Flagellin Suppresses the Inflammatory Response and Enhances Bacterial Clearance in a Murine Model of *Pseudomonas aeruginosa* Keratitis[▽]

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Pseudomonas aeruginosa **is a common organism associated with bacterial keratitis, especially in extended-wear contact lens users. In the present study, we determined that pretreatment of cultured human corneal epithelial cells with flagellin isolated from the *P. aeruginosa* PAO1 strain attenuated cytokine production when the cells were challenged with a cytotoxic strain (ATCC 19660), suggesting a potential use of bacterial flagellin to downregulate infection-associated inflammation in vivo. Administration of flagellin via the subconjunctival and intraperitoneal routes 24 h prior to *Pseudomonas* inoculation significantly improved the disease outcome, preserved structural integrity and transparency, and thus maintained vision in otherwise perforated corneas of C57BL/6 (B6) mice. The flagellin pretreatment resulted in suppression of polymorphonuclear leukocyte infiltration at a late stage of infection but not at an early stage of infection, decreased the expression of proinflammatory cytokine genes (genes encoding interleukin-1β [IL-1β], macrophage inflammatory protein 2, IL-12, and gamma interferon), and greatly enhanced bacterial clearance in the corneas of B6 mice probably through induced expression of the cathelicidin-related antimicrobial peptide and inducible nitric oxide synthase. This is the first report that describes the protective mechanisms induced by a Toll-like receptor agonist that not only curbs the host inflammatory response but also eliminates invading bacteria in the B6 mouse cornea.**

Pseudomonas aeruginosa keratitis is a potentially vision-threatening condition that requires prompt diagnosis and treatment to prevent vision loss (19, 48). Although an antibiotic regimen can eliminate the infectious organisms, the host inflammation that occurs, if not suppressed, can cause corneal scarring that may lead to permanent vision loss in some patients (13, 26). At present, only corticosteroids in ophthalmic solutions are available to suppress the ongoing inflammatory response after bacterial corneal infection. The effect (beneficial or detrimental) of these preparations on host-mediated tissue damage in bacterial keratitis has not been proven conclusively (47). Therefore, the controversial role of corticosteroids and the emerging resistance of *P. aeruginosa* to antibiotics warrant development of new adjunctive therapeutic modalities.

Previous studies in our laboratory and other laboratories have shown that corneal epithelial cells (CECs) express several Toll-like receptors (TLRs) and play an important role in recognition of keratitis-causing pathogens, including bacteria, fungi, and viruses (21, 24, 30, 40, 56). Recognition of microbial products by TLRs elicits a cascade of signal transduction pathways, resulting in the production of proinflammatory cytokines and chemokines that recruit polymorphonuclear leukocytes (PMN) to the site of infection and in the production of antimicrobial molecules that kill the invading pathogens at the

mucosal surface (6, 21, 25, 40, 44). Although the production of proinflammatory cytokines is important for mediating the initial host defense against invading pathogens, an excessive host inflammatory response can be detrimental. Thus, TLR-mediated corneal inflammation is a double-edged sword that must be precisely regulated. The TLR signaling pathways leading to an inflammatory response have been well documented (20, 52), but the underlying cellular mechanisms that directly control cytokine production after TLR stimulation are largely unresolved. Endotoxin tolerance was initially described in the 1960s and 1970s, when it was observed that animals could survive a lethal dose of bacterial endotoxin if they had been previously treated with a sublethal injection of lipopolysaccharide (LPS) (12, 33, 46). Recent studies revealed that modification of TLR4-mediated signal transduction is the underlying cellular mechanism responsible for altered responsiveness to bacterial endotoxin (9, 32, 35). Other TLR agonists, including mycobacterial products (31), flagellin,(34) CpG DNA (51), and lipoteichoic acid from gram-positive bacteria (28), also induce TLR tolerance. Since tolerance is associated with impaired NF-κB activation and suppression of proinflammatory cytokine and chemokine production (32), ligand-induced tolerance may represent a negative feedback mechanism invoked to induce inflammation resolution and to restore homeostasis after TLR activation. Preexposure of animals to TLR ligands such as LPS (27, 39, 42), TLR2 ligand bacterial lipoproteins (3, 10, 29), and flagellin (43) has been shown to provide protection against infection and sepsis in animal models. Since the corneal visual properties are exquisitely sensitive to inflammation-mediated damage (22), it is of paramount importance to identify ways to

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enhance the innate defenses in order to reduce or avoid inflammatory, adaptive immune responses to pathogen attack.

In the present study, we demonstrated that in the C57BL/6 (B6) mouse model of *P. aeruginosa* keratitis flagellin administration prior to bacterial infection prevented corneal tissue destruction and perforation and significantly improved the disease outcome. The flagellin-induced protection may result from augmented expression of antimicrobial and protective genes, unaffected PMN infiltration at the early stage of infection, and significantly suppressed production of proinflammatory cytokines in the cornea.

MATERIALS AND METHODS

Bacterial strains and flagellin. *P. aeruginosa* strain ATCC 19660 (cytotoxic) was used in this study. This strain is a standard laboratory strain that provides a reproducible inflammatory response in the cornea in the B6 mouse (15, 55). Flagellin was prepared from *P. aeruginosa* PAO1 by ammonium sulfate precipitation, followed by DEAE-Sephadex A-50 chromatography. LPS was removed by using Detoxi-Gel Affinity Pak columns (Pierce, Rockford, IL). The amount of LPS in the flagellin samples, determined with a quantitative *Limulus* amebocyte lysate kit, was 0.0027 endotoxin unit/μg protein after the two chromatography steps. In addition, up to 1,000 ng/ml purified flagellin failed to elicit activation of HeLa cells expressing functional TLR4, CD14, and MD2 (37) that were fully activated by 1 ng/ml purified *P. aeruginosa* LPS (Sigma).

Cell line. Human telomerase-immortalized corneal epithelial (HUCL) cells (11) were maintained in a defined keratinocyte-serum-free medium (Invitrogen Life Technologies, Carlsbad, CA) in a humidified 5% CO_2 incubator at 37°C. Before treatment, cells were cultured in growth factor-free and antibiotic-free keratinocyte basic medium (BioWhittaker, Walkersville, MD) for 16 h (growth factor starvation).

Cytokine ELISA. Secretion of tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) in cultured cells and secretion of macrophage inflammatory protein 2 (MIP-2) and IL-1β in B6 mouse sera and corneas were determined by an enzyme-linked immunosorbent assay (ELISA). Human CECs were plated at a concentration of 1×10^6 cells/well in six-well plates. After growth factor starvation, cells were pretreated with or without flagellin and then challenged with live bacteria for 4 h. At the end of the culture time, the media were harvested for measurement of cytokines. To measure mouse MIP-2 and IL-1β, sera were prepared from clotted blood, and corneal extracts were processed by homogenization in 1.0 ml phosphate-buffered saline (PBS) with a glass micro tissue grinder, followed by centrifugation at $14,000 \times g$ for 10 min. The ELISA was performed according to the manufacturer's instructions (R & D Systems).

Infection protocol. Female B6 mice (age, 8 weeks; weight, 20 to 24 g) were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained at an animal facility under pathogen-free conditions. Animals were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee of Wayne State University approved all animal procedures. B6 mice (five mice per group per treatment) were injected with 100 ng (in 5 μl) *P. aeruginosa* flagellin subconjunctivally and 125 ng (in 100 μl) *P. aeruginosa* flagellin intraperitoneally per mouse, and the same amount of PBS was injected as a control. B6 mice were anesthetized with ether and placed beneath a stereoscopic microscope at a magnification of ×40, and the cornea of the left eye of each mouse was wounded with three 1-mm incisions using a sterile 25-gauge needle. A bacterial suspension (5 μl) containing 1×10^6 CFU of *P. aeruginosa* strain ATCC 19660, prepared as described previously (15), was applied to the surface of the scarified cornea. Eyes were examined daily to monitor the disease progression.

Clinical examination. For assessment of clinical scores, mice were color coded and examined in a blinded fashion by two independent observers at 1, 3, and 5 days postinfection (p.i.) to visually grade the severity of disease after *P. aeruginosa* infection. The ocular disease was graded, and clinical scores were assigned using the following previously (15, 17) described scale: 0, clear; 1, slight opacity partially or fully covering the pupil; 2, slight opacity fully covering the anterior segment; 3, dense opacity partially or fully covering the pupil; and 4, dense opacity covering the anterior segment and corneal perforation. On day 5 p.i., all infected corneas were photographed with a slit lamp microscope to illustrate the disease response.

Histopathology. For histopathological examination, eyes from both the PBS- and flagellin-pretreated groups infected with strain ATCC 19660 were enucleated at 5 days p.i. The eyes were immersed in PBS, rinsed, and placed in a fixative containing 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorenson's phosphate buffer (pH 7.4) at a 1:1:1 ratio at 4°C for 3 h. The eyes were transferred into a fresh fixative after 1.5 h and then dehydrated in a graded ethanol series and embedded in Epon-araldite; sections were cut, stained with a modified Richardson's stain, and photographed as previously described (15).

PMN infiltration assay. Measurement of myeloperoxidase (MPO) activity was employed to determine PMN infiltration in the cornea as previously described (49). The corneas were excised from enucleated B6 mouse eyes at 5 days p.i. and homogenized in 1 ml of hexadecyltrimethylammonium bromide buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0). The samples were then subjected to three freeze-thaw cycles, followed by centrifugation at $16,000 \times g$ for 20 min. Each supernatant was mixed with 50 mM phosphate buffer (pH 6.0) containing 16.7 mg/ml *O,O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide at a 1:30 ratio to obtain a total volume of 3 ml. The change in absorbance at 460 nm was monitored continuously for 5 min. The results were expressed in units of MPO activity/cornea. One unit of MPO activity corresponded to approximately 2.0×10^5 PMN (49).

Bacterial load in the cornea. At 1, 3, and 5 days p.i., corneas (five corneas per group) from both groups of mice infected with the ATCC 19660 strain were collected, and the numbers of viable bacteria were determined. Individual corneas were homogenized in sterile PBS, and aliquots (100 μl) of serial dilutions were plated onto *Pseudomonas* isolation agar (Difco, Detroit, MI) plates in triplicate. The plates were incubated for 24 h at 37°C. The results were expressed as the mean number of CFU/cornea ± standard error of the mean.

Mouse corneal real-time RT-PCR. Real-time reverse transcription (RT)-PCR of mouse corneas was performed as described previously (18) using either SYBR green or TaqMan technology. Briefly, mouse corneas were homogenized in RNA (STAT-60; Tel-Test, Friendsville, TX), and total RNA was isolated according to the manufacturer's instructions to produce a cDNA template for PCR. The real-time primers used were either the primers described previously (17, 41) or primers purchased from Applied Biosystems. For PCR amplification, 1 μl each cDNA sample was used for each 25-μl PCR mixture. Real-time measurements were analyzed in duplicate in three independent runs (MyiQ system; Bio-Rad, Hercules, CA). Relative mRNA levels were calculated after normalization with β-actin (18).

Statistical analysis. An unpaired, two-tailed Student t test was used to determine statistical significance for data from the MPO assay. A nonparametric Mann-Whitney U test was performed for the bacterial count, clinical score, and real-time PCR analyses of treated and control groups. Mean differences were considered significant at a P value of <0.05. Experiments were repeated at least twice to ensure reproducibility; pooled data from two separate experiments are shown below.

RESULTS

Flagellin pretreatment reduces *P. aeruginosa*-induced proinflammatory cytokine production in cultured CECs. In a recent study (23), we showed that prolonged activation of TLR5 by flagellin purified from *P. aeruginosa* PAO1 induced human CECs to become tolerized, as manifested by impaired NF-κB activation and greatly decreased production of proinflammatory cytokines in response to PAO1 challenge. To determine whether PAO1 flagellin also induces tolerance to a different strain of *P. aeruginosa* in human CECs, we pretreated HUCL cells with several doses (10 to 100 ng/ml) of flagellin for 24 h and then challenged them with ATCC 19660, a cytotoxic strain; PAO1 was used as the control. Both strains induced secretion of TNF-α and IL-8 (Fig. 1) in HUCL cells. The bacterium-induced production of both TNF-α and IL-8 was retarded by pretreatment of the cells with flagellin in a concentration-dependent manner; 50 ng/ml flagellin was sufficient to completely block the cytokine secretion induced by both the PAO1 and ATCC 19660 strains of *P. aeruginosa* ($P < 0.001$). Of note, 50 ng/ml flagellin did not induce significant proinflammatory cytokine production in HUCL cells (23). Furthermore, *Staph-*
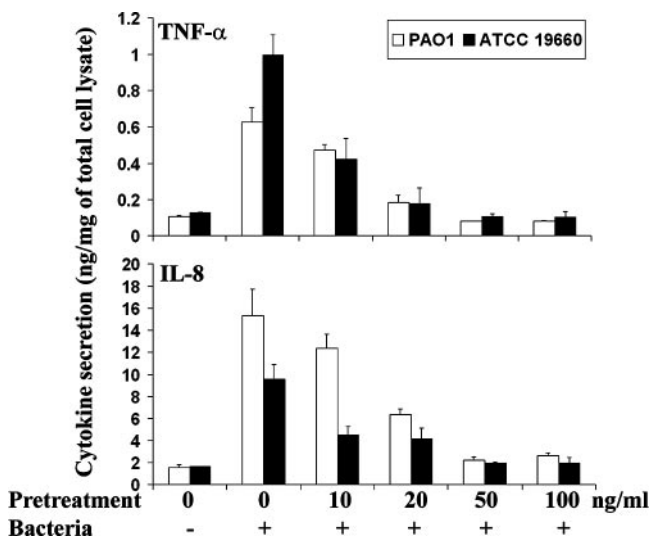
FIG. 1. Flagellin pretreatment dampens production of TNF-α and IL-8 in response to bacterial challenge in human CECs. HUCL cells were cultured without flagellin (0) or with 10, 20, 50, or 100 ng/ml flagellin for 24 h. After two washes with PBS, cells were challenged with *P. aeruginosa* PAO1 (solid bars) or ATCC 19660 (gray bars) at a multiplicity of infection of 100 for 4 h. TNF-α and IL-8 secretion into culture supernatants was assayed by ELISA. The amounts of cytokines in culture media were expressed in ng of cytokine per mg of cell lysate. The bars indicate means, and the error bars indicate standard deviations.

ylococcus aureus-induced cytokine production was not significantly affected by flagellin pretreatment (data not shown).

Flagellin pretreatment protects the cornea from *P. aeruginosa* keratitis in B6 mice. We hypothesized that flagellin pretreatment can suppress inflammation in vivo and induce protective mechanisms in the cornea, and we tested this hypothesis using the B6 mouse model of *P. aeruginosa* keratitis (15, 41). Flagellin was administrated subconjunctivally (100 ng/eye) and intraperitoneally (125 ng/per mouse) 24 h prior to bacterial infection. The next day, mice were inoculated with 1.0×10^6 CFU of *P. aeruginosa* (strain ATCC 19660) after needle injury of the cornea (15). The corneal disease response was graded on different days after infection using the previously described scale (15). No significant difference in mean clinical scores was observed at 1 day p.i. (1.47 versus 1.21 for PBS- and flagellin-pretreated mice, respectively; $P = 0.35$). On the other hand, the average scores for the flagellin-pretreated mice did not change from day 1 to day 5 (~1.2) and were significantly lower than the scores for the PBS controls (1.22 versus 2.9, respectively, at day 3 [$P < 0.0002$]; 1.21 versus 3.89, respectively, at day 5 [$P < 0.0001$; $n = 19$]).

Figure 2B (left panel) shows the results of slit lamp microscopy for a flagellin-pretreated cornea at day 5 p.i. (clinical score, 1) displaying only slight opacity in the peripheral region due to residual infiltrating cells in the cornea and/or the anterior chamber, whereas the placebo-treated eye was perforated (clinical score, 4) (Fig. 2B, right panel). Histopathological analysis revealed that the eyes of PBS-treated B6 mice (clinical score, 4) showed a heavy cellular infiltrate in the cornea and anterior chamber along with complete destruction of the corneal epithelium, central stromal degradation, severe edema,

severe anterior chamber inflammation, and perforation (Fig. 2C, right panel). On the other hand, flagellin pretreatment preserved the morphological integrity of the cornea, and little infiltration was detected in the anterior chamber (clinical score, 1) (Fig. 2C, left panel).

Flagellin pretreatment reduces the bacterial load in the cornea. Having demonstrated that a significant number of corneas had scores of 0 and 1 at 5 days p.i., we next examined the effect of flagellin pretreatment on bacterial clearance in the cornea (Fig. 3A). At 1 day p.i. 5×10^4 CFU (average) per cornea were detected in the control PBS-pretreated mice, whereas the number of bacteria in flagellin-pretreated mice was 8×10^3 CFU/cornea, a value that was sixfold lower. At 3 days p.i., the average levels in the control PBS-pretreated mice and flagellin-pretreated mice were 4×10^7 and 7×10^5 CFU/cornea, respectively. The differences between these two groups were more striking at 5 days p.i.; the average CFU in the flagellin-pretreated mice was 1×10^3 CFU/cornea, whereas 8×10^7 CFU/cornea was detected in the corneas of control, PBS-pretreated mice. Of note, three of five corneas were sterile with no bacterial growth found by the plate counting assay (Fig. 3A). Thus, pretreatment of mice with the TLR5 ligand enhanced the innate bacterial killing ability of the cornea, leading to control of *P. aeruginosa* infection in B6 mice.

Figure 3B shows the effects of flagellin pretreatment on PMN infiltration in the cornea determined by assessing MPO activity. While there was no measurable MPO activity in non-infected corneas with or without flagellin pretreatment, MPO activity was detected in *P. aeruginosa*-infected corneas at 1 day p.i. However, there was no significant difference in MPO activity between the PBS-pretreated and flagellin-pretreated mice at this time. A much higher level of MPO activity was observed in the control, PBS-pretreated corneas on days 3 and 5 p.i. than on day 1 p.i. The increase in PMN infiltration, however, was muted by flagellin pretreatment. Moreover, in flagellin-pretreated mice, the PMN infiltration was significantly less on day 5 p.i. than that on days 1 and 3 p.i., suggesting that enhanced resolution of inflammation was associated with a reduced bacterial load in the cornea.

Flagellin pretreatment suppresses the inflammatory response and induces antimicrobial gene expression in vivo. To determine if flagellin pretreatment suppresses the expression of inflammatory cytokines in vivo, we next assessed the expression of IL-1β, IFN-γ, MIP-2, and IL-12 in B6 corneas before and after *P. aeruginosa* infection (Fig. 4). While flagellin pretreatment alone did not elicit expression of these cytokines in the cornea or in the blood (data not shown), it significantly attenuated their expression at the mRNA level induced by *P. aeruginosa* at day 1 p.i. ($P < 0.05$). At day 5 p.i. the levels of these cytokines were further elevated in the control mouse corneas but had declined in flagellin-pretreated corneas, resulting in significant differences ($P < 0.001$) in the levels of all four cytokines assessed. The inhibition of IL-1β and MIP-2 expression by flagellin pretreatment was also verified at the protein level in *P. aeruginosa*-infected corneas on day 1 p.i. (Fig. 5).

The enhanced bacterial clearance in the mouse corneas suggests that flagellin pretreatment may influence the expression of antimicrobial genes that can lead to the direct killing of invading bacteria. Hence, we investigated the mRNA expres-
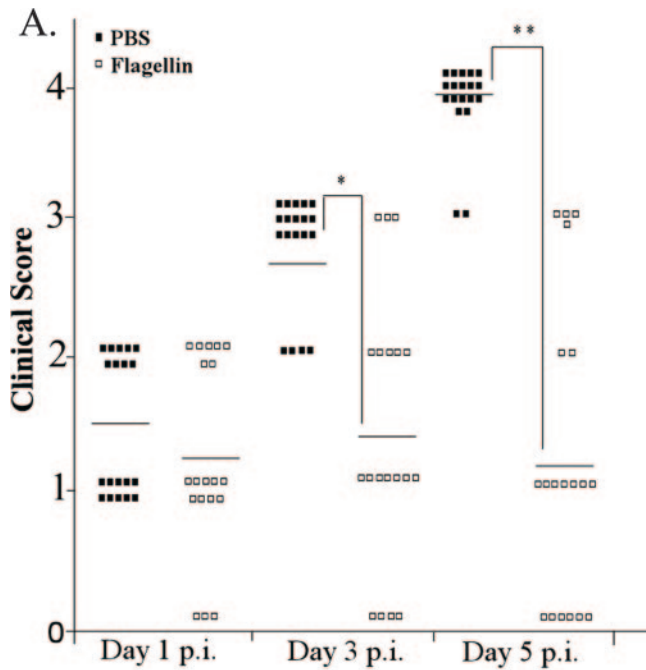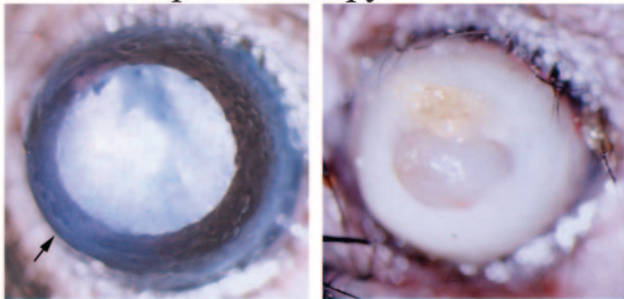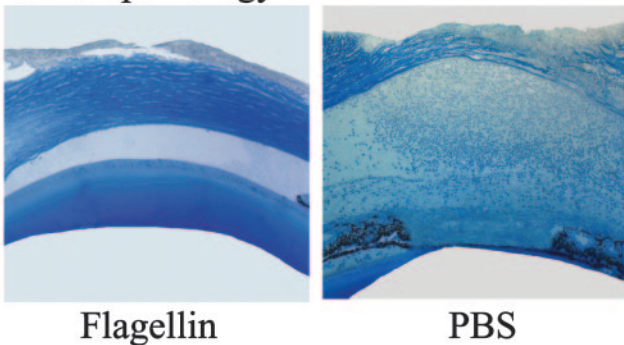
FIG. 2. Flagellin pretreatment diminishes clinical symptoms of keratitis in B6 mice. B6 mice ($n = 19$) were injected with either PBS or flagellin subconjunctivally (100 ng/eye) and intraperitoneally (125 ng/mice). One day after flagellin injection, the cornea was scarified with three parallel 1-mm incisions using a sterile 25-gauge needle, and the scarified corneas were challenged topically with 1.0×10^6 CFU of *P. aeruginosa* strain ATCC 19660. (A) Clinical scores for the ocular disease response assigned on days 1, 3, and 5 p.i. Two asterisks, $P > 0.0001$. (B) Slit lamp examination of infected corneas performed on day 5 p.i. The arrow indicates sedimentation of residual infiltrated cells in the anterior chamber. Magnification, $\times 40$. (C) Histopathological analysis performed with representative infected corneas (score for PBS-treated mice, 4; score for flagellin-treated mice, 1) at 5 days p.i. Magnification, $\times 165$.
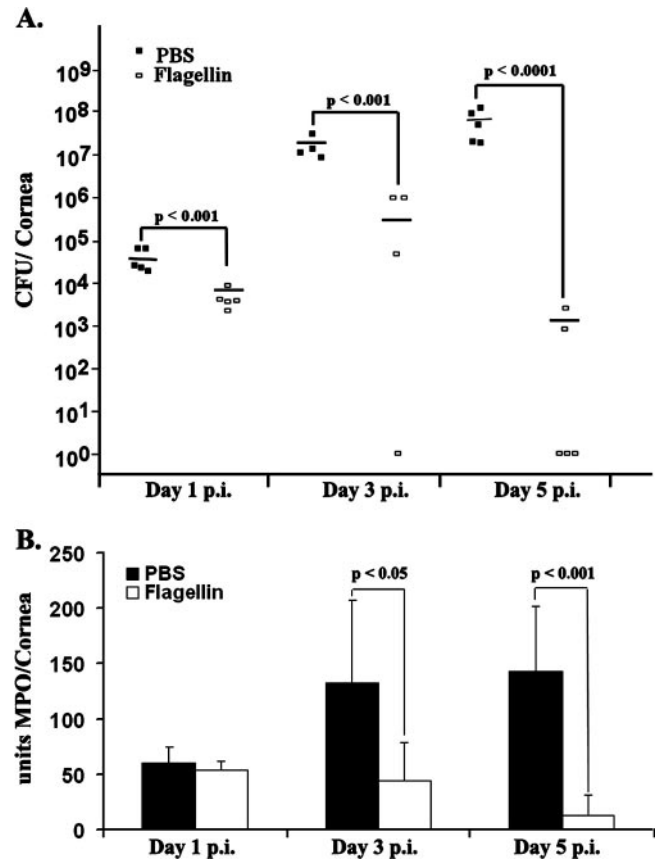

FIG. 3. PMN infiltration and bacterial clearance in flagellin-pretreated corneas. Mice were treated with PBS ($n = 5$) or flagellin ($n = 5$) as described in the text, followed by ocular inoculation of 1×10^6 CFU of *P. aeruginosa* strain ATCC 19660 per cornea as described in the legend to Fig. 2. At the indicated times after infection, the corneas were excised and subjected to plate bacterial counting (A) or determination of MPO activity as a parameter for PMN infiltration (B). In panel B the bars indicate the arithmetic mean and the error bars indicate the standard error of the mean for five mice in each group. *P* values were generated using paired Student's *t* test for the MPO assay and the Mann-Whitney test for bacterial counts.

sion of cathelicidin-related antimicrobial peptide (CRAMP) (a mouse homolog of human CAP-18) and inducible nitric oxide synthase (iNOS) in flagellin-pretreated corneas using TaqMan real-time PCR. In the corneas that were not infected with *P. aeruginosa*, flagellin pretreatment induced a threefold increase in CRAMP mRNA expression compared with the PBS-pretreated controls 24 h after injection (Fig. 6). *P. aeruginosa* infection caused upregulation of CRAMP mRNA, and flagellin pretreatment had no effect on the infection-induced CRAMP expression at 1 day p.i. On day 3, CRAMP expression continued to rise and flagellin pretreatment further increased CRAMP expression in *P. aeruginosa*-infected corneas. At 5 days p.i., the CRAMP mRNA levels were lower in the corneas pretreated with or without flagellin. Unlike CRAMP expression, the expression of iNOS was not induced by flagellin alone. However, at 1 day p.i. flagellin pretreatment dramatically increased the iNOS expression in B6 mouse corneas compared to the control PBS-pretreated corneas (threefold increase compared with PBS-treated corneas). On day 3 p.i., the
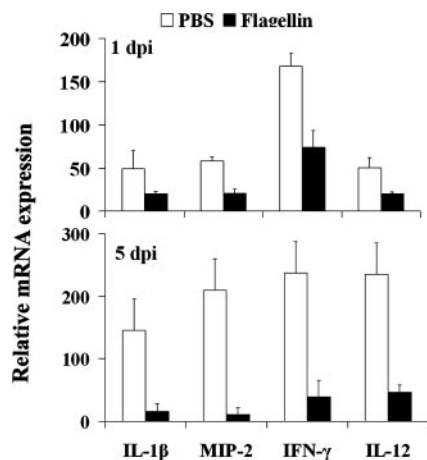
FIG. 4. Expression of cytokine mRNA in *P. aeruginosa*-infected and flagellin-pretreated corneas. Corneas (five corneas per group) of B6 mice were removed at 1 and 5 days p.i. as described in the legend to Fig. 2. Real-time RT-PCR was used to assess cytokine mRNA levels in flagellin- and PBS-pretreated corneas. The mRNA expression levels of IL-1β, MIP-2, IFN-γ, and IL-12 were normalized to β-actin levels and expressed as relative ratios. Flagellin pretreatment significantly ($P < 0.001$) reduced the expression of these cytokines. dpi, days postinfection.
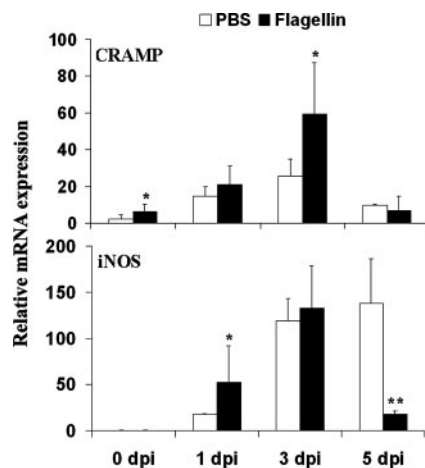

FIG. 6. Expression of CRAMP and iNOS mRNA in *P. aeruginosa*-infected and flagellin-pretreated corneas. Corneas (five corneas per group) of B6 mice were removed after pretreatment on days 0 (without infection), 1, 3, and 5 p.i. Real-time RT-PCR was used to assess mRNA levels of CRAMP and iNOS in flagellin-pretreated and PBS-pretreated corneas. mRNA expression was normalized to β-actin expression and expressed as relative expression. Flagellin pretreatment significantly (one asterisk, $P < 0.05$; two asterisks, $P < 0.001$) induced the expression of CRAMP and iNOS in mouse corneas. dpi, days postinfection.

iNOS mRNA levels were further increased in both flagellin-pretreated and control corneas, and no significant difference between two groups was observed. At 5 days p.i., while elevated levels of iNOS mRNA persisted in PBS-pretreated corneas, the levels were dramatically lower in flagellin-pretreated B6 mice ($P < 0.0001$).

DISCUSSION

In the present study, we demonstrated a novel protective role of bacterial flagellin, a major pathogen-associated molecular pattern of gram-negative bacteria recognized by TLR5, in prevention of *Pseudomonas* keratitis. A single administration of flagellin prior to bacterial inoculation greatly attenuated the expression of proinflammatory cytokines but not CRAMP and
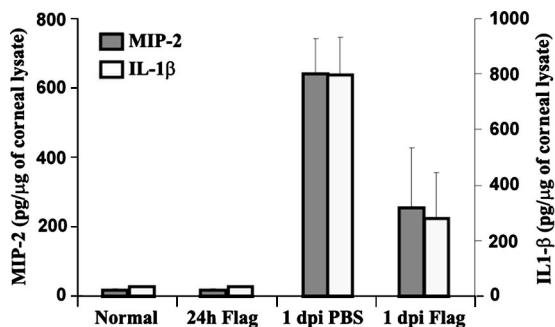

FIG. 5. ELISA analysis of corneal proinflammatory cytokine protein levels after *P. aeruginosa* infection. Corneas of B6 mice were removed at 24 h after flagellin pretreatment and 1 day p.i. as described in the legend to Fig. 2. Protein levels for IL-1β and MIP-2 were determined by the ELISA. The bars indicate the means, and the error bars indicate the standard errors of the means (five mice/group/time). dpi, days postinfection; Flag, flagellin treatment.

iNOS, significantly reduced PMN infiltration at a late stage of infection but not at an early stage of infection, and, most strikingly, greatly enhanced bacterial clearance in the corneas of B6 mice, leading to functional recovery of corneas that would otherwise be perforated. Thus, flagellin can be exploited as a novel anti-inflammatory and/or anti-infection therapy for bacterial keratitis and other infectious diseases.

The cornea is an immune-privileged avascular tissue in the human body and possesses strong innate defenses which are essential for rapid elimination of invading pathogens, as well as for reducing the destructive effects precipitated by pathogens and by the host inflammatory reaction. Our previous study showed that activation of TLR5 by flagellin resulted in production of proinflammatory cytokines in human CECs (56). To identify negative feedback mechanisms invoked to induce the resolution of inflammation and to restore homeostasis, we found that preexposure of cultured CECs to flagellin dampened the production of proinflammatory cytokines but enhanced the expression of antimicrobial genes induced by live *P. aeruginosa* (23) (Fig. 1). The dramatic effects of flagellin on the epithelial response to live *P. aeruginosa* in vitro suggest a potential use of the approach to control corneal infection in vivo. In this study, we used a well-characterized and accepted mouse model of *Pseudomonas* keratitis (13, 15) and found that flagellin administration prior to bacterial infection prevents corneal perforation and significantly improves the disease outcome, as manifested by the preserved structural integrity of the cornea and a vast reduction in PMN infiltration within the cornea and in the anterior chamber at day 5 p.i. when the control corneas are perforated. Consistent with in vitro suppression of *P. aeruginosa*-induced cytokine expression in cultured CECs, our data also show that a single exposure of B6 mice to flagellin 24 h prior to bacterial inoculation reduced the expression of

several key proinflammatory cytokines significantly at day 1 p.i. and greatly at day 5 p.i. The reduction in the cytokine expression levels in flagellin-pretreated corneas at the late stage of infection correlated to greatly reduced infiltration and tissue damage, suggesting that pretreatment induced resolution of the inflammation. Thus, TLR ligands may be used as pharmacological reagents to control corneal injury and inflammation caused by microbial infection.

Significantly reduced inflammation and tissue destruction in flagellin-pretreated B6 mouse corneas is associated with enhanced bacterial clearance. We showed that flagellin pretreatment of B6 mice resulted in a sixfold reduction in the viable bacterial count in the cornea at 1 day p.i. More strikingly, by day 5 p.i., when the control corneas were perforated with $\sim 10^8$ CFU bacteria per cornea, flagellin-treated corneas were either sterile or contained a very limited number of bacteria compared to the number observed at 1 day p.i. This suggests that bacterial growth was inhibited or eliminated in an environment where an otherwise rapidly proliferating bacterial population is expected. To our knowledge, this is the first report that a nonantibiotic regimen leads to total elimination of *P. aeruginosa* in the corneas of B6 mice, a susceptible strain (15). We suggest that the greatly enhanced bacterial clearance induced by flagellin is related to the induced antimicrobial gene expression in the corneas. The role of antimicrobial peptides, especially those produced by epithelial linings, in limiting infection has been the subject of several recent studies (53, 54). We reported that flagellin pretreatment resulted in upregulation of CRAMP (a homolog of human LL-37) prior to *P. aeruginosa* infection at the ocular surface. As at this stage there was no significant PMN infiltration as assessed by MPO determination, we suggest that the epithelium is a major source of CRAMP. The presence of more CRAMP molecules at the ocular surface may increase corneal innate bacterial killing when pathogens are invading the flagellin-exposed epithelial cells, leading to a rapid reduction in the bacterial load. Moreover, *P. aeruginosa* infection triggers a great increase in CRAMP expression, a potential innate defense mechanism against infection in the immune-privileged cornea. While flagellin pretreatment-induced CRAMP expression may be masked at 1 day p.i., a much high level of CRAMP mRNA was observed at day 3 p.i. in flagellin-pretreated corneas compared to the control. This continued upregulation of CRAMP may contribute to bacterial clearance in the cornea by functioning synergistically with other antibacterial agents, such as β-defensins and iNOS (see below) found at the ocular surface, to kill invading pathogens (5). LL-37 has previously been shown to bind LPS. This may also help reduce the detrimental effects of infection and inflammation. The role of CRAMP as part of the corneal innate defense mechanism was recently confirmed by use of CRAMP-deficient mice that became susceptible (corneas perforated) in an otherwise resistant (no corneal perforation) mouse strain (16). In addition to CRAMP, we also observed strong induction of iNOS, which produces NO, a free radical gas with important antimicrobial activity (1, 4), in the B6 corneas at day 1 p.i. The levels of iNOS at day 1 p.i. in flagellin-pretreated corneas were severalfold higher than the levels in the control group, and the levels were even higher in both groups by day 3 p.i. Importantly, as excessive production of NO is known to cause tissue damage, rapid downregulation

of iNOS was observed in the flagellin-treated group but not in the control group in B6 corneas at 5 days p.i. Interestingly, a recent study revealed that, in contrast to the general conjecture, the dramatically elevated levels of NO during septic shock are not produced by hematopoietic cells rather by parenchymal cells in the liver, kidney, and gut (2). Hence, we suggest that CECs are a major source of iNOS in the corneas of B6 mice at least at 1 day p.i., when macrophage infiltration is not yet predominant (14). Recently, studies with other systems suggested that epithelium-derived cathelicidin contributed substantially to protection of the urinary tract against infection (7) and that stimulation of epithelial cells to produce LL-37-like protein with sodium butyrate prevented infection of the colon by *Shigella* (36). Thus, augmented expression of antimicrobial molecules due to flagellin pretreatment may contribute to the increased bacterial clearance observed in the corneas of B6 mice.

Another contributing factor for bacterial clearance in the cornea is PMN infiltration. Interestingly, although the bacterium-induced expression of chemokines such as MIP-2 in B6 mouse corneas or IL-8 in cultured human CECs was downregulated (23), PMN infiltration was not significantly affected in flagellin-pretreated corneas on day 1 p.i compared with the control. Flagellin present in the cornea and/or blood before infection may prime the PMN and increased their bacterial recognition and bactericidal activity, as shown in bacterial lipoprotein-pretreated mice (45). The nature of the chemokines recruiting PMN to the infected corneas remains to be determined. One potential candidate is KC, another mouse homolog of human IL-8 that, along with MIP-2, has been shown to play a role in PMN infiltration in infected corneas (8, 50). Together, the combined effects of elevated antimicrobial activity, significantly reduced expression of proinflammatory cytokines, and unaffected PMN infiltration contributed to the increase in bacterial clearance at the early stage on infection, leading to significant improvement in the outcome of *P. aeruginosa* infection in B6 mice.

Although effective, the use of flagellin given systemically and simultaneously subconjunctivally as a keratitis prophylactic is not likely to gain clinical approval. For potential ocular application, we recently observed that subconjunctival injection of flagellin, a procedure routinely performed in ophthalmologists' offices, ameliorated corneal pathology and reduced the numbers of *P. aeruginosa* cells in the eye. Hence, subcoinjunctival injection of flagellin might be used as a prophylactic measure to reduce the potential for bacterial infection after ocular surgeries such as corneal transplantation and diabetic vitrectomy. Furthermore, premixing of isolated flagella with *P. aeruginosa* was also found to reduce the rate of corneal perforation (38), a phenomenon that was confirmed using purified flagellin (A. Kumar and F.-S. X. Yu, unpublished results). Considering the fact that most bacterial infections of the cornea occur only when the epithelial barrier is breached (e.g., infections caused by wearing contact lenses), flagellin present at the ocular surface likely diffuses rapidly into the basal or wing layer of epithelial cells that express TLR5 and induces TLR signaling, leading to tolerance and the production of antimicrobial peptides. Thus, addition of flagellin at the ocular surface through eye drops or in the contact lens solution may have prophylactic effects for prevention of contact lens-associated infection.

In summary, we identified and characterized protective and anti-inflammatory mechanisms induced by prolonged activation of TLR5 in vivo using a mouse *P. aeruginosa* keratitis model. Our findings reveal that manipulation of the tolerance induced by TLR ligands can be exploited as a prophylatic or therapeutic approach to dampen inflammation and control infection. Ongoing studies by our group are exploring the specific role of the epithelium in corneal innate immunity and mechanisms for inducing endogenous antimicrobial peptides to prevent contact lens-associated corneal infections.

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