Activation of Toll-Like Receptor (TLR)2, TLR4, and TLR9 in the Mammalian Cornea Induces MyD88-Dependent Corneal Inflammation

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Purpose. Toll-like receptors (TLRs), which recognize microbial products, have an important role in the host innate immune response. The purpose of the present study was to determine whether activation of these receptors leads to development of keratitis and to assess the role of the common adaptor molecule myeloid differentiation factor-88 (MyD88).

Methods. Corneal epithelium of C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$, and MyD88$^{-/-}$ mice was abraded and treated with Pam_3Cys, LPS, or CpG DNA, which bind TLR2, -4, and -9, respectively, and neutrophil recruitment to the corneal stroma, development of corneal haze, and chemokine production were measured.

Results. Activation of TLR2 and -9 stimulated neutrophil recruitment to the corneal stroma of C57BL/6 mice, but not TLR2$^{-/-}$ or -9$^{-/-}$ mice, respectively. In marked contrast, neutrophil migration to the corneal stroma of MyD88$^{-/-}$ mice challenged with Pam_3Cys, LPS, or CpG DNA was completely ablated. Activation of TLR2, -4, and -9 also caused a significant increase in corneal thickness and haze, indicative of disruption of corneal clarity; however, this response was ablated in MyD88$^{-/-}$ mice, which were not significantly different from untreated corneas. Production of CXC chemokines MIP-2 and KC, which mediate neutrophil recruitment to the corneal stroma, was elevated in the corneal epithelium and stroma of control, but not MyD88$^{-/-}$ mice.

Conclusions. Together, these findings demonstrate that the corneal epithelium has functional TLR2 and -9, and that TLR2, -4, and -9 signal through MyD88. This pathway is therefore likely to have an important role in the early events leading to microbial keratitis. (*Invest Ophthalmol Vis Sci.* 2005;46: 589–595) DOI:10.1167/iovs.04-1077

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Epithelial cells that comprise the outer layer of the mammalian cornea function as a critical barrier of the eye to the external environment. These cells therefore have an important role in protecting the visual axis from microbial pathogens and from microbial products on the ocular surface. Physical or chemical injury to the corneal surface can compromise the integrity of the epithelial barrier and allow entry of live organisms and microbial products to underlying epithelium and to the stroma, where they can induce an inflammatory response that can lead to visual impairment and possibly blindness.

Toll-like receptors play an important role in the innate immune response to invading pathogens.[1,2] At least 11 toll-like receptors (TLRs) have been identified to date that recognize ligands from viral, bacterial, protozoan, and helminth pathogens.[2–6] Bacterial products activate the TLR family of proteins, including bacterial lipopolysaccharide (LPS), characteristic of Gram-negative bacteria, of which most forms are recognized by TLR4[7–9]; bacterial peptidoglycan, more highly represented in Gram-positive bacteria, which is recognized by TLR2[10–12]; and unmethylated DNA containing CpG motifs, present in Gram-positive and -negative bacteria, which binds TLR9.[13]

TLRs signal through several adaptor molecules, including the common adaptor protein myeloid differentiation factor (MyD)88.[14,15] These adaptor molecules are thought to provide a platform for binding of kinases such as IRAK-1 and -4 that ultimately lead to NF-κB translocation to the nucleus and transcription of proinflammatory and chemotactic cytokines.[1,2,14,16,17] Although TLR2 and -9 signal through MyD88, TLR4 also signals through a MyD88-independent pathway that involves the TIR domain containing adaptor inducing IFN-β (TRIF) and the IRF-3 transcription factor.[15,18–20]

Whereas TLR4 and -5 are expressed in human corneal epithelial cells[21,22] and TLR4 activation induces corneal inflammation in mice,[23] the presence and functional significance of other TLRs and the role of MyD88 in the cornea has yet to be determined. Therefore, in the present study, we used TLR2$^{-/-}$, TLR9$^{-/-}$, and MyD88$^{-/-}$ mice to examine their role in the development of corneal inflammation. Our findings demonstrate that epithelial cells express functional TLR2 and -9 in addition to TLR4, and that keratitis induced by these receptors is entirely dependent on the presence of MyD88.

Materials and Methods

Source of Animals and Reagents

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, and MyD88$^{-/-}$ mice were kindly provided by Shizao Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR9$^{-/-}$ were fully backcrossed to C57BL/6 mice, whereas MyD88$^{-/-}$ mice were backcrossed for six generations, and wild-type littermates were used as the control. TLR9 agonist CpG ODN (1826, 5′-TCC ATG A*CG* TTC CTG A*CG* TT-3′) and control, non-CpG oligonucleotide (1911, 5′-TCC A*GG* A*CT* TTC CT*C* AG*G* TT-3′) were obtained from

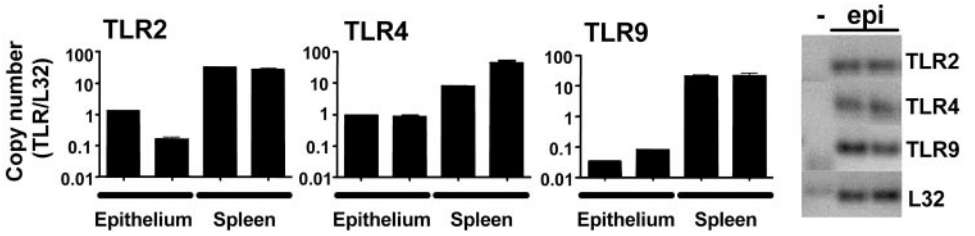

FIGURE 1. TLR expression in normal corneal epithelium. Corneas were dissected from naïve C57BL/6 mice, and epithelial sheets were separated after incubation with Na EDTA. Two groups of five epithelial sheets were pooled, RNA was extracted and reverse transcribed, and the number of TLR2, -4, and -9 transcripts was determined by quantitative PCR and presented as a ratio to the expression of the L32 housekeeping gene. TLR transcripts in splenocytes were calculated for comparison. For corneal epithelial sheets, PCR was continued for 40 cycles, and products were visualized after agarose gel electrophoresis. The no-template control (−) and TLR expression in two separate pools of epithelial sheets (epi) are shown.

Operon, Qiagen (Alameda, CA). The synthetic bacterial lipopeptide Pam_3Cys-Ser-$(Lys)_4$ (Pam_3Cys), which is a ligand for TLR2,[24,25] was purchased from EMC Microcollections (Tübingen, Germany). TLR4 agonist LPS from *Pseudomonas aeruginosa* serotype 10 (99.7% purity) was purchased from Sigma-Aldrich (St. Louis, MO). TLR4 specificity of this compound was confirmed using human embryonic kidney cells transfected with TLR2 or -4.

Animal Model of Keratitis

Six- to 10-week-old C57BL/6 mice from The Jackson Laboratory were anesthetized by intraperitoneal injection of 0.4 mL 2,2,2-tribromoethanol (1.2%), and the central corneal epithelium was abraded with three contiguous scratches made with a 26-gauge needle, and Pam_3Cys, LPS, or CpG DNA was applied to the surface, as described.[23] Histologic examination of the abraded cornea showed that the wound was limited to the epithelial layer. Dose–response studies (not shown) indicated that 20 μg CpG, 20 μg LPS, and 5 μg Pam_3Cys induced maximum levels of corneal inflammation. The animals used in these studies were raised in specific pathogen-free conditions in microisolater cages and were treated in accordance with the guidelines provided in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Quantitative Reverse Transcription–PCR

Corneas were carefully dissected to ensure that conjunctival and iris tissues were not included. Tissues were then placed in 30 μL RNA stabilization reagent (RNAlater; Qiagen, Valencia, CA) and later incubated in 250 μL 20 mM EDTA (sterile, pH 7.4) at 37°C for 30 minutes. The epithelial layer was then teased from the stromal layer, placed in 1 mL extraction reagent (TRIzol; Invitrogen Corp., Carlsbad, CA), homogenized by passing three times through a 26-gauge needle, and RNA was extracted according to the manufacturer's directions.

For reverse transcription (RT), 2 μg oligo dT (Midland Certified Reagent Co., Inc., Midland, TX) was added to the RNA (2 μg sample) and incubated at 95°C for 3 minutes. Samples were then incubated for 1.5 hours at 42°C in RT reaction mix (4 μL of 5 × first-strand buffer (Invitrogen Corp.), 2 μL of 10 mM dNTP mix (Invitrogen Corp.), 30 U RNase inhibitor (Prime; Eppendorf, Hamburg, Germany), 2 μL 0.1 M dithiothreitol (DTT; Invitrogen Corp.), and 200 U reverse transcriptase (Superscript II RNase H^-; Invitrogen Corp.), followed by 5 minutes 95°C to inactivate the enzyme.

For quantitative PCR, PCR master mix (Sybr Green; Applied Biosystems, Foster City, CA), PCR-grade water, and 20 mM reverse and forward primers were added to cDNA samples, and amplified using a sequence-detection system (GeneAmp 5700; Applied Biosystems). The reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Primers used were as follows: TLR2: sense, 5′-TGGAATGTCACCAGGCTGC-3′, and antisense, 5′-GTCCGTGGAAATGGTGGC-3′; TLR4: sense, 5′-AGGAAGTTTCTCTGGACTAACAAGTTTAGA-3′, and antisense, 5′-AAATTGTGAGCCACATTGAGTTTC-3′; TLR9: sense, 5′-TTCTCAAGACGGTGGATCGC-3′, and antisense, 5′-GCAGAGGGTTGCTTCTCACG-3′; L32: sense, 5′-TGTGCAACAAATCTTACCGTGC-3′, and antisense, 5′-GGATTGGTGACTCTGATGGCC-3′. PCR products were separated on a 4% agarose gel, and stained with fluorescent nuclear stain (Sybr Green I; Molecular Probes, Eugene, OR).

Detection of Neutrophils in the Cornea by Immunohistochemistry

Eyes were enucleated and snap frozen in liquid nitrogen, and 5-μm sections were fixed in 4% formaldehyde for 30 minutes. The slides were then washed with 0.05 M Tris buffer (TBS; pH 7.6), and the sections were incubated for 2 hours with anti-neutrophil antibody NIMP-R14 diluted 1:100 in 1% fetal calf serum/TBS (1% FCS/TBS) as described.[23] Sections were incubated 45 minutes with FITC-conjugated rabbit anti-rat antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in 1% FCS/TBS. After they were washed, the slides were mounted in antifade medium (Vectashield; Vector Laboratories), and the number of neutrophils per section was counted (from limbus to limbus at 400×) by fluorescence microscopy (Olympus Optical Co. Ltd., Tokyo, Japan).

Examination of Stromal Thickness and Haze

In vivo analysis of cellular infiltration was accomplished by in vivo confocal microscopy (Confoscan; Nidek Technologies America, New Orleans, LA). Briefly, mice were anesthetized and immobilized on a secure platform. A 40× objective was maneuvered into place on the corneal surface, by using transparent gel (Genteal; Novartis Ophthalmics, Duluth, GA) as a medium between the corneal surface and the objective, and the software (NAVIS; Lucent Technologies, Murray Hill, NJ) captured images every 5 μm and stored them as a stack for analysis of corneal thickness and haze.

Stromal thickness was determined as the area between basal epithelium and corneal endothelium, and stromal haze was defined as stromal thickness × combined light intensity of each image of the corneal stroma. To obtain this, the series of intensity values for each corneal stroma was saved on a computer spreadsheet (Excel; Microsoft, Redmond, WA), and exported into another program (Prism; Graph Pad Software, San Diego, CA) to generate a curve using the "curves and regression" function. The total area under the curve was then calculated by using this software. Baseline measurements were determined from naïve mouse corneas.

Chemokine Analysis

The corneal epithelium of mice was abraded, and exposed to TLR ligands as described. At the indicated time points, corneas were dissected and the epithelial layers separated by EDTA and placed into 200 μL RPMI-1640. Samples were then sonicated for 90 seconds with 50% duty cycle (VibraCell; Sonics and Materials, Danbury, CT). Tissue chemokine levels were assayed by a sandwich ELISA according to the manufacturer's directions (R&D Systems, Minneapolis, MN). The limit of detection of the assays was 1.5 pg/mL.

Statistics

Statistical analysis was performed with an unpaired *t*-test (Prism; Graph Pad Software). $P < 0.05$ was considered significant.

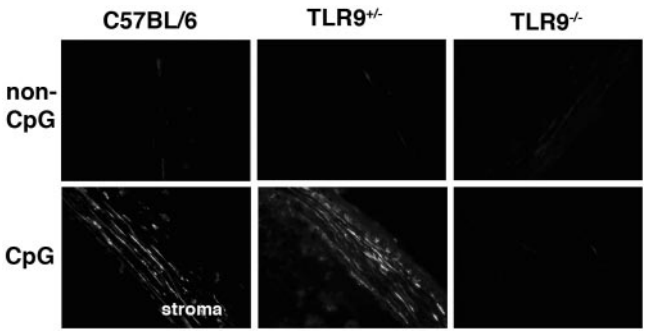

FIGURE 2. TLR9-induced neutrophil migration into the corneal stroma. Corneas of C57BL/6, TLR9$^{+/-}$, or TLR9$^{-/-}$ mice were abraded, and 20 μg CpG oligodeoxynucleotide (ODN) or control ODN was applied to the wound. After 24 hours, eyes were removed, sectioned, immunostained with anti-neutrophil mAb NIMP-R14 and FITC anti-rat antibody. Representative 5-μm sections of the corneal stroma show neutrophils in the corneal stroma of C57BL/6 and TLR9$^{+/-}$ mice by fluorescence microscopy in response to CpG DNA (*bottom*) but not in TLR9$^{-/-}$ mice, and no neutrophils were detected in response to control ODN (*top*).

RESULTS

TLR Expression in the Corneal Epithelium

To determine whether TLRs are expressed in the corneal epithelium, corneas were dissected from naïve C57BL/6 mice, the epithelial layer was separated from the stroma, and TLR expression was assessed by quantitative RT-PCR. As shown in Figure 1, TLR2, -4, and -9 were expressed in normal corneal epithelial cells, with transcript numbers of each of the TLRs within the range of the standard curve generated from cloned TLRs. When normalized to the copy number of the L32 housekeeping gene, TLR9 expression was lower than TLR2 and -4, and all were lower in the epithelium compared with the spleen. To confirm expression of TLR transcripts, products from Q-PCR (after 40 cycles) were examined by agarose gel electrophoresis. As shown in Figure 1 (right), TLR2, -4, and -9 products were present in corneal epithelium, but not in the no-template control.

TLR9-Induced Neutrophil Infiltration of the Corneal Stroma

To determine whether expression of TLR9 on corneal epithelium has functional significance, corneas of C57BL/6, TLR9$^{+/-}$, and TLR9$^{-/-}$ mice received a superficial abrasion and were treated with either the TLR9 ligand CpG ODN or with a control ODN, as described in the Methods section. After 24 hours, eyes were removed and sectioned, and the number of neutrophils in the corneal stroma was determined by immunohistochemistry using NIMP-R14, which is highly specific for neutrophils.[23,26] As shown in Figures 2 and 3A, neutrophils were present in the corneal stroma of CpG ODN-treated C57BL/6 and TLR9$^{+/-}$ mice, but not in mice treated with control, non-CpG ODN. Similar results were noted at other time points, although as shown previously with LPS,[23] the maximum neutrophil infiltration was at 24 hours (not shown).

In marked contrast, neutrophil recruitment was ablated in corneas of TLR9$^{-/-}$ mice after treatment with CpG ODN or control ODN. TLR9$^{-/-}$ mice exposed to LPS had a neutrophil infiltrate similar to that in C57BL/6 mice (Fig. 3B), indicating that corneas of these mice respond to TLR4 stimulation. Taken together, these findings demonstrate the presence of functional TLR9 on the corneal epithelium and indicate that activation of TLR9 induces keratitis as measured by neutrophil migration to the corneal stroma.

TLR2-Induced Neutrophil Infiltration of the Corneal Stroma

Bacterial lipoproteins and synthetic lipopeptide from the N-terminal region are potent immune activators that signal through TLR2.[24,25,27] To determine whether TLR2 activation of corneal epithelium induces neutrophil infiltration of the corneal stroma, the epithelial layer of C57BL/6 and TLR2$^{-/-}$ mouse corneas was abraded as described earlier, and treated with the synthetic lipopeptide Pam_3Cys. As shown in Figure 4, there was a pronounced neutrophil infiltrate in C57BL/6 mice exposed to Pam_3Cys. In contrast, TLR2$^{-/-}$ mice did not respond to this ligand, indicating that activation of TLR2 on the corneal epithelium induces neutrophil infiltration of the corneal stroma. As with TLR9$^{-/-}$ mice, LPS stimulation of TLR2$^{-/-}$ corneas was identical with C57BL/6 mice, indicating that these mice respond normally to bacterial products.

TLR2, -4, and -9–Mediated Keratitis Is MyD88 Dependent

Although MyD88 is a common adaptor molecule in TLR signaling, MyD88-independent pathways have been described for TLR4.[14,15] Our previous study showed that LPS keratitis is diminished in C3H/HeJ mice, indicating TLR4 dependence.[23] Therefore, to determine whether TLR2, -4, and -9–induced keratitis is MyD88 dependent, corneas of control and MyD88$^{-/-}$ mice were abraded and exposed to Pam_3Cys, LPS, or CpG ODN. After 24 hours, neutrophil infiltration of the corneal stroma was determined by immunohistochemistry, as before.

As shown in Figure 5, there was a pronounced neutrophil infiltration of the corneal stroma in control, wild-type mice in response to each of these agonists. In contrast, neutrophils were not detected in MyD88$^{-/-}$ mice treated with either

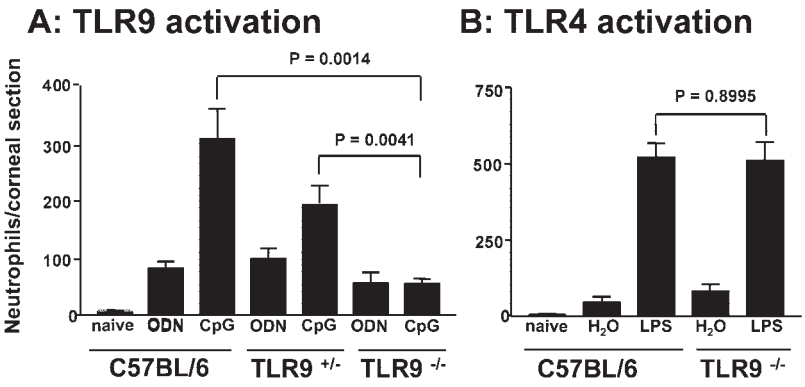

FIGURE 3. CpG and LPS-induced neutrophil migration to the corneas of C57BL/6 and TLR9$^{-/-}$ mice. (**A**) Corneas of C57BL/6, TLR9$^{+/-}$ and TLR9$^{-/-}$ mice were abraded and treated with CpG ODN (CpG) or control ODN (ODN) to activate TLR9. (**B**) Corneas of C57BL/6 and TLR9$^{-/-}$ mice were treated with LPS to activate TLR4. Neutrophil numbers were determined by direct counting of 5-μm corneal sections. Data presented are the mean ± SEM of counts in five mice per group and are representative of three experiments.

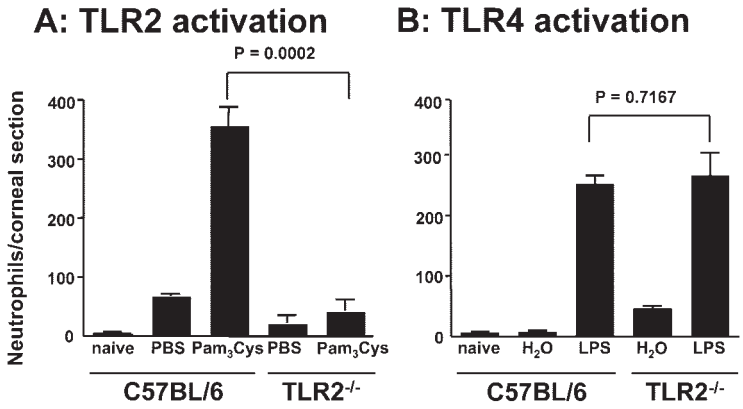

FIGURE 4. TLR2-dependent neutrophil infiltration into the corneal stroma. The corneal epithelium of C57BL/6 and TLR2 gene knockout mice was abraded and exposed to Pam_3Cys (**A**) or LPS (**B**). After 24 hours, eyes were removed, sectioned, and immunostained with NIMP-R14. The number of neutrophils was determined by direct counting of 5-μm corneal sections. Data presented are the mean ± SEM of counts in five mice per group. These results are representative of two repeat experiments.

Pam_3Cys, LPS or CpG DNA, demonstrating that TLR-induced neutrophil migration into the corneal stroma is MyD88 dependent.

To determine whether MyD88 expression is essential for cellular infiltration throughout the corneal stroma and for loss of corneal transparency, as measured by increased corneal thickness and haze, we examined the corneas of wild-type and $MyD88^{-/-}$ mice by in vivo confocal microscopy (Confoscan; Nidek), which produced a series of images through the cornea at 5-μm intervals. Representative images from the central corneal stroma showed a pronounced cellular infiltrate in wild-type mice in response to Pam_3Cys, LPS, and CpG ODN compared with PBS (Fig. 6A); however, as shown in the bottom panels, there was no detectable cellular infiltrate in $MyD88^{-/-}$ mice.

Sequential images of the corneal stroma were also used to generate corneal thickness and haze measurements, as described in the Methods section. Stromal thickness (Fig. 6B) and haze (Fig. 6C) were elevated in wild-type mice treated with Pam_3Cys, LPS or CpG ODN compared with PBS-treated or naïve corneas (*dotted lines*). In contrast, stromal thickness and haze in $MyD88^{-/-}$ mice were significantly lower than in Pam_3Cys, LPS or CpG ODN-treated wild-type mice, indicating that keratitis mediated by TLR2, -4, and -9 is entirely dependent on MyD88.

MyD88-Dependent Production of CXC Chemokines

Neutrophil recruitment to the corneal stroma in *Pseudomonas aeruginosa* keratitis, herpes simplex keratitis, *Onchocerca volvulus* keratitis (river blindness) and LPS keratitis is mediated by CXC chemokines and CXCR2.[28–31] To determine the relative production of MIP-2 and KC in the corneal epithelium and stroma in response to TLR2, -4, and -9 activation, and to determine the role of MyD88, the corneal epithelium of C57BL/6 mice was abraded and treated with Pam_3Cys, LPS, or CpG DNA. Corneas were dissected after 2, 4, and 6 hours (before detectable neutrophil infiltration), separated into epithelium and stroma, and MIP-2 and KC in sonicates were measured by ELISA.

As shown in Figure 7A, TLR2- and -4–induced MIP-2 production was elevated in corneal epithelium at 2 and 4 hours after exposure to Pam_3Cys or LPS and then decreased after 6 hours. In contrast, MIP-2 detected in the corneal stroma was lower than in the epithelial layer at 2 and 4 hours, and then increased by 6 hours after exposure to these TLR ligands. The time course of KC production in the corneal epithelium and stroma paralleled that of MIP-2, indicating that corneal epithelium is the earliest source of these chemokines.

To determine whether TLR2- and -4–mediated CXC chemokine production is MyD88 dependent, $MyD88^{-/-}$ mice and wild-type littermates were treated with Pam_3Cys and LPS, as described earlier. Corneas were dissected after 2 hours, and chemokines in corneal epithelium and stroma was determined as before. TLR2- and -4–induced MIP-2 and KC production was ablated in corneal epithelium and stroma of $MyD88^{-/-}$ mice compared with wild-type littermates, indicating an essential role for MyD88 in production of CXC chemokines.

Similarly, TLR9 activation induced KC production in corneal epithelial cells of C57BL/6 mice, but not $TLR9^{-/-}$ mice (Fig. 7C). However, MIP-2 was not detected in corneal epithelium of CpG DNA-treated C57BL/6 mice, and neither chemokine was detected in the corneal stroma (data not shown).

DISCUSSION

Results from the current study extend previous observations by demonstrating that TLR2, -4, and -9 mRNA is expressed in normal corneal epithelium, and that activation stimulates production of CXC chemokines by corneal epithelium, leading to

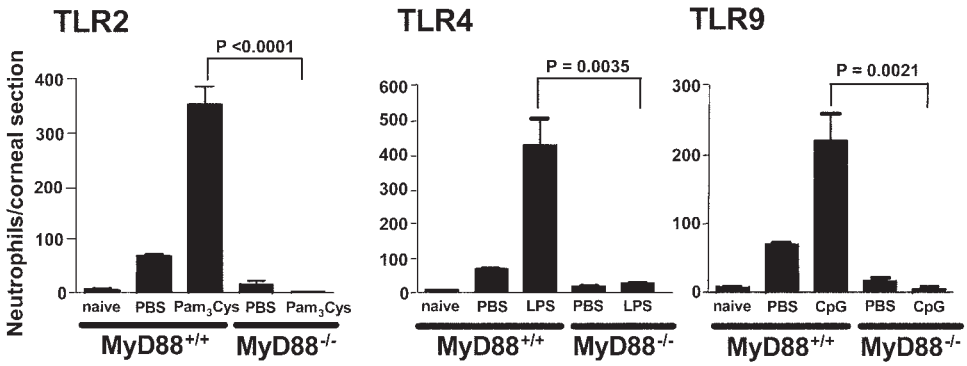

FIGURE 5. MyD88-dependent neutrophil migration to the cornea in response to TLR2, -4, or -9 activation. The corneal epithelium of MyD88 wild-type or $MyD88^{-/-}$ mice was abraded and exposed to Pam_3Cys (5 μg), LPS (20 μg), or CpG ODN (20 μg). After 24 hours, eyes were dissected, and the number of neutrophils was determined as described earlier. Data presented are the mean ± SEM of counts in four mice per group. These results are representative of two repeat experiments.

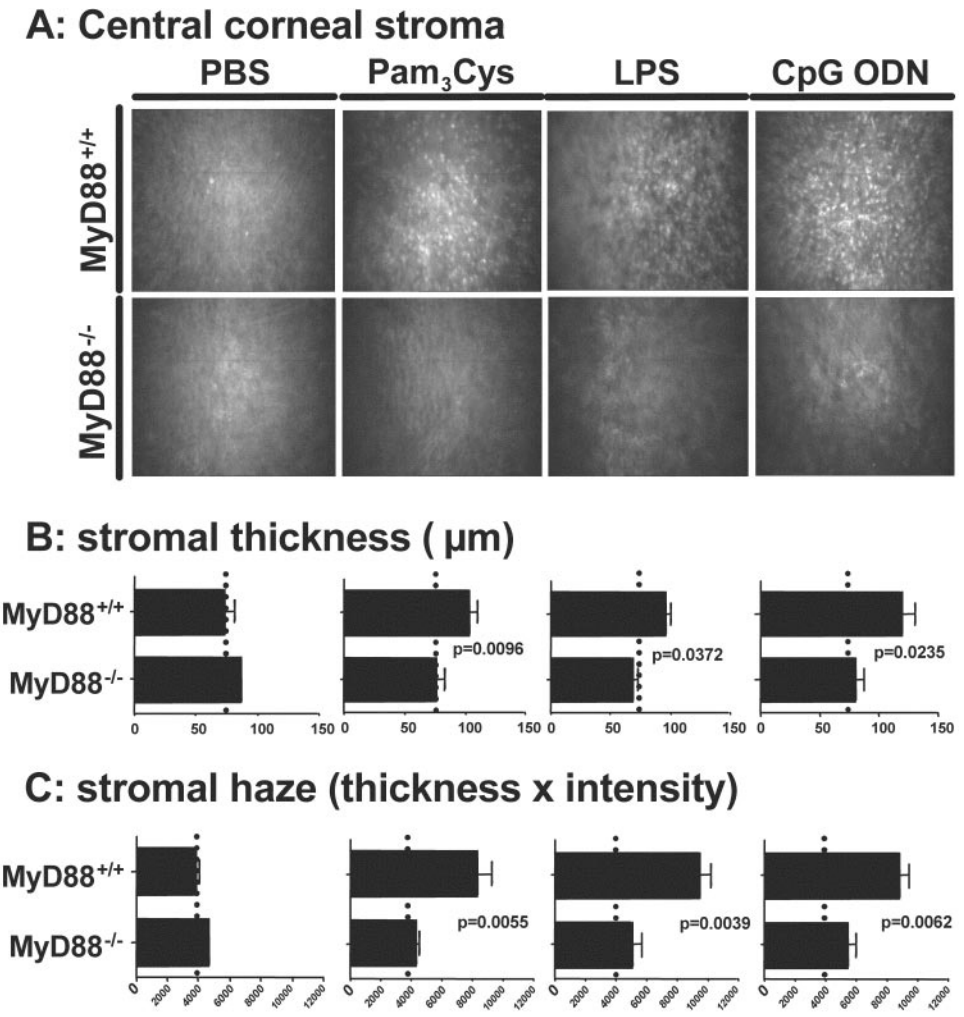

FIGURE 6. MyD88-dependent corneal thickness and haze. Corneas of control and MyD88$^{-/-}$ mice were abraded and treated with PBS, Pam_3Cys, LPS, or CpG ODN. After 24 hours, cellular infiltration of the corneal stroma and stromal thickness and haze were determined by in vivo confocal microscopy. (**A**) Representative images of the central corneal stroma showing a cellular infiltrate in wild-type mice (*top*) but not in MyD88$^{-/-}$ mice (*bottom*). (**B**) Stromal thickness and (**C**) stromal haze in MyD88$^{-/-}$ mice and wild-type littermates were calculated. Results are (mean ± SEM) of four mice per group, showing one of two representative experiments. *Dotted lines*: mean stromal thickness and haze of naïve mice.

neutrophil recruitment to the corneal stroma and subsequent increased corneal thickness and haze. Furthermore, as each of these stages in development of keratitis are ablated in MyD88$^{-/-}$ mice, the data indicate that TLR2, -4, and -9–induced keratitis is a MyD88-dependent process, and imply that the MyD88-independent pathway that has been described for TLR4[14] has no apparent role in this inflammatory process. Further studies will determine whether TLR3, which signals through TRIF rather than MyD88,[32] can also induce keratitis.

Epithelial cells have a key role in host defense and immune surveillance as they are constantly exposed to bacteria and bacterial products; however, the response to microbial products must be regulated to avoid continuous cellular activation. For example, TLR5 expression on intestinal epithelial cells is localized to the basal rather than the luminal side of epithelial cells.[33] Similarly, TLR4 expression in intestinal epithelial cells is intracellular and interacts with LPS in the Golgi apparatus, which is in contrast to TLR4 surface expression on macrophages and dendritic cells.[34,35]

Corneal epithelial cells are also exposed to bacteria and bacterial products on the ocular surface, and although TLRs are expressed on epithelial cells, the cornea is not normally inflamed. Indeed, an ongoing inflammatory response to commensal bacterial would probably result in development of corneal opacification and loss of vision. The underlying mechanisms that regulate corneal epithelial cell activation are therefore important in development of keratitis. Song et al.[21] demonstrated that TLR4 is localized on the cell surface of human corneal epithelial cells and that LPS induces production of proinflammatory and chemotactic cytokines. In contrast, Ueta et al.[36] reported that TLR2 and -4 are intracellular, and showed that LPS and peptidoglycan do not stimulate cytokine production above background levels. Although the discrepancy between these studies has yet to be resolved, the results of the present study clearly indicate that activation of TLRs on corneal epithelial cells in vivo stimulates cytokine production and development of keratitis.

Zhang et al.[22] showed that TLR5, which is activated by bacterial flagellin, is localized to basal epithelium and intermediate cells of the cornea and was not detected on the apical surface. This observation is consistent with the notion that TLRs are activated only when there is a breach of the squamous cell layer, and may be a more general mechanism for limited stimulation of intact corneal epithelium.

In the present study, TLR2, -4, and -9 were expressed in corneal epithelium recovered from normal mice. Although this is the first report of functional TLR2 and -9 in corneal epithelial cells, human respiratory epithelial cells express TLR2 and low levels of TLR9, and activation of these receptors with bacterial lipopeptides or bacterial DNA leads to production of IL-8 and β-defensin 2.[37,38]

Corneal epithelial cells recovered after stimulation with TLR2 and -4 ligands produced the CXC chemokines MIP-2 and KC within 2 hours. Chemokines were also detected in sonicates of the corneal stroma, although at later time points, indicating either that stromal fibroblasts are also a source of

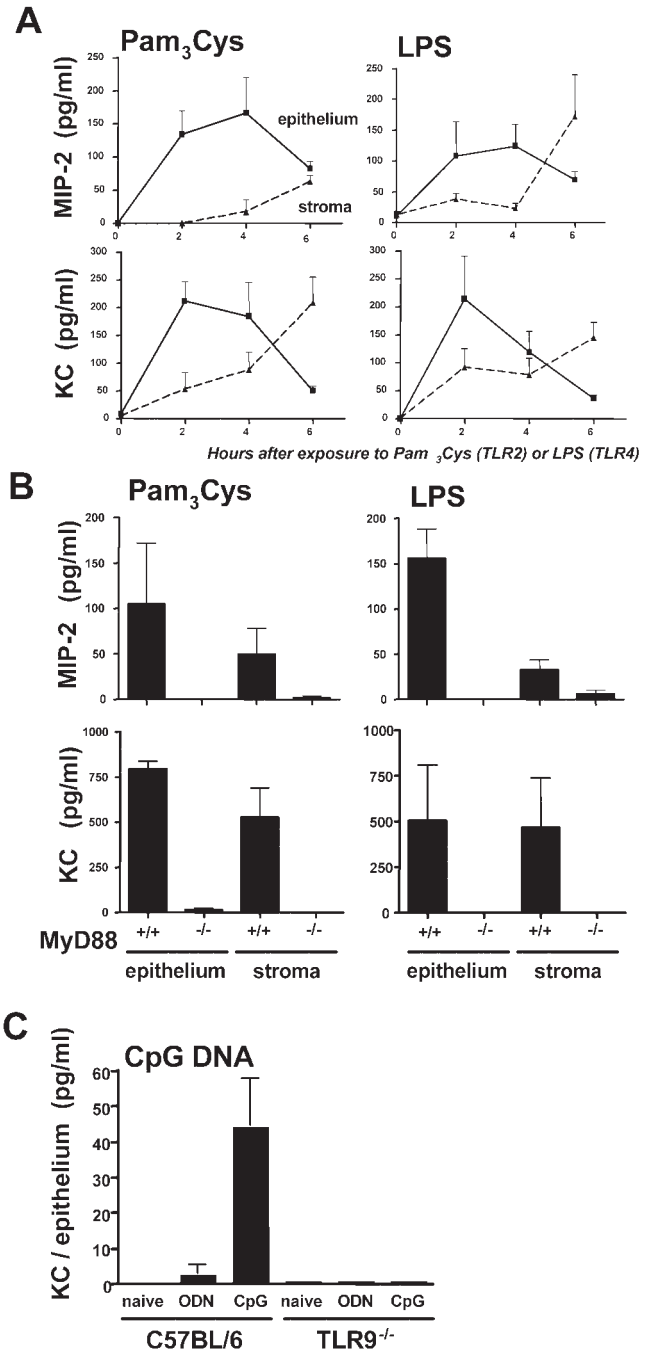

FIGURE 7. TLR-induced CXC chemokine production in the cornea. Corneas from C57BL/6 mice were abraded and treated with LPS or Pam_3Cys. After 2, 4, and 6 hours, corneas were dissected, and corneal epithelial sheets were separated from the stroma after incubation in EDTA. Tissues were sonicated, and the concentrations (in picograms per milliliter) of KC and MIP-2 were determined by ELISA. (**A**) MIP-2 and KC in sonicates of corneal epithelium (*solid lines*) and stroma (*dotted lines*) after TLR2 or -4 activation. (**B**) MIP-2 and KC production in corneas of $MyD88^{-/-}$ (−/−) and wild-type littermates (+/+) 2 hours after treatment with Pam_3Cys or LPS. (**C**) Corneas of C57BL/6 and $TLR9^{-/-}$ mice were treated with CpG DNA (CpG) or with control oligonucleotide (ODN) without the CpG motif. Results for all data points are the mean ± SEM of five individual corneal epithelial sheets per time point.

chemokines or that chemokines produced by epithelial cells diffused into the corneal stroma. Further studies will determine whether stromal fibroblasts express chemokine mRNA in response to these ligands, which would be consistent with reports that corneal keratocytes express chemokine mRNA in culture in response to proinflammatory cytokines.[39,40] It is likely that both resident cell types contribute to chemokine production and recruitment of neutrophils. Furthermore, neutrophils themselves express most TLRs, the activation of which stimulates production of chemotactic and proinflammatory cytokines.[41,42] Therefore, once activated in the corneal stroma, neutrophils may also produce CXC chemokines, leading to further neutrophil recruitment and exacerbation of the inflammatory response.

TLR2, -4, and -9 signal through the common MyD88 adaptor molecule, which signals through a cascade of intracellular events to NF-κB translocation and subsequent transcription of proinflammatory cytokines and neutrophil chemokines.[14,15] However, TLR4 also signals through a MyD88-independent pathway, using the TRIF adaptor molecule, which generates a type I IFN-dependent response that is essential to host defense against viral infection.[14,43] Although the ligands used in the present study indicate that there is no role for the TLR4/MyD88-independent pathway in development of keratitis, future studies will examine the effect of other ligands on keratitis, including the role of MyD88-independent receptors such as TLR3.[15,43]

In conclusion, the results of the present study demonstrate that the TLR/MyD88 pathway in corneal epithelial cells which is activated by ligands representative of Gram-negative and -positive bacteria can induce keratitis that may result in visual impairment. This pathway could therefore be a key target for therapeutic intervention in bacterial keratitis.

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References

1. Janssens S, Beyaert R. Role of Toll-like receptors in pathogen recognition. *Clin Microbiol Rev*. 2003;16:637–646.
2. Takeda K, Akira S. Microbial recognition by Toll-like receptors. *J Dermatol Sci*. 2004;34:73–82.
3. Akira S. Toll receptor families: structure and function. *Semin Immunol*. 2004;16:1–2.
4. van der Kleij D, Latz E, Brouwers JF, Kruize YC, et al. A novel host-parasite lipid cross-talk: schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem*. 2002;277:48122–48129.
5. Brattig NW, Bazzocchi C, Kirschning CJ, et al. The major surface protein of Wolbachia endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4. *J Immunol*. 2004;173:437–445.
6. Zhang D, Zhang G, Hayden MS, et al. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science*. 2004;303:1522–1526.
7. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*. 1999;274:10689–10692.
8. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*. 1999;162:3749–3752.
9. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282:2085–2088.
10. Takeuchi O, Kawai T, Muhlradt PF, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol*. 2001;13:933–940.

11. Takeuchi O, Sato S, Horiuchi T, et al. Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol*. 2002;169:10–14.
12. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*. 1999;11:443–451.
13. Hemmi H, Takeuchi O, Kawai T,et al. A toll-like receptor recognizes bacterial DNA. *Nature*. 2000;408:740–745.
14. Vogel SN, Fenton M. Toll-like receptor 4 signalling: new perspectives on a complex signal-transduction problem. *Biochem Soc Trans*. 2003;31:664–668.
15. Takeda K, Akira S. TLR signaling pathways. *Semin Immunol*. 2004;16:3–9.
16. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003;21:335–376.
17. Vogel SN, Fitzgerald KA, Fenton MJ. TLRs: differential adapter utilization by toll-like receptors mediates TLR-specific patterns of gene expression. *Mol Interv*. 2003;3:466–477.
18. Kawai T, Takeuchi O, Fujita T, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol*. 2001;167:5887–5894.
19. Serbina NV, Kuziel W, Flavell R, Akira S, Rollins B, Pamer EG. Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection. *Immunity*. 2003;19:891–901.
20. Yamamoto M, Sato S, Hemmi H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*. 2003;301:640–643.
21. Song PI, Abraham TA, Park Y, et al. The expression of functional LPS receptor proteins CD14 and toll-like receptor 4 in human corneal cells. *Invest Ophthalmol Vis Sci*. 2001;42:2867–2877.
22. Zhang J, Xu K, Ambati B, Yu FS. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to Pseudomonas aeruginosa flagellin. *Invest Ophthalmol Vis Sci*. 2003;44:4247–4254.
23. Khatri S, Lass JH, Heinzel FP, et al. Regulation of endotoxin-induced keratitis by PECAM-1, MIP-2, and toll-like receptor 4. *Invest Ophthalmol Vis Sci*. 2002;43:2278–2284.
24. Aliprantis AO, Weiss DS, Zychlinsky A. Toll-like receptor-2 transduces signals for NF-kappa B activation, apoptosis and reactive oxygen species production. *J Endotoxin Res*. 2001;7:287–291.
25. Muller MR, Pfannes SD, Ayoub M, Hoffmann P, Bessler WG, Mittenbuhler K. Immunostimulation by the synthetic lipopeptide P3CSK4: TLR4-independent activation of the ERK1/2 signal transduction pathway in macrophages. *Immunology*. 2001;103:49–60.
26. Pearlman E, Hall LR, Higgins AW, et al. The role of eosinophils and neutrophils in helminth-induced keratitis. *Invest Ophthalmol Vis Sci*. 1998;39:1176–1182.
27. Hoffmann P, Heinle S, Schade UF, et al. Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. *Immunobiology*. 1988;177:158–170.
28. Hall LR, Diaconu E, Patel R, Pearlman E. CXC chemokine receptor 2 but not C-C chemokine receptor 1 expression is essential for neutrophil recruitment to the cornea in helminth-mediated keratitis (river blindness). *J Immunol*. 2001;166:4035–4041.
29. Hazlett LD. Corneal response to Pseudomonas aeruginosa infection. *Prog Retin Eye Res*. 2004;23:1–30.
30. Kernacki KA, Barrett RP, Hobden JA, Hazlett LD. Macrophage inflammatory protein-2 is a mediator of polymorphonuclear neutrophil influx in ocular bacterial infection. *J Immunol*. 2000;164:1037–1045.
31. Yan XT, Tumpey TM, Kunkel SL, Oakes JE, Lausch RN. Role of MIP-2 in neutrophil migration and tissue injury in the herpes simplex virus-1-infected cornea. *Invest Ophthalmol Vis Sci*. 1998;39:1854–1862.
32. Backhed F, Hornef M. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? *Microbes Infect*. 2003;5:951–959.
33. Ortega-Cava CF, Ishihara S, Rumi MA,et al. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. *J Immunol*. 2003;170:3977–3985.
34. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate toll-like receptors at distinct cellular compartments. *Eur J Immunol*. 2002;32:1958–1968.
35. Latz E, Schoenemeyer A, Visintin A, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol*. 2004;5:190–198.
36. Ueta M, Nochi T, Jang MH, et al. Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. *J Immunol*. 2004;173:3337–3347.
37. Hertz CJ, Wu Q, Porter EM, et al. Activation of toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol*. 2003;171:6820–6826.
38. Platz J, Beisswenger C, Dalpke A, et al. Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *J Immunol*. 2004;173:1219–1223.
39. Cubitt CL, Tang Q, Monteiro CA, Lausch RN, Oakes JE. IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. *Invest Ophthalmol Vis Sci*. 1993;34:3199–3206.
40. Fillmore RA, Nelson SE, Lausch RN, Oakes JE. Differential regulation of ENA-78 and GCP-2 gene expression in human corneal keratocytes and epithelial cells. *Invest Ophthalmol Vis Sci*. 2003;44:3432–3437.
41. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood*. 2003;102:2660–2669.
42. Sabroe I, Prince LR, Jones EC, et al. Selective roles for toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J Immunol*. 2003;170:5268–5275.
43. Fitzgerald KA, Rowe DC, Barnes BJ, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med*. 2003;198:1043–1055.