1-mm incisions with a sterile 25-gauge needle. A bacterial sus-

From the Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan.

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Corresponding author: Xi Huang, Department of Anatomy & Cell Biology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201; xhuang@med.wayne.edu.

**TABLE 1.** Primer Sequences for Real-Time PCR

| Gene | | Primer Sequences |
|---|---|---|
| TLR4 | Forward | CGC TTT CAC CTC TGC CTT CAC TAC AG |
| | Reverse | ACA CTA CCA CAA TAA CCT TCC GGC TC |
| IL-1$\beta$ | Forward | CGC AGC AGC ACA TCA ACA AGA GC |
| | Reverse | TGT CCT CAT CCT GGA AGG TCC ACG |
| MIP-2 | Forward | TGT CAA TGC CTG AAG ACC CTG CC |
| | Reverse | AAC TTT TTG ACC GCC CTT GAG AGT GG |
| IL-18 | Forward | GCC ATG TCA GAA GAC TCT TGC GTC |
| | Reverse | GTA CAG TGA AGT CGG CCA AAG TTG TC |
| IFN-$\gamma$ | Forward | GTT ACT GCC ACG GCA CAG TCA TTG |
| | Reverse | ACC ATC CTT TTG CCA GTT CCT CCA G |
| iNOs | Forward | TCC TCA CTG GGA CAG CAC AGA ATG |
| | Reverse | GTG TCA TGC AAA ATC TCT CCA CTG CC |
| mBD-2 | Forward | TCT CTG CTC TCT GCT GCT GAT ATG C |
| | Reverse | AGG ACA AAT GGC TCT GAC ACA GTA CC |
| $\beta$-Actin | Forward | GAT TAC TGC TCT GGC TCC TAG C |
| | Reverse | GAC TCA TCG TAC TCC TGC TTG C |

pension (5 $\mu$L) containing $1 \times 10^6$ colony-forming units (CFU)/$\mu$L of *P. aeruginosa* (ATCC strain 19660), prepared as described elsewhere,[3] was topically applied to the scarified cornea. Eyes were examined macroscopically at 1 day postinfection (PI) and/or at times described later in the article, 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

After bacterial infection, corneal disease was graded as described elsewhere[3]: 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 calculated for each group of mice ($n = 5$ per group per treatment) to express disease severity. Five mice from each group, together with a similar number of control animals were examined at 1 to 7 days PI, and slit lamp photography was used to illustrate the disease response.

## Real-Time PCR

Corneas removed from normal, uninfected mice and at 1, 3, 5, and 7 days PI, were immediately frozen in liquid nitrogen, homogenized (RNA STAT-60; Tel-Test, Friendsville, TX), and total RNA isolated per the manufacturer's instruction, as described before.[19] Then, 1 $\mu$g of total RNA was reverse transcribed to produce a cDNA template for PCR reaction. For real-time PCR amplification, 1 $\mu$L of each cDNA sample was used per 25 $\mu$L of PCR reaction. Sequences of primer sets for real-time PCR are shown in Table 1. PCR measurements were analyzed in duplicate in three independent runs (Smart Cycler System; Cepheid Inc., Sunnyvale, CA). Relative mRNA levels of TLR4, IL-1$\beta$, MIP-2, IL-18, IFN-$\gamma$, iNOS, and $\beta$-defensin-2 were calculated after normalization to $\beta$-actin.[22]

## Histopathology

For histopathology, whole infected eyes ($n = 3$/group) were enucleated from BALB/c and TLR4$^{lps\text{-}d}$ mice at 5 days PI, immersed in PBS, rinsed, and fixed in 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorenson's phosphate buffer (pH 7.4) (1:1:1) at 4°C for 3 hours. Eyes were rinsed with 0.1 M phosphate buffer, dehydrated in graded ethanols and propylene oxide, and then infiltrated and embedded in Epon-araldite. Thick sections (1.5 $\mu$m) were cut, stained, observed, and photographed as described before[24,25] with a photomicroscope (Axiophot; Carl Zeiss Meditec, Dublin, CA).

## Quantitation of PMNs

Samples were assayed for myeloperoxidase (MPO) activity as described before.[26] Corneas from BALB/c and TLR4$^{lps\text{-}d}$ mice ($n = 5$ per group per time) were collected at 3 and 5 days PI and homogenized in 1.0 mL of 50 mM phosphate buffer (pH 6.0) containing 0.5% HTAB (hexadecyltrimethylammonium bromide). Samples were freeze thawed three times and centrifuged at 14,000*g* for 10 minutes. Supernatant (0.1 mL) was added to 2.9 mL of the 50 mM phosphate buffer containing *o*-dianisidine dihydrochloride (16.7 mg/100 mL) and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm was monitored for 5 minutes (Helios-$\alpha$; Thermo Spectronics, Rochester, NY) and the results expressed as units of MPO per cornea. One unit of MPO activity = $\sim 2.0 \times 10^5$ PMNs.[27,28]

## Quantitation of Viable Bacteria in the Cornea

Bacteria were quantitated in the infected cornea of BALB/c and TLR4$^{lps\text{-}d}$ mice at 3 and 5 days PI ($n = 5$ per group per time point). Corneas were collected from both experimental groups and individually homogenized in sterile 0.9% saline containing 0.25% BSA. A 0.1 mL aliquot of the corneal homogenate was serially diluted 1:10 in sterile PBS-BSA. Serial 10-fold dilutions of the samples were plated on *Pseudomonas* isolation agar (Difco, Detroit, MI) in triplicate, and the plates were incubated overnight at 37°C. The number of viable bacteria in an individual cornea was determined by counting individual colonies on plates that contained the various dilutions. Results are reported as $\log_{10}$ number of CFU per cornea ± SEM.

## ELISA Analysis of Cytokines

Protein levels for proinflammatory cytokines and chemokines were quantitated using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), as described previously.[19] Infected corneas of BALB/c and TLR4$^{lps\text{-}d}$ mice ($n = 5$/group/time) were collected at 3 and 5 days PI and tested for IL-1$\beta$ and MIP-2 protein levels. Individual samples were homogenized with a glass pestle (Fischer, Itasca, IL) in 1.0 mL PBS with 0.1% Tween 20 and centrifuged at 13,000 rpm for 10 minutes. A 50-$\mu$L aliquot of the supernatant was assayed per the manufacturer's instruction. The reported sensitivity of these assays is $<3$ pg/mL for IL-1$\beta$ and $<1.5$ pg/mL for MIP-2.

## Capture ELISA for IFN-$\gamma$ Protein

Protein levels for IFN-$\gamma$ were measured by capture ELISA. The infected corneas of BALB/c and TLR4$^{lps\text{-}d}$ mice ($n = 10$ per group) were collected at 6 days PI and tested for IFN-$\gamma$ protein levels. A pooled sample (10 corneas per group) was homogenized with a glass pestle in 1.0 mL PBS with 0.1% Tween 20, centrifuged at 13,000 rpm for 10 minutes. An aliquot of the supernatant was used to quantitate IFN-$\gamma$ protein levels. For this, plates were coated with 100 $\mu$L/well of capture antibody (R&D Systems) overnight at room temperature. After the plates were washed three times with wash buffer (0.05% Tween-20 in PBS, pH7.4), they were blocked with 300 $\mu$L/well blocking buffer (PBS containing 1% BSA, 5% sucrose, and 0.05% sodium azide) for at least 30 minutes. After three washes with wash buffer, 100 $\mu$L/well standard recombinant mouse IFN-$\gamma$ (R&D Systems) or supernatant samples were added to the plates and incubated for 2 hours at room temperature. A biotinylated secondary antibody (100 $\mu$L/well) was added and incubated for 2 hours at room temperature after three washes. Then, 100 $\mu$L/well streptavidin/horseradish-peroxidase (HRP; 1:200 in PBS) was added to the plates and incubated for 20 minutes at room temperature after three washes. Finally, 100 $\mu$L/well substrate solution was added to the plates for 20 to 30 minutes, and 50 $\mu$L/well blocking solution (1 M $H_2SO_4$) was added and absorbance read at 450 nm.

## Statistical Analysis

An unpaired, two-tailed Student's *t*-test was used to determine statistical significance for real-time PCR, ELISA, clinical score, MPO, and bacterial plate counts. Data were considered significant at $P < 0.05$. All

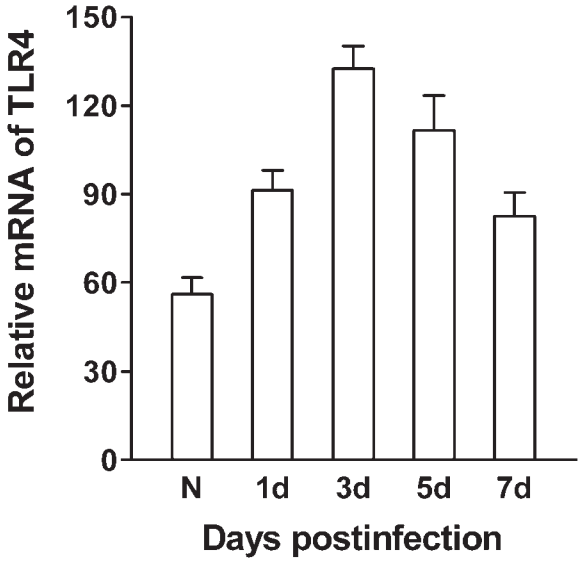


**FIGURE 1.** TLR4 expression in the cornea of resistant BALB/c mice. TLR4 mRNA expression levels (normalized to β-actin) in normal uninfected and infected cornea of BALB/c mice at 1 to 7 days PI were tested by real-time PCR. When compared with normal cornea (N), TLR4 mRNA levels were significantly upregulated after bacterial infection. ($P < 0.001$, $< 0.0001$, $< 0.0001$, and $< 0.01$ at 1, 3, 5, and 7 days PI, respectively).

experiments were repeated at least once to ensure reproducibility, and the data from a single typical experiment are shown.

## RESULTS

### TLR4 Expression in *P. aeruginosa*–Infected Cornea

To determine whether TLR4 is present in the normal cornea of BALB/c mice and changes after corneal infection, we tested the TLR4 mRNA expression (Fig. 1) in the cornea of normal uninfected and *P. aeruginosa*–infected BALB/c mice by real-time PCR. We found that TLR4 was constitutively expressed in the uninfected normal cornea of these mice and that mRNA levels were significantly upregulated versus normal cornea at 1 to 7 days PI ($P < 0.001$, $< 0.0001$, $< 0.0001$, and $< 0.01$ at 1, 3, 5, and 7 days PI, respectively) peaking at 3 days PI. These data indicate that TLR4 is upregulated at the gene level in bacterial keratitis.

### A Protective Role of TLR4 in Host Defense against Corneal Infection

Because TLR4 mRNA expression was detected and upregulated in the cornea of infected BALB/c mice, the next series of in vivo studies investigated whether TLR4 was critical for host resistance against bacterial infection. Corneas of $TLR4^{lps\text{-}d}$ and wild-type BALB/c mice were challenged with *P. aeruginosa*, and corneal disease was compared between the two mouse groups. Clinical score data (Fig. 2A) showed that disease was significantly increased and that corneas perforated in $TLR4^{lps\text{-}d}$ versus wild-type mice ($P < 0.001$, $< 0.0001$, and $< 0.0001$ at 1, 3, and 5 days PI). A representative slit lamp photograph at 5 days PI revealed more corneal opacity in the $TLR4^{lps\text{-}d}$ (Fig. 2B) versus wild-type (Fig. 2C) mouse. To further confirm these data, we enucleated eyes from both mouse groups for histopathology at 5 days PI. $TLR4^{lps\text{-}d}$ mice (Fig. 2D) exhibited a more swollen cornea, numerous infiltrating cells in the corneal stroma and anterior chamber, and perforation. In contrast, the cornea of wild-type mice (Fig. 2E) was less swollen, exhibited a relatively intact epithelium, showed fewer infiltrated inflammatory cells in the stroma and anterior chamber, and did not perforate. These results clearly demonstrate that $TLR4^{lps\text{-}d}$ mice are susceptible to corneal infection by *P. aeruginosa*.

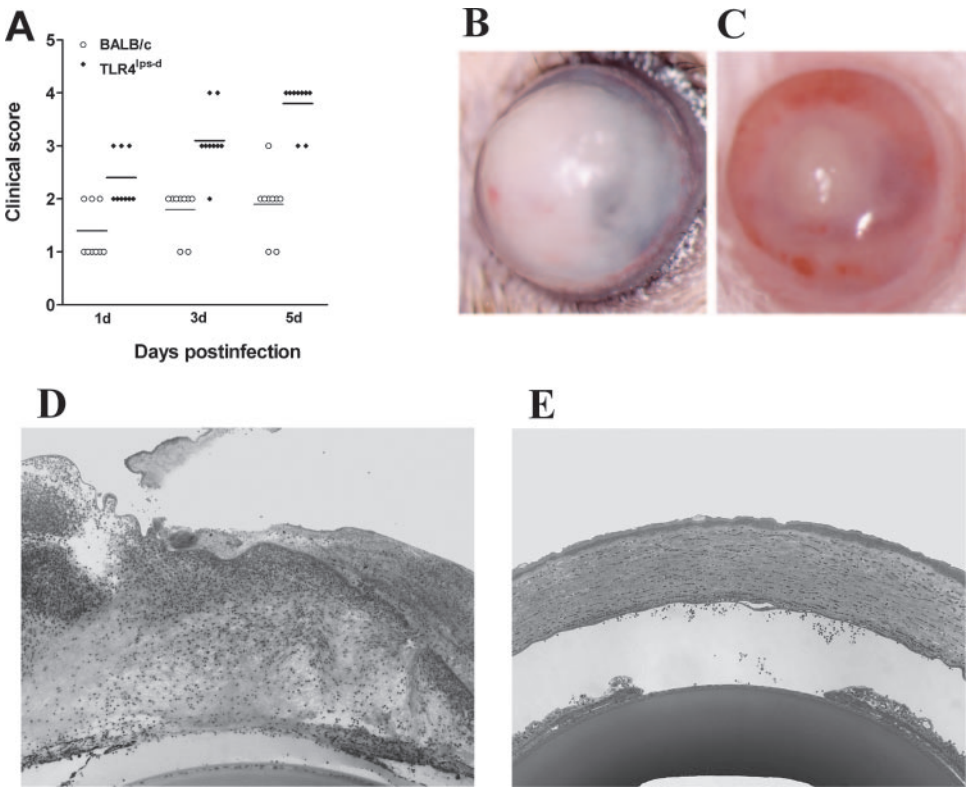


**FIGURE 2.** In vivo studies of TLR4 in host resistance. Clinical score (**A**) shows worse disease and perforated corneas in $TLR4^{lps\text{-}d}$ versus wild-type control mice ($P < 0.001$, $< 0.0001$, and $< 0.0001$ at 1, 3, and 5 days PI, respectively). Slit lamp at 5 days PI (**B**, **C**) shows more opacity in $TLR4^{lps\text{-}d}$ (**B**) versus control (**C**) cornea. Histopathology at 5 days PI shows that the cornea of $TLR4^{lps\text{-}d}$ mice (**D**) was more swollen, contained numerous infiltrating cells in the stroma and anterior chamber, and was perforated, whereas the cornea of control mice (**E**) had more intact cytoarchitecture, less swelling, and fewer infiltrated cells. Magnification: (**B**, **C**) ×25; (**D**, **E**) ×40.

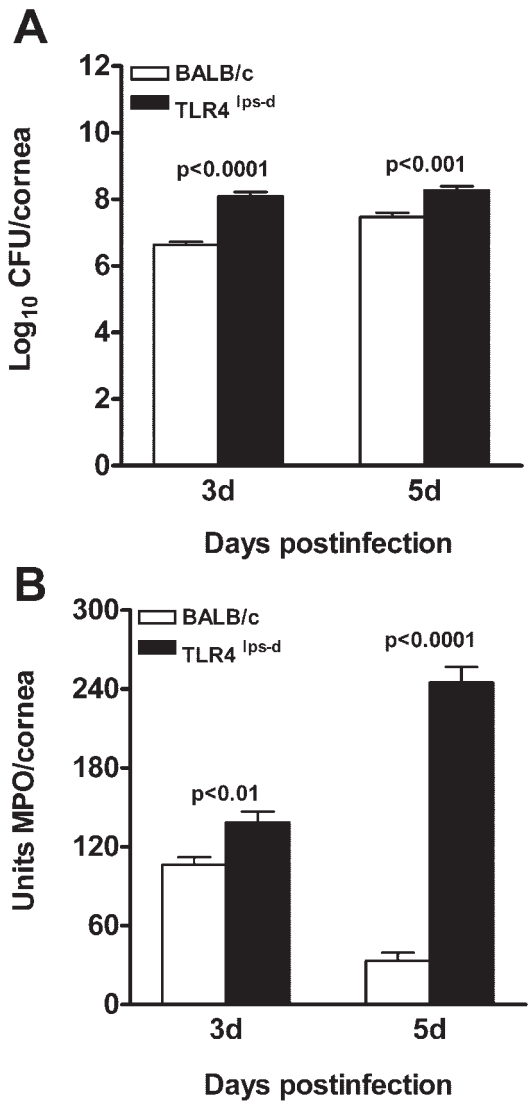


**FIGURE 3.** Bacterial load and PMN infiltration of the cornea of infected mice. Bacterial load (**A**) in the cornea at 3 and 5 days PI was reported as $\log_{10}$ the number of viable bacteria per cornea ± SEM. Significantly increased bacterial counts were observed in the cornea of $TLR4^{lps\text{-}d}$ versus wild-type control mice. A significantly increased recruitment of PMN (MPO activity) was also detected in the cornea of $TLR4^{lps\text{-}d}$ versus control mice at 3 and 5 days PI.

## Bacterial Killing, PMN Infiltration, and Cytokine Production

Because TLR4 deficiency confers susceptibility to corneal infection, we next investigated the mechanisms involved. To determine whether TLR4 deficiency impairs bacterial killing, viable bacterial counts were performed. Bacterial load (Fig. 3A) was significantly upregulated (approximately 1 log increase, $P < 0.0001$ and $< 0.001$) at 3 and 5 days PI in the cornea of $TLR4^{lps\text{-}d}$ versus BALB/c mice. We also tested the effect of TLR4 deficiency on PMN infiltration by quantitation of MPO activity in the cornea (Fig. 3B). A significantly increased recruitment of PMN ($P < 0.01$ and $< 0.0001$ at 3 and 5 days PI, respectively) was detected in the corneas of $TLR4^{lps\text{-}d}$ versus wild-type mice challenged with *P. aeruginosa*. In addition, TLR4 deficiency resulted in a significant increase in mRNA levels for IL-1$\beta$ (Fig. 4A, $P < 0.001$, $< 0.01$, and $< 0.05$ at 1, 3, and 5 days PI, respectively) and MIP-2 (Fig. 4B, $P < 0.05$, $< 0.05$, and $< 0.001$ at 1, 3, and 5 days PI, respectively) when compared with levels of these cytokines in the wild-type mouse cornea. The mRNA data were confirmed by ELISA analysis, which showed that protein levels for IL-1$\beta$ (Fig. 4C, $P < 0.01$ and $< 0.001$ at 3 and 5 days PI, respectively) and MIP-2 (Fig. 4D, $P < 0.0001$ and $< 0.001$ at 3 and 5 days PI, respectively) also were significantly upregulated in the cornea of $TLR4^{lps\text{-}d}$ versus wild-type mice. To further investigate the immunoregulatory role of TLR4 in bacterial keratitis, we also tested type-1–related cytokines in the infected corneas. Significantly elevated mRNA expression levels for IFN-$\gamma$ (Fig. 5A, $P < 0.01$ and $< 0.01$ at 3 and 5 days PI, respectively) and IL-18 (Fig. 5C, $P < 0.01$, $< 0.01$, and $< 0.01$ at 1, 3, and 5 days PI, respectively) were detected in the cornea of $TLR4^{lps\text{-}d}$ versus BALB/c mice challenged with *P. aeruginosa* Capture ELISA also was used to detect IFN-$\gamma$ protein at 6 days PI ($P < 0.01$). A significantly enhanced level of IFN-$\gamma$ protein in $TLR4^{lps\text{-}d}$ versus BALB/c cornea confirmed the mRNA data. Taken together, these results show that TLR4 deficiency impairs bacterial clearance and leads to increased PMN infiltration and upregulation of proinflammatory cytokine and chemokine production.

## Impaired iNOS and $\beta$-Defensin-2 Production in TLR4-Deficient Mice

Because TLR4 deficiency reduced bacterial killing and resulted in enhanced bacterial growth in the cornea with enhanced PMN infiltration, we next tested the possible mechanisms involved in bacterial clearance. After corneal infection, mRNA levels for iNOS at 1, 3, and 5 days PI (Fig. 6A, $P < 0.05$, $< 0.05$, and $< 0.01$, respectively), and defensin-$\beta$-2 at 3 and 5 days PI (Fig. 6B, $P < 0.05$ and $< 0.05$, respectively), were significantly reduced in the cornea of $TLR4^{lps\text{-}d}$ versus BALB/c mice. These results indicate that the impaired expression and activation of antimicrobial molecules such as NO and antimicrobial peptides such as defensin-$\beta$-2 may contribute to increased bacterial growth in the TLR4-deficient cornea.

## DISCUSSION

Innate immunity provides the first line of host defense against bacterial growth and spread in the early phase of microbial invasion.[19,29] TLRs initiate innate immunity through recognition of microbial products and activation of stress-activated protein kinases and transcription factors, which lead to the expression of immune and proinflammatory genes.[30] At least 10 human and 13 murine TLRs have been identified so far.[7] Among the best characterized TLRs are TLR3,[16] TLR4,[31] TLR5,[18] and TLR9,[32] that sense dsRNA,[33] LPS,[34] flagellin,[35] and CpG-DNA,[19] respectively. Although TLR4 was the first defined pathogen recognition receptor and has been studied extensively, its functional role remains incompletely defined in bacterial keratitis. Results presented herein revealed that murine TLR4 is constitutively expressed in the uninfected normal corneas of resistant BALB/c mice and that, after *P. aeruginosa* infection, its corneal mRNA levels were significantly upregulated. These data, indicating that TLR4 signaling is activated and involved in bacterial keratitis, are consistent with data obtained from in vitro studies of human corneal cells, which showed that protein for TLR4 was constitutively expressed and augmented after LPS stimulation.[2] Data also are consistent with a previous study showing that TLR4 activation was detected in the cornea using a sterile keratitis model and LPS from *P. aeruginosa*. In that study, Johnson et al.[15] examined mRNA expression of TLR2, TLR4, and TLR9 in corneal epithelial sheets of C57BL/6 (B6) mice, and demonstrated that the corneal epithelium has functional TLR2, TLR4, and TLR9 activa-

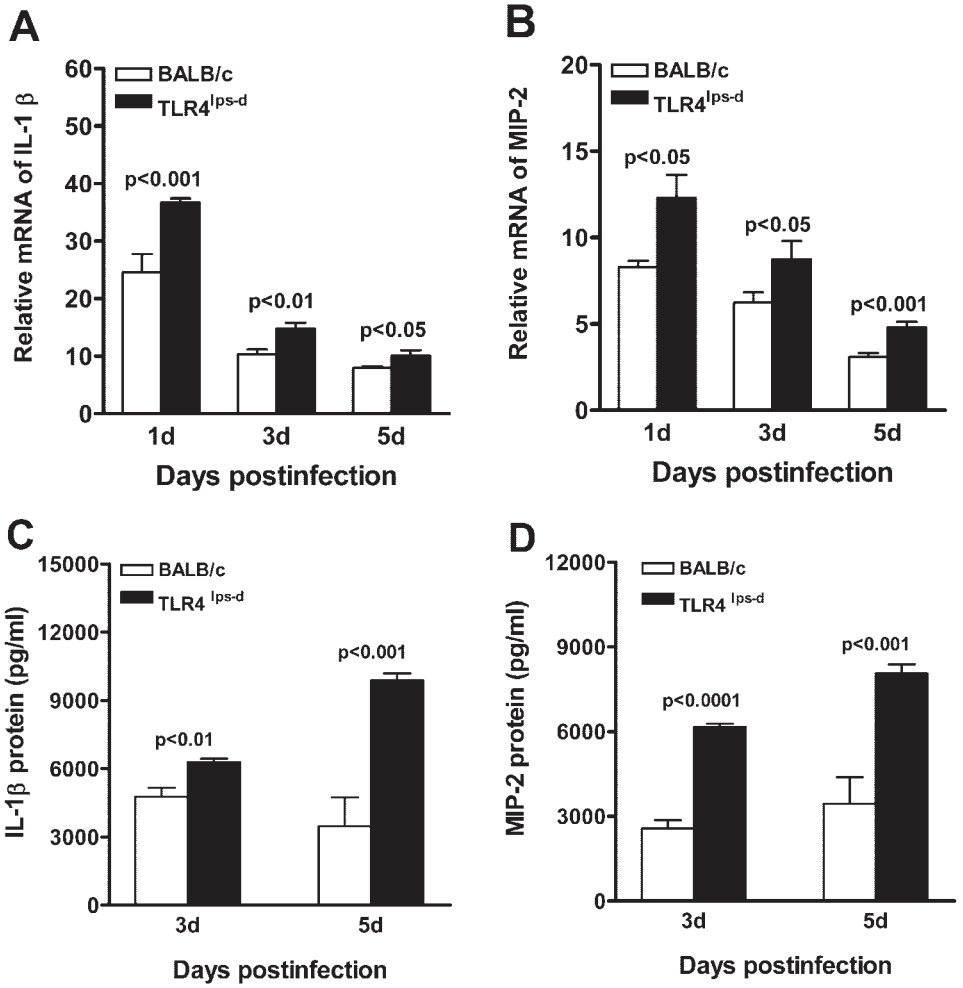


**FIGURE 4.** Proinflammatory cytokine and chemokine production in the cornea. Quantification of real-time PCR showing that mRNA levels for IL-1$\beta$ (**A**) and MIP-2 (**B**), and of ELISA assay of protein levels showing that IL-1$\beta$ (**C**) and MIP-2 (**D**) were significantly upregulated in the corneas of TLR4$^{lps-d}$ versus wild-type control mice after infection.

tion when stimulated with Pam3Cys (TLR2 ligand), LPS (TLR4 ligand), and CpG-DNA (TLR9 ligand), respectively. Activation of these three TLRs caused a significant increase in corneal thickness and haze through a MyD88-dependent signaling pathway. Khatri et al.[17] also examined the role of TLR4 in a sterile keratitis model using TLR4-deficient mice challenged with LPS. When C3H/HeJ (TLR4 point mutation) versus C3H/HeN (control) mice were treated with LPS, a significant increase in stromal thickness and haze was seen in the cornea of the control, but not in TLR4 mutant mice, and the severity of disease coincided with PMN stromal infiltration. Data from these sterile keratitis models clearly provided the inference that TLR4 has a crucial role in the pathogenesis of bacteria-induced cornea disease.

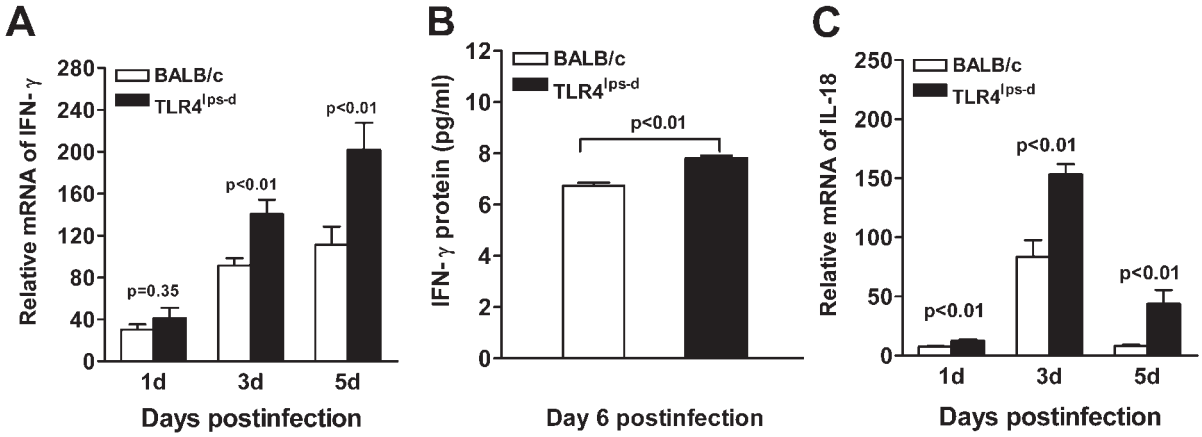


**FIGURE 5.** Production of IFN-$\gamma$ and IL-18 in infected cornea. IFN-$\gamma$ mRNA expression levels (**A**) at 1, 3, and 5 days PI by real-time PCR, and protein levels (**B**) at 6 days PI by ELISA, were significantly elevated in the cornea of TLR4$^{lps-d}$ versus wild-type control mice. IL-18 (**C**) mRNA expression levels also were markedly upregulated at 1, 3, and 5 days PI in TLR4$^{lps-d}$ versus control mice.

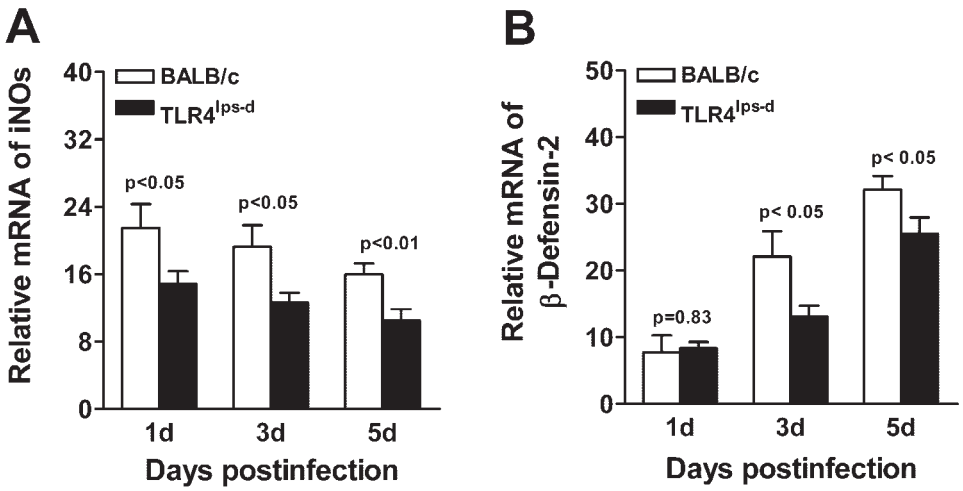


**FIGURE 6.** Reduction of iNOS and defensin-β-2 in infected cornea. mRNA expression levels of iNOS (**A**) and defensin-β-2 (**B**) were significantly downregulated at 1, 3, and 5 days PI (iNOS) and at 3 and 5 days PI (defensin-β-2) in the infected cornea of $TLR4^{lps-d}$ versus wild-type control mice.

In our study, TLR4 mRNA expression was markedly increased in the cornea of resistant BALB/c mice after bacterial infection. These data led us to test corneas from TLR4-deficient and wild-type control (BALB/c) mice after challenge with *P. aeruginosa*, to determine the role of TLR4 in bacterial keratitis. Given that TLR4 deficiency was suggested to be protective in a model of sterile keratitis,[15,17] we might predict that corneas of TLR4-deficient mice would be less susceptible and exhibit a decreased inflammatory response to bacterial infection. In marked contrast, TLR4-deficient versus control mice exhibited significantly increased inflammation and corneal perforation instead of healing after infection. Furthermore, data from clinical scoring, slit lamp observations, and histopathology confirmed that TLR4-deficient versus wild-type control mice exhibited significantly increased corneal disease with more opacity and more severe stromal swelling and destruction. In addition, bacterial load (>10-fold higher) and PMN recruitment (MPO activity) were markedly upregulated in the infected cornea of TLR4-deficient versus control mice. These findings are consistent with those in a previous study that provided evidence that TLR4-deficient mice are more susceptible to pulmonary tuberculosis and exhibited increased mycobacterial outgrowth and more cellular infiltrate than controls.[36] Our data provide strong evidence that TLR4 is essential for the resistance response of BALB/c mice to *P. aeruginosa* challenge and, unlike the sterile keratitis model, that TLR4 is necessary for disease resolution.

All in all, it appears contradictory that TLR4 is critical in the pathologic course of corneal disease in sterile keratitis, whereas it is protective in bacterial keratitis and is necessary for host resistance. In fact, these data illustrate the characteristics of a double-edged sword mechanism of action of TLR4 activation. On the one hand, in our model, TLR4 recognized an endotoxin (LPS), a component of *P. aeruginosa*, and initiated an innate immune response that was essential for bacterial clearance.[30] TLR4 deficiency impaired bacterial clearance, led to overgrowth of bacteria, overwhelming infiltration of PMNs, and excessive proinflammatory cytokine production. These effects in turn contributed to corneal destruction and perforation. On the other hand, in the sterile keratitis model, activation of LPS-TLR4 signaling leads to proinflammatory cytokine production and PMN infiltration, which increases stromal thickness and contributes to haze production and increases, albeit transiently, corneal perturbation.[17]

Innate immunity leads to proinflammatory cytokine production and PMN infiltration and induces expression of antimicrobial peptides that may contribute to bacterial clearance. In this regard, data presented in this study showed that expression levels of proinflammatory cytokines and chemokines such as IL-1β, MIP-2, IFN-γ, and IL-18 were significantly increased in the cornea of TLR4-deficient versus wild-type control mice. Generally, IL-1β and MIP-2 are chemoattractants for PMN infiltration, and prolonged PMN infiltration, routinely seen in the B6 susceptible response, has been shown to contribute to corneal immunopathology.[26] In addition, IL-18 often induces costimulation of IFN-γ production by synergizing with IL-12 to drive Th1-like responses.[24] IFN-γ plays a critical role in inflammation and regulates the antimicrobial potential of macrophages by upregulation of MHC class I and class II protein expression, enhanced production of macrophage-derived mediators such as TNF-α, IL-1, IL-6, IL-12, and IL-18, and downregulation of the synthesis of anti-inflammatory mediators such as IL-10.[29] TLR4 deficiency enhanced the production of corneal proinflammatory cytokines such as IFN-γ, which results in detrimental side effects contributing to corneal destruction and breakdown of stromal proteins. This in turn could contribute to an enhanced nutrient source for the bacteria in the cornea and lead to an increase in bacterial count, as we have shown in this study.

Furthermore, mRNA expression levels for iNOS and β-defensin-2 were markedly reduced in the infected cornea of TLR4-deficient versus control mice. This finding is consistent with those of a previous study in this laboratory that showed that iNOS is constitutively expressed in the resistant BALB/c mouse cornea and that iNOS-derived NO is required for bacterial killing/stasis in *P. aeruginosa* keratitis.[37] In addition, this is the first study to show that the expression of β-defensin-2, an antimicrobial peptide, is impaired in TLR4-deficient mice and agrees with in vitro studies in human corneal epithelial cells, where expression of β-defensin-2 was TLR2 and TLR4 dependent.[9,21] Defensin-β-2, a small cationic peptide, is synthesized by various epithelial cells, such as those in the skin,[38] the respiratory tract,[39] and the intestine.[40] Its effects are exerted by damaging bacterial cell membranes, especially in low salt conditions. However, in salty solutions such as in tears and serum, defensins effects are impaired and in addition, high concentrations are often toxic.[41] Together, these pose obvious problems when trying to develop defensins for use as a topical antimicrobial agent. The latter was not examined in the present study.

In summary, the data presented herein demonstrate that TLR4 is constitutively produced and activated in the cornea of resistant BALB/c mice after bacterial infection. We also present direct evidence that TLR4 deficiency confers susceptibility to bacterial infection by impairing bacterial killing and stasis, increasing PMN infiltration, enhancing proinflammatory cyto-

kine/chemokine expression, and inhibiting iNOS and β-defensin-2 production in cornea. These data suggest that TLR4 plays a protective role in the corneal defense against bacterial infection. Elucidating the mechanism of action of TLR4 in *P. aeruginosa* keratitis may provide a better therapeutic approach to the control of this disease.

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