Flagellin Suppresses Inflammatory Response and Enhances Bacterial Clearance in Murine Model of *Pseudomonas* Keratitis

Ashok Kumar¹,², Linda D. Hazlett² and Fu-Shin X. Yu¹,²*

¹The Kresge Eye Institute/Department of Ophthalmology, ²Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, MI 48201

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*Corresponding Author

Kresge Eye Institute
Wayne State University School of Medicine
4717 St. Antoine, Detroit, MI 48201
Tel: (313) 577-1657
Fax: (313) 577-7781
E-mail: fyu@med.wayne.edu
Abstract

*Pseudomonas aeruginosa* is a common organism associated with bacterial keratitis, especially in extended wear contact lens users. In the present study, we report that pre-treatment of cultured human corneal epithelial cells (CECs) with flagellin, isolated from *P. aeruginosa* PA01 strain, attenuated cytokine production when challenged with a cytotoxic strain (ATCC19660), suggesting a potential use of bacterial flagellin to downregulate infection associated inflammation in vivo. Administration of flagellin via subconjunctival and intraperitoneal routes 24 h prior to *Pseudomonas* inoculation significantly improved disease outcome, preserved structural integrity and transparency, and thus maintained vision in otherwise perforated corneas of C57BL/6 mice. The flagellin pretreatment resulted in the suppression of PMN infiltration in late, but not in early stage of infection, decreased the expression of proinflammatory cytokine genes (IL-1β, MIP-2, IL-12, IFN-γ), 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 TLR agonist that not only curbs the host inflammatory response, but also eliminates the invading bacteria in the B6 mouse cornea.
Introduction

*P. 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 are available in ophthalmic solutions to suppress the ongoing inflammatory response after bacterial corneal infection. Their effect (beneficial or detrimental) in reducing host-mediated tissue damage has not been proven conclusively on bacterial keratitis (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 from our laboratory and others have shown that corneal epithelial cells 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/chemokines that recruit polymorphonuclear leukocyte (PMN) to the site of infection and 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. To date, the TLR signaling pathways leading to 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 1960-1970s 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 (12, 33, 46). Recent studies revealed that the 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 pro-inflammatory 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. Pre-exposure 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 offer protection against infection and sepsis in animal models. Since the corneal visual properties are exquisitely sensitive to inflammation-mediated damage, it is of paramount importance to identify ways to 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 C57BL/6 (B6) mouse model of *P. aeruginosa* keratitis flagellin administration prior to bacterial infection
prevented corneal tissue destruction and perforation and significantly improved 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.
Material and Methods

Bacterial strains and flagellin

*P. aeruginosa* strain, ATCC19660 (cytotoxic), was used for the studies. This is a standard laboratory strain that provides a reproducible inflammatory response in corneal pathology in the B6 mouse (15, 55). Flagellin was prepared from PA01 by ammonium sulfate precipitation, followed by DEAE-Sephadex A-50 chromatography. LPS was removed by Detoxi-Gel Affinity Pak columns (Pierce, Rockford, IL). The amount of LPS in the flagellin samples, determined with quantitative Limulus Amebocyte lysate (LAL) kit, was 0.0027 endotoxin units (EU)/µg protein after the two steps of chromatography. In addition up to 1000 ng/ml purified flagellin failed to elicit the activation of Hela cells expressing functional TLR4, CD14 and MD2 (37) that was 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 (SFM, Invitrogen Life Technologies, Carlsbad, CA) in a humidified 5% CO₂ incubator at 37 °C. Before treatment, cells were cultured in growth factor-free and antibiotic-free keratinocyte basic medium (KBM, BioWhittaker, Walkersville, MD) for 16 h (growth factor starvation).

Cytokine ELISA
Secretion of TNF-α and IL-8 in cultured cells and macrophage inflammatory protein 2 (MIP-2) and IL-1β in B6 mouse sera and corneas was determined by ELISA. Human CECs were plated at $1 \times 10^6$ cells/well in six-well plates. After growth factor starvation, cells were pretreated with or without flagellin and then further challenged with live bacteria for 4 h. At the end of culture, 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 PBS with a glass micro tissue grinder, followed by centrifugation at 14,000g for 10 minutes. The ELISA was performed according to the manufacturer's instructions (R & D Systems).

**Infection protocol**

Female C57BL/6 mice (6–8 weeks old, weighing 20–24 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 ($n = 5$ per group per treatment) were injected with 100 ng (in 5 µL) *P. aeruginosa* flagellin subconjunctivally and 125 ng (in 100 µL) intraperitoneally (IP) per mouse, with the same amount of PBS injected as control. B6 mice were anesthetized with ether and placed beneath a stereoscopic microscope at 40 x magnification, and the cornea of the left eye was wounded with three 1-mm incisions using a sterile 25-gauge needle. A bacterial suspension (5 µL) containing $1 \times 10^6$ colony-forming units (CFU) of *P. aeruginosa* ATCC 19660.
strain, prepared as described earlier (15), was applied to the surface of the scarified cornea. Eyes were examined on daily basis to monitor the disease progression.

**Clinical Examination**

For clinical score assessment, mice were color-coded and examined in a blinded fashion by two independent observers at 1, 3, and 5, days p.i. to visually grade the severity of disease after *P. aeruginosa* infection. Ocular disease was graded and clinical scores were assigned using the previously (15, 17) described scale: 0, clear or slight opacity, +1, 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 PBS and flagellin-pretreated groups infected with strain 19660 were enucleated at 5 days p.i. 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) in 1:1:1 ratio at 4°C for 3 hours. Eyes were transferred into a fresh fixative after 1.5 hours and then dehydrated in graded ethanols, 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 hexadecyl trimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). The samples were then subjected to three freeze–thaw cycles, followed by centrifugation at 16 x g for 20 min. The supernatant was mixed with 50 mM phosphate buffer (pH 6.0), containing 16.7 mg/ml O-dianisidine hydrochloride and 0.0005% hydrogen peroxide at a 1:30 ratio for a total volume of 3 ml. The change in absorbance at 460 nm was continuously monitored for 5 minutes. The results were expressed as units of MPO/cornea. One unit of MPO activity corresponds to approximately 2.0 x 10^5 PMN (49).

Bacterial load in the Cornea

At 1, 3 and 5 days p.i., corneas (n = 5/group) from both groups of mice infected with ATCC 19660 strain were collected and the number 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. Plates were incubated for 24 hours at 37°C. Results were expressed as number of CFU/cornea ± standard error of the mean (SEM).

Mouse Corneal Real-Time RT-PCR

Real-time RT-PCR of mouse corneas was performed either as described (18) using Syber green or TaqMan technologies. 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 reaction. The real time primers used were either as previous described (17, 41) or purchased from Applied Biosystems. For PCR amplification, 1 µL each cDNA sample was used for each 25-µL PCR reaction. Real-time measurements were analyzed in duplicate in three independent runs (BioRad MyiQ system; Hercules, CA). Relative mRNA levels were calculated after normalizing to β-actin (18).

Statistical Analysis
An unpaired, two-tailed Student’s t-test was used to determine statistical significance for data from MPO assay. A nonparametric, Mann-Whitney U test was performed for bacterial counts, clinical score, and real-time PCR analyses between treated and control groups. Mean differences were considered significant at $P < 0.05$. Experiments were repeated at least twice to ensure reproducibility; pooled data from two separate experiments are shown.
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* PA01, induced human CECs to become tolerized, as manifested by impaired NF-κB activation and greatly decreased production of pro-inflammatory cytokines in response to *PA01* challenge. To determine whether PA01 flagellin also induces tolerance of human CECs to a different strain of *P. aeruginosa*, we pretreated HUCL cells with several dosages (10 to 100 ng/ml) of flagellin for 24 h and then challenged them with ATCC 19660, a cytotoxic strain with PA01 as the control. Both strains induced the 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 the pretreatment of the cells with flagellin in a concentration-dependent manner; with 50 ng/ml flagellin being sufficient to completely block the cytokine secretion induced by both *PA01* 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, *Staphylococcus 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 tested this hypothesis using C57BL/6 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 x 10^6 CFU of P. aeruginosa (strain ATCC19660) after needle-injury of the cornea (15). The corneal disease response was graded on different days post infection (p.i.) using the previously described scale (15). No significant difference was observed in clinical scores at 1 day post infection (1.47 vs. 1.21, PBS vs. flagellin pretreated, 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 PBS injected controls (1.22 vs. 2.9 at day 3, p < 0.0002; 1.21 vs. 3.89 at day 5, P< 0.0001, n=19).

Figure 2B shows the slit lamp microscopy of a flagellin pre-treated cornea at Day 5 p.i. (left panel, clinical score +1), displaying only slight corneal opacity seen in the peripheral corneal region due to residual infiltrating cells in the cornea and/or the anterior chamber whereas the placebo-treated eye perforated (Fig. 2B, right panel, clinical score +4). Histopathological analysis revealed that the eye of PBS-treated B6 mice (clinical score +4) showed a heavy cellular infiltrate in the cornea and anterior chamber 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 with little infiltration detected in the anterior chamber (left panel, Fig. 2C, clinical score +1).
Flagellin pretreatment reduces bacterial load in the cornea

Having demonstrated that significant number of corneas was scored 0 and 1 at 5 days p.i., we next examined the effect of flagellin-pretreatment on the bacterial clearance in the cornea (Fig. 3A). At 1 day p.i there were $5 \times 10^4$ CFU detected per cornea in the control PBS-injected mice, whereas the number of bacteria per cornea in flagellin-pretreated mice was $8 \times 10^3$, a 6 fold decrease. At 3 days p.i., average $4 \times 10^7$ CFU were found per cornea in the control PBS-injected mice and $7 \times 10^5$ CFU in flagellin-pretreated mice. The differences between these two groups were more striking at 5 days p.i.; the average CFU per cornea of the flagellin-pretreated mice was $1 \times 10^3$ whereas $8 \times 10^7$ CFU/cornea was detected in the corneas of control, PBS-injected mice. Of note, 3 out of 5 corneas were sterile with no bacterial growth found by plate counting assay (Fig. 3A). Thus, pretreatment of mice with TLR5 ligand enhances innate bacterial killing ability of the cornea, leading to the control of *P. aeruginosa* infection in B6 mice.

Figure 3B shows the effects of flagellin pretreatment on PMN infiltration in the cornea by assessing myeloperoxidase (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 in both the PBS-treated and flagellin-treated mice at this time. On day 3 and day 5 p.i., a much higher level of MPO activity was observed in the control, PBS-pretreated cornea compared to that at 1 day p.i. This increase in PMN infiltration, however, was muted by flagellin-pretreatment. Moreover, at 5 days in flagellin-pretreated
mice, the PMN infiltration was significantly less than that at 1 day and 3 days p.i., suggesting an enhanced resolution of inflammation associated with reduced bacterial load in the cornea.

**Flagellin pretreatment suppresses 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 the B6 cornea before and after *P. aeruginosa* infection (Fig. 4). While flagellin pretreatment alone did not elicit the expression of these cytokines in the cornea or in the blood (data not shown), it significantly attenuated their expression at the mRNA levels induced by *P. aeruginosa* at day 1 p.i. (p<0.05). At day 5 p.i. the levels of these cytokine were further elevated in the control mouse corneas, but declined in flagellin-pretreated ones, resulting in significant difference (p<0.001) in the levels of all 4 cytokines assessed. The inhibition of IL-1β and MIP-2 expression by flagellin pretreatment was also verified at the protein levels 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 expression of CRAMP (mouse homolog of hCAP-18) and iNOS in flagellin pretreated corneas using TaqMan Real-Time-PCR. In the corneas without *P. aeruginosa* infection, flagellin pretreatment induced 3-fold increase in CRAMP mRNA expression over PBS-treated controls 24 h post injection (Fig. 6). *P. aeruginosa* infection caused
an up-regulation of CRAMP mRNA and flagellin pretreatment exhibited no effects 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., CRAMP mRNA levels were declined in the corneas pretreated with or without flagellin. Unlike CRAMP, 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 when compared to the control PBS pretreated ones (3 fold increase over PBS-treated corneas). On day 3 p.i., the iNOS mRNA levels were further increased in both flagellin pretreated and control corneas with no significant difference observed between two groups. At 5 days p.i., while elevation in iNOS mRNA persisted in PBS treated corneas, it was dramatically declined in flagellin-treated 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 (PAMP) of Gram-negative bacteria recognized by TLR5, in preventing Pseudomonas keratitis. An one-time-administration of flagellin prior to bacterial inoculation greatly attenuated the expression of proinflammatory cytokines but not CRAMP and iNOS, significantly reduced PMN infiltration at late, but not early stage of infection and, most strikingly, greatly enhanced bacterial clearance in the cornea of B6 mice, leading to functional recovery of the corneas that would otherwise be perforated. Thus, flagellin can be exploited as novel anti-inflammatory and/or anti-infection therapy for bacterial keratitis and other infectious diseases.

The cornea is an immune-privilege avascular tissue in human body and possesses strong innate defenses which are essential for the 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 pre-exposure of cultured CECs to flagellin dampened the production of proinflammatory cytokines but enhanced the expression of antimicrobial genes induced by live *P. aeruginosa* ([23] and 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 disease outcome, as manifested by the preserved structural integrity of the cornea and 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 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 induce 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 6 fold reduction of viable bacterial count in the cornea at 1 day p.i. More strikingly, by day 5 when the control corneas were perforated with \(\sim 10^8\) CFU bacteria per cornea, flagellin-treated corneas were either sterile or contained very limited number of bacteria as compared to that 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 non-antibiotic regimen leads to a total elimination of *P. aeruginosa*
in the cornea of B6 mice, a susceptible strain (15). We suggest that the greatly enhanced bacterial clearance induced by flagellin is related to the induced expression of 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 the up-regulation of CRAMP (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 PMO determination, we suggest that 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 is invading the flagellin-exposed epithelial cells, leading to rapid reduction of bacterial loading. Moreover, *P. aeruginosa* infection triggers a great increase in CRAMP expression, a potential innate defense mechanism against infection in the immune-privilege 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 when 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). The role of CRAMP as part of corneal innate defense mechanism is recently confirmed by the use of CRAMP-deficient mice that become susceptible (cornea perforates) in otherwise resistant (no corneal perforation) mouse strain (16). In addition to CRAMP, we also observed a strong induction of iNOS that produces NO, a free radical gas with
important anti-microbial action (1, 4), in the B6 cornea at day 1 p.i.. The levels of iNOS at day 1 p.i. in flagellin pretreated corneas was several folds higher than that in the control group and elevated to a higher level in both group by day 3. Importantly, as excessive production of NO is known to cause tissue damage, rapid down-regulation of iNOS was observed in flagellin treated, but not the control 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 liver, kidney and gut (2). Hence, we suggest that CECs are a major source of iNOS in the cornea of B6 corneas at least at 1 day p.i. when macrophage infiltration is not yet predominant (14). Recently, studies in other systems suggested that epithelium-derived cathelicidin substantially contributed to the protection of the urinary tract against infection (7) and that stimulation of epithelial cells to produce LL-37-like protein with sodium butyrate prevented the colon Shigella infection (36). Thus, augmented expression of antimicrobial molecules by flagellin pretreatment may contribute to increased bacterial clearance observed in the corneas of B6 mice.

Another contributing factor for bacterial clearance in the cornea is the PMN infiltration. Interestingly, although the bacterial induced expression of chemokine such as MIP-2 in B6 mouse cornea or IL-8 in cultured human CECs was downregulated (23), the PMN infiltration was not significantly affected in flagellin pretreated corneas on day 1 p.i as compared with the control. Flagellin present in the cornea and/or blood before infection may prime the PMNs and increased their bacterial recognition and bactericidal activity, as that shown in bacterial lipoprotein-pretreated mice (45). It remains to be determined the nature of
chemokines recruiting PMN to the infected corneas. 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). Taken together, the combined effects of elevated antimicrobial activity, significant reduced expression of proinflammatory cytokines, and unaffected PMN infiltration contribute to the increase in bacterial clearance at the early stage on infection, leading to significant improvement in the outcome of PA infection in B6 mice.

Although effective, the use of flagellin as a keratitis prophylactic given systemically and simultaneously subconjunctivally is not likely to gain clinical approval. For potential ocular application, we recently observed that subconjunctival injection, a routinely performed procedure in ophthalmologists’ office, of flagellin ameliorated corneal pathology and reduced *P. aeruginosa* numbers in the eye. Hence, subcoinjunctival injection of flagellin might be used as a prophylactic measure to reduce the potential of bacterial infection after ocular surgeries such as corneal transplantation and diabetic vitrectomy. Furthermore, premix of isolated flagella with *P. aeruginosa* was also found to reduce rate of corneal perforation (38), a phenomenon that was confirmed using purified flagellin (Kumar and Yu, unpublished results). Considering the fact that most bacterial infection of the cornea only occurs when epithelial barrier is breached, such as that caused by contact lenses wearing, 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 AMPs. Thus, flagellin presence at the ocular surface through eye drop or in the
contact lens solution may have prophylactic effects for preventing contact lens associated infection.

In summary, we have 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 manipulating tolerance induced by TLR ligands can be exploited as a prophylactic or therapeutic approach to dampen inflammation and control infection. Ongoing studies from our group are exploring the specific role of epithelium in corneal innate immunity and the mechanisms of inducing endogenous antimicrobial peptides to prevent contact lens associated corneal infections.
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Figure Legends

Figure 1. Flagellin pretreatment dampens production of TNF-α in response to bacterial challenge in human CECs. HUCL cells were cultured without (0) or with 10, 20, 50, 100 ng/ml flagellin for 24 h. After being washed twice with PBS, cells were challenged with P. aeruginosa PA01 (solid bars) or ATCC 19660 (gray bars) at an MOI of 100 for 4 h. TNF-α and IL-8 secretion into culture supernatants was assayed by ELISA. The amount of cytokines in cultured media was expressed as ng of cytokine per mg cell lysate. All values were expressed as mean ± standard deviation (SD).

Figure 2. Flagellin Pretreatment diminishes Clinical Symptoms of Keratitis in C57BL/6 mice. C57BL/6 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 5/8 gauge needle and the scarified corneas were challenged topically with 1.0 x 10^6 CFU of P. aeruginosa ATCC 19660 strain. Clinical score (A) of ocular disease response was assigned on day 1, 3 and 5 p.i and slit lamp examination (B) was performed on day 5 p.i. on each infected corneas. Histopathology was performed on representative infected cornea (score +4 for PBS treated and score +1 for PBS treated) at 5 days p.i. (C). Arrow:
Figure 3. PMN infiltration and bacterial clearance in flagellin-pretreated corneas. Mice were treated with PBS (n = 5) or flagellin (n = 5) as described, followed by ocular inoculation of 1 x 10^6 CFU of *P. aeruginosa* strain ATCC 19660 per cornea as described in Fig 2. At the indicated times after infection, the corneas were excised and subjected plate bacterial counting (A) or the determination of MPO activity as the parameter for PMN infiltration (B). Data are expressed as the arithmetic mean ± SEM from five mice in each group. *P* values were generated using paired Student’s *t*-test for MPO assay and Mann-Whitney test for bacterial count.

Figure 4. The expression of cytokine mRNA in *P. aeruginosa* infected and flagellin-pretreated corneas. Corneas (n=5 each group) of C57BL/6 mice were taken at 1 and 5 days p.i. as described in legend of Figure 2. Real-time RT-PCR was used to assess cytokine mRNA levels in flagellin vs. PBS pretreated corneas. mRNA expression levels of IL-1β, MIP-2, IFN-γ, and IL-12 were normalized to β-actin and presented as relative ratio. Flagellin pretreatment significantly (*P* <0.001) reduced the expression of these cytokines.

**FIGURE 5.** ELISA analysis of corneal pro-inflammatory cytokine protein levels after *P. aeruginosa* infection. Corneas of C57BL/6 mice were taken at 24h after flagellin pretreatment and 1 day p.i. as described in legend of
figure 2. Protein levels for IL-1β and MIP-2 were assayed by ELISA. All data are mean ± SEM (n = 5/group/time).

Figure 6. The expression of CRAMP and iNOS mRNA in *P. aeruginosa* infected and flagellin-pretreated corneas. Corneas (n=5 each group) of C57BL/6 mice were taken after pretreatment on day 0 (without infection), 1, 3 and 5 p.i. Real-time RT-PCR was used to assess mRNA levels of CRAMP and iNOS in normal, flagellin and PBS pretreated corneas. mRNA expression were normalized to β-actin and presented relative expression. Flagellin pretreatment significantly (*P < 0.05, **P < 0.001) induced the expression of CRAMP and iNOS in mouse corneas.
Figure 1

The figure shows the expression levels of TNF-\(\alpha\) and IL-8 cytokines in cell lysates following treatment with different concentrations of PAO1 and ATCC 19560 bacteria. The x-axis represents the concentrations of PAO1 and ATCC 19560 (0, 10, 20, 50, 100 ng/ml), and the y-axis represents the cytokine secretion levels (ng/mg of total cell lysate).
Figure 2A
Figure 2B and C

B. Slit Lamp Microscopy

C. Histopathology

Flagellin

PBS
Figure 3

A.

B.

Day 1 p.i.  Day 3 p.i.  Day 5 p.i.

Day 1 p.i.  Day 3 p.i.  Day 5 p.i.
Figure 4

The figure shows the relative mRNA expression of various genes (IL-1β, MIP-2, IFN-γ, IL-12) at 1 dpi and 5 dpi after treatment with PBS or Flagellin. The y-axis represents the relative mRNA expression, while the x-axis lists the genes under observation. The bars indicate the mean expression levels with error bars showing the standard deviation. The figure highlights the differential expression patterns in response to the two treatments at different time points.
Figure 5

The figure shows a bar graph comparing the levels of MIP-2 and IL1-β (pg/μg of cornreal lysate) across different conditions. The conditions include Normal, 24h Flag, 1 dpi PBS, and 1 dpi Flag. The y-axis represents the concentration in pg/μg of cornreal lysate, ranging from 0 to 800 for MIP-2 and from 0 to 1000 for IL1-β.
Figure 6

Relative mRNA expression

CRAMP

- PBS
- Flagellin

iNOS

- 0 dpi
- 1 dpi
- 3 dpi
- 5 dpi

* Significant difference
** Highly significant difference