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Modulation of Corneal Epithelial Innate Immune Response to *Pseudomonas* Infection by Flagellin Pretreatment

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Abstract

Purpose—A prior study showed that Toll-like receptor (TLR)-5 recognizes *Pseudomonas aeruginosa* flagellin and triggers the production of proinflammatory cytokines in human corneal epithelial cells (HCECs). The present study was conducted to determine how the inflammatory response is modulated after TLR activation in HCECs.

Methods—HUCL cells, a telomerase-immortalized HCEC line, and primary cultures of HCECs were pretreated with low-dose flagellin and then challenged, with either a high dose of flagellin or with *Pseudomonas*. NF- κ B activation was determined by the extent of I κ B- α phosphorylation and degradation and of nuclear p65 DNA binding. The amount of cytokines in the culture media was assessed by ELISA. The activation of p38 and JNK and the cellular expression of TLR5 were determined by Western blot analysis. Cell surface distribution of TLR5 was assessed by flow cytometry. The expression and secretion of antimicrobial peptides were assessed by semiquantitative RT-PCR and slot– blot analysis, respectively.

Results—Pre-exposure (12–24 hours) of HCECs to low-dose flagellin induced a state of tolerance, characterized by impaired activation of the NF- κ B, p38, and JNK pathways and reduced production of IL-8 and TNF- α on subsequent challenge with a high dose of flagellin. Flagellin-induced tolerance did not alter the cellular level and surface distribution of TLR5. Furthermore, flagellin priming of HCECs dampened the inflammatory response of HCECs to live *Pseudomonas*. *Pseudomonas*-induced upregulation of antimicrobial genes such as *hBD2* and *LL-37* was augmented, even in tolerized HCECs.

Conclusions—Pre-exposure of the ocular surface to TLR agonists may induce protective mechanisms that not only modulate the host inflammatory response but also provide an innate defense against bacterial infection in the cornea.

Keratitis caused by *Pseudomonas aeruginosa* is a potentially vision-threatening condition that requires prompt diagnosis and treatment to prevent vision loss.¹ The corneal epithelium functions as a barrier that separates the eye from the outside environment and contributes to homeostasis and host defense of the cornea.^{2,3} Like in other tissues, corneal epithelial cells constitute the first line of defense against microbial pathogens and therefore must possess the ability to detect the presence of pathogenic bacteria. The ability of epithelial cells to recognize and respond to microbial components is attributed largely to the family of Toll-like receptors

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(TLRs).^{4,5} Among the TLRs, TLR5, via recognition of flagellin, is a sensor for detecting Gram-negative bacteria in a variety of cells and tissues.^{3,6–8} In HCECs, purified flagellin induces NF- κ B activation and proinflammatory cytokine secretion in a TLR5-dependent manner,⁹ but recognition of lipopolysaccharide (LPS) by these cells is controversial.^{10,11} TLR5-knockout and other studies have highlighted the importance of this pattern-recognizing receptor in microbial recognition, particularly at a mucosal surface.^{7,12,13}

The recognition of microbial products by TLRs elicits a cascade of signal transduction pathways, resulting in the production of proinflammatory cytokines/chemokines and the expression of antimicrobial molecules that kill the invading pathogens at the mucosal surface.^{14–16} 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.² To date, the TLR signaling pathways leading to inflammatory response have been well documented,³ but the underlying cellular mechanisms that directly control cytokine production after TLR stimulation are largely unresolved. Recent studies revealed that pre-exposure to a TLR ligand can result in impaired NF- κ B activation and greatly decreased production of proinflammatory cytokines in response to subsequent high-dose TLR ligand challenge in a variety of cells including epithelial cells,^{17–22} a phenomenon resembling endotoxin tolerance.²³ In the present study, we used a dose of flagellin that causes NF- κ B activation, but only minimal proinflammatory cytokine production, to induce tolerance or reprogramming of HCECs to the subsequent high-dose flagellin and live *P. aeruginosa* challenge. Our results suggest that flagellin-induced tolerance and reprogramming represent a negative-feedback mechanism invoked to induce resolution of inflammation and to restore homeostasis after TLR activation triggered by exposure to invading pathogens in the cornea.

Materials and Methods

Bacterial Strains and Flagellin

PA01, an invasive strain of *P. aeruginosa*, was used for the studies. Flagellin was prepared from PA01 by ammonium sulfate precipitation, followed by DEAE-Sephadex A-50 chromatography. LPS was removed by gel-affinity separation columns (Detoxi-Gel Affinity Pak columns; Pierce, Rockford, IL). The amount of LPS in the flagellin samples was determined with a quantitative limulus amoebocyte lysate (LAL) kit and, after two steps of chromatography, the levels were 0.0027 endotoxin units (EU)/ μ g protein. As TLR5 recognizes all types of flagellin except that prepared from *Helicobacter pylori*,^{24,25} it is thought that flagellin prepared from other flagellated bacteria also induces cell tolerance, as shown in this study and by other groups.^{18,26}

Cell Line

Human telomerase-immortalized corneal epithelial (HUCL) cells,²⁷ 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, the cells were cultured in growth factor-free and antibiotic-free keratinocyte basic medium (KBM; Bio-Whittaker, Walkersville, MD) for 16 hours (growth factor starvation). To verify the results obtained from HUCL cells, HCECs were isolated from human donor corneas obtained from the Michigan Eye Bank. The epithelial sheet was separated from the underlying stroma after overnight Dispase treatment. The dissected epithelial sheet was trypsinized, and the epithelial cells were collected by centrifugation (500g, 5 minutes). HCECs were cultured in keratinocyte growth medium (KBM supplemented with growth factors; BioWhittaker) in T25 flasks coated with fibronectin-collagen (FNC) and used at passage 3.

Western Blot Analysis

Cells challenged with either flagellin or bacteria were lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 100 mM Tris-HCl [pH 7.5], 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, and 3.5 mM sodium orthovanadate). A protease inhibitor cocktail (aprotinin, pepstatin A, leupeptin, and antipain, 1 mg/mL each) and 0.1 M phenylmethylsulfonyl fluoride were added to the RIPA buffer (1: 1000 dilution) before use. The protein concentration in cell lysates was determined with the bicinchoninic acid detection assay (MicroBCA; Pierce). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) and electroblotted onto nitrocellulose membranes (0.45- μ m pores; Bio-Rad, Hercules, CA). After blocking for 2 hours in Tris-buffered saline/Tween (TBST; 20 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween) containing 5% nonfat milk, the blots were probed with primary antibodies overnight at 4°C. The membranes were washed with 0.05% (vol/vol) Tween 20 in TBS (pH 7.6) and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 60 minutes at room temperature. Protein bands were visualized by chemiluminescence (Supersignal reagents; Pierce).

NF- κ B DNA-Binding Activity

HUCL or primary cells were grown to 80% confluence in 100-mm culture dishes and treated with or without 50 ng/mL flagellin for 24 hours. For nuclear extracts, the cells were rinsed in cold PBS, scraped, and centrifuged. The cells were lysed by incubation on ice for 15 minutes in a hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol [DTT], and protease inhibitor mixture) and centrifuged. The cell pellets were resuspended in the same buffer. Further cell disruption was achieved by repetitive filling and flushing of cell suspensions with a syringe having a narrow-gauge needle. The resultant suspensions were centrifuged. The crude nuclear pellets were then resuspended in extraction buffer (20 mM HEPES, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, and 1 mM DTT) containing the protease inhibitor cocktail, and the nuclei were disrupted with a fresh syringe. The nuclear suspensions were incubated on ice for 30 minutes with occasional tapping and centrifuged. A total of 40 ng of the nuclear extracts was added to assay wells (Mercury TransFactor NF- κ B p65 Kit; BD Biosciences, Franklin Lakes, NJ), which were blocked with 1 $\times$ transcription factor/blocking buffer, and incubated for 60 minutes at room temperature. After washing three times with 1 $\times$ transcription factor/blocking buffer, p65 Ab (1:500 diluted) was added to the wells and incubated for 60 minutes at room temperature, followed by 30 minutes' incubation of HRP-conjugated secondary Ab. Then, 1 $\times$ transcription factor buffer was applied to wash the wells. Finally, tetra-methylbenzidine substrate was added and incubated for 10 minutes at room temperature, and the reaction was then stopped by 1 M H₂SO₄. The absorbance was read at 450 nm on a microplate reader.

ELISA Measurement of Cytokines

Secretion of TNF- α and IL-8 was determined by ELISA. It should be mentioned that other cytokines such as IL-6 and IL-1 β , the expression of which was also blunted in flagellin pretreatment (data not shown), can also serve as markers for the epithelial inflammatory response. HCECs were plated at 1 $\times$ 10⁶ cells/well in six-well plates. After growth factor starvation, the cells were pretreated with or without flagellin and then further challenged for various periods either with high-dose flagellin or with live *P. aeruginosa* (10⁸ CFU in 1 mL culture medium per well). At the end of the culture period, the media were harvested for measurement of cytokines. The ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The amount of cytokines in cultured media was expressed as nanogram per milligram of cell lysate. All values are expressed as the mean

± SD. Statistical analysis was performed with analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from HUCL cells (TRIzol solution; Invitrogen) according to the manufacturer's instructions, and 2 μ g of total RNA was reverse-transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). cDNA was amplified by PCR by using primers for human hBD2, LL-37 IL-8, TNF- α , and GAPDH, as described earlier.^{16,28} The PCR products and internal control GAPDH were subjected to electrophoresis on 1% agarose gels containing ethidium bromide. Stained gels were captured by using a digital camera, and band intensity was quantified with 1-D image-analysis software (EDAS 290 system; Eastman Kodak, Rochester, NY).

Slot-Blot Analysis of hBD2 and LL-37 Protein

The accumulation of hBD-2 and LL-37 in the culture media of pathogen-challenged HCECs was detected by slot-blot analysis, as described previously.¹⁶ Briefly, culture media were collected at 0, 2, and 4 hours after infection and centrifuged, and 100 μ L was applied to a nitrocellulose membrane (0.2- μ m pores; Bio-Rad) by vacuum with a slot-blot apparatus (Bio-Rad). The membrane was fixed by incubation with 10% formalin for 2 hours at room temperature followed by blocking in TBS containing 5% nonfat powdered milk for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with rabbit anti-hBD-2 and LL-37 antibodies diluted 1:1000 in TBS containing 5% nonfat powdered milk, 5% goat serum, 0.05% Tween-20, and 0.02% sodium azide. After washing, the membrane was incubated for 1 hour at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:2000 with 5% nonfat powdered milk. Immunoreactivity was visualized with chemiluminescence (Supersignal reagents; Pierce).

Flow Cytometry Analysis

HUCL cells were dispensed (1×10^6 cells/mL) into conical plastic tubes and centrifuged at 100g at 4°C for 10 minutes. The cells were washed with Hanks' balanced salt solution containing 0.5% bovine serum albumin, incubated with the same buffer containing anti-TLR5 antibody or isotype-matched IgG (5 μ g/mL) for 30 minutes at 4°C, and washed and incubated for 30 minutes at 4°C with the corresponding secondary FITC-conjugated antibody (5 μ g/mL). A flow cytometer (FACScan; Immunocytometry Systems, BD Biosciences) was used for cytometric analysis.

Results

Impairment of NF- κ B Activation Induced by Flagellin in HCECs

To determine whether pre-exposure to flagellin induces a state of tolerance in corneal epithelial cells, we pretreated HUCL cells with different dosages of flagellin and assessed cell response to subsequent challenge with a higher dose of flagellin using I κ B- α phosphorylation and degradation as markers for NF- κ B activation. When rechallenged with 100 ng/mL flagellin (Fig. 1A), cells pretreated with flagellin as low as 5 ng/mL for 24 hours had reduced I κ B- α phosphorylation compared with cells without pretreatment. Incubation of HCECs with 50 ng/mL flagellin for 24 hours almost completely abolished I κ B- α phosphorylation/degradation when rechallenged with 100 ng/mL flagellin. The time needed for HCECs to acquire tolerance to secondary flagellin challenge was determined with 50 ng/mL flagellin (Fig. 1B). A decrease in responsiveness, as determined by I κ B- α phosphorylation/degradation, was observed as early as 4 hours after the initial exposure to flagellin. I κ B- α phosphorylation/degradation stimulated by 100 ng/mL flagellin was significantly reduced after 12 hours of flagellin incubation and

almost completely diminished after 18 hours or longer pretreatment with flagellin. Furthermore, we tested the effect of flagellin pretreatment on DNA binding activity of the p65 subunit of NF- κ B using ELISA. In naive cells, flagellin challenge increased the DNA binding activity of p65 subunit, whereas its binding activity was completely attenuated in HCECs pre-exposed to 50 ng/mL flagellin (Fig. 1C).

The effects of different concentrations of flagellin on the activation of NF- κ B and on proinflammatory cytokine production were assessed (Fig. 2). Detectable levels of I κ B- α phosphorylation were induced by flagellin at a concentration as low as 10 ng/mL; at 50 ng/mL or higher, flagellin significantly induced I κ B- α phosphorylation and degradation (Fig. 2A). The densitometry analysis showed that 50 ng/mL flagellin decreased I κ B- α levels by twofold, compared with the effect of 20 ng/mL flagellin, whereas phosphorylation of I κ B- α was increased by 3-fold. To assess proinflammatory response, we used ELISA to measure the production of IL-8. Although 10 ng/mL flagellin essentially exhibited no effect on IL-8 production, 50 ng/mL flagellin stimulation resulted in a 1-fold increase in IL-8 secretion when compared with the control. In contrast, 7.1 times more IL-8 was produced by cells treated with 250 ng/mL flagellin (Fig. 1B). Thus, 50 ng/mL may represent a dosage that is sufficient to induce NF- κ B activation but not proinflammatory cytokine production.

In addition to NF- κ B, JNK, and p38 are other major pathways activated on TLR activation.²⁹ Figure 3 shows the effects of flagellin pretreatment on TLR-mediated MAPK activation. Challenge of HCECs with 250 ng/mL flagellin induced the phosphorylation of P38 and JNK. This effect was markedly reduced or even abolished by pre-exposure of cells to 50 ng/mL flagellin for 24 hours, suggesting that prolonged incubation with low-dose flagellin impairs TLR5-mediated proinflammatory signaling pathways. As expected, the high-dose flagellin-induced I κ B- α phosphorylation and degradation were also inhibited by flagellin pretreatment. Taken together, these results demonstrate that the tolerized corneal epithelium becomes insensitive to flagellin in activation of the TLR-mediated proinflammatory signaling pathways.

Effect of Flagellin Pretreatment on Expression of TLR5 in HCECs

To understand the mechanisms underlying flagellin-induced tolerance, we next investigated whether flagellin suppresses expression and/or surface distribution of TLR5. Western blot analysis showed that the levels of immunoreactivity of TLR5-specific antibody remained unchanged during the development of tolerance (Fig. 4A). Furthermore, the surface expression of the TLR5 detected by flow cytometry was not altered in HUCL cells by flagellin pretreatment (Fig. 4B). Thus, flagellin-induced tolerance does not occur through the downregulation of TLR5 expression or the alteration of surface distribution.

Effect of Flagellin Pretreatment on the Production of Proinflammatory Cytokines

Having demonstrated that prior exposure of HCECs to flagellin resulted in impaired activation of NF- κ B and MAPKs in response to a secondary high-dose flagellin challenge, we next sought to determine the effect of flagellin pretreatment on production of proinflammatory cytokines. Both HUCL cells and primary HCECs were incubated with or without 50 ng/mL flagellin for 24 hours and then were rechallenged with 250 ng/mL flagellin. Both HUCL cells and primary HCECs secreted significantly high levels of TNF- α and IL-8 in response to 250 ng/mL flagellin challenge (Fig. 5A, 5C). This elevated production was retarded by the pretreatment of the cells with 50 ng/mL flagellin. Challenge with live *P. aeruginosa* (PA01 strain) also stimulated the secretion of TNF- α and IL-8 in these cells, and pre-exposure to flagellin muted the proinflammatory response to live bacterial challenge (Figs. 5B, 5D). Thus, flagellin pretreatment induced the development of the tolerance phenotype in HCECs, not only in response to TLR5 ligand but also in response to live bacteria with multiple virulence factors.

Effect of Flagellin Pretreatment on the Expression of Antimicrobial Genes Induced by *P. aeruginosa* in HCECs

We have shown in a prior study that activation of HCECs via TLRs leads to an increase in bactericidal–static activity in the culture media.¹⁶ To determine whether innate antimicrobial activity is regulated differently from that of proinflammatory cytokines by tolerance development, we investigated the effects of flagellin pretreatment on the expression of antimicrobial peptides in HCECs in response to live *P. aeruginosa* challenge. As expected, the expression of IL-8, TNF- α , and the antimicrobial genes, human- β -defensin2 (*hBD2*) and cathelicidin, which produces antimicrobial peptide LL-37, at the mRNA levels was significantly increased in HUCL cells after *P. aeruginosa* challenge. The upregulated expression of proinflammatory cytokines was attenuated by flagellin pretreatment 24 hours before bacterial challenge (Fig. 6A). In marked contrast, the *P. aeruginosa*–induced expression of *hBD2* and cathelicidin was augmented by flagellin preexposure. The secretion of these inducible antimicrobial peptides was also measured in the conditioned media of HUCL cells. Concomitant with elevated expression of *hBD-2* and LL-37 mRNA, *hBD2* and LL-37 secretion was induced by *P. aeruginosa* challenge, and flagellin pretreatment clearly augmented their secretion in PA-challenged cells.

Discussion

In the present study, we demonstrated that prolonged exposure of corneal epithelial cells to flagellin, a major pathogen-associated molecular pattern (PAMP) of Gram-negative bacteria recognized by TLR5, leads to the development of tolerance in HCECs, characterized by an impaired inflammatory response, not only to subsequent high-dose flagellin, but also to live bacteria. Moreover, whereas the expression of proinflammatory cytokines was suppressed, the expression of antimicrobial genes was not affected or even augmented, suggesting that TLR5-mediated tolerance to bacteria reflects a functional switch of HCECs rather than a general hyporesponsiveness to flagellin and that the tolerance phenotype is the result of cell reprogramming. As the expression of antimicrobial molecules in epithelial cells has been shown as a key to control infection,^{30,31} modulation of corneal tolerance may be exploited as a novel approach for anti-inflammatory therapy.

In an intact epithelial barrier, the epithelium must remain hyporesponsive to its normal flora, including bacteria and their products in the tears or on the mucosal surface of the cornea.³ Once the epithelial barrier is breached, however, bacteria may invade the epithelium. The pattern recognizing receptors, such as TLR5 and TLR2 expressed in the metabolically active cells of corneal epithelium, are now exposed to the pathogens or PAMPs such as flagellin or lipoproteins.^{9,32} We have shown in earlier work that HCECs express multiple TLRs and that they recognize and respond to Gram-positive and -negative bacteria, possibly through TLR2 and TLR5, respectively.^{16,32} Indeed, Sun et al.³³ showed that the exposure of corneal epithelium to heat- or UV-inactivated *Staphylococcus aureus* induces neutrophil recruitment to the corneal stroma in a TLR2 and MyD88 dependent manner, suggesting a role of epithelium, via the action of TLRs, in PMN recruitment. As for Gram-negative bacteria such as *P. aeruginosa*, we observed that HCECs do not recognize LPS as they lack MD2 (Yu, unpublished results, 2006), in line with other mucosal epithelial cells.^{10,34} We showed that the initial challenge of cells with flagellin induces a proinflammatory response and proposed that TLR5 is a major sensor on the ocular surface for detecting *P. aeruginosa*. In line with this, a recent *in vivo* study revealed that TLR5 regulates the innate immune response in the urinary tract.¹² In the present study, we explored ways to manipulate the TLR5-mediated inflammatory response, and we report that pre-exposure of HCECs to flagellin impaired NF- κ B activation and attenuated the production of proinflammatory cytokines, reminiscent of TLR-mediated cell tolerance.²³ Moreover, we observed that this tolerance could be induced with a flagellin

dosage that is sufficient to induce NF- κ B activation, as evidenced by the increased phosphorylation and degradation of I κ B- α , but not robust production of proinflammatory cytokines in vitro. This is of clinical relevance, as induction of inflammation is an undesired effect. Remarkably, cells exposed to this flagellin dosage also showed a blunted cytokine response to live *P. aeruginosa*. Thus, muting of TLR5-mediated signaling pathways appears sufficient to attenuate the inflammatory response of corneal epithelial cells to Gram-negative bacteria, and targeting TLR5 with flagellin may be a useful pharmacologic approach to the modulation of inflammation associated with infection in the cornea.

The role of antimicrobial peptides (AMPs), especially those produced by epithelial linings, in limiting infection has been the subject of several recent studies.^{30,31} We showed in an earlier study that activation of TLR2 results in induction of hBD2 production in HCECs and in significantly enhanced bactericidal activity in their conditioned medium.¹⁶ In the present study, we showed that the expression of hBD2 and LL-37 was upregulated by *P. aeruginosa* challenge. Upregulation of hBD2 in HCECs was also induced by IL-1, *P. aeruginosa*-conditioned medium, and injury, as reported by other groups.^{35–37} LL-37, the only cathelicidin-derived antimicrobial peptide found in humans, is a 37-residue, amphipathic, helical peptide with a broad spectrum of antimicrobial activity.³⁸ Its expression in HCECs is upregulated by wounding.³⁹ LL-37 kills *P. aeruginosa* in vitro³⁹ and provides an innate defense against corneal *P. aeruginosa* infection in 129/SVJ mice (Huang L, et al. *IOVS* 2007;48:ARVO E-Abstract 4725). In a striking contrast to the suppressed cytokine expression, we showed that the *P. aeruginosa*-induced expression and secretion of hBD2 and LL-37 were augmented by flagellin pre-exposure. Thus, innate antimicrobial activity can be triggered independent of the release of proinflammatory molecules in the cornea. Furthermore, detection of LL-37 in the conditioned media of *P. aeruginosa*-challenged HUCL cells suggests that the mechanism for processing cathelicidin to antimicrobial peptide LL-37 is also activated in HCECs. The role of LL-37 and hBD2, individually and in combination for their synergistic and additive effects,^{40,41} in epithelial innate killing of bacteria¹⁶ is currently under investigation in our laboratory. Recent studies revealed that epithelium-derived cathelicidin substantially contributes to the protection of the urinary tract⁴² and the cornea against bacterial infection and that stimulation of epithelial cells to produce LL-37 with sodium butyrate prevents the colon *Shigella* infection.⁴³ TLR5 was found to be a key pattern-recognizing receptor for regulating the innate immune response in the urinary tract.¹² Hence, it is of interest to note that the stimulation of the epithelial linings to produce endogenous AMPs has been proposed as a radical and revolutionary alternative to the conventional way of treating acute infectious diseases.^{30,31} Thus, as *P. aeruginosa* is an opportunistic pathogen and its flagellin is a predominant immunostimulant for the epithelial cells, administration of flagellin could serve as a prophylactic and/or therapeutic means of preventing corneal infection, particularly in patients who are known to be at risk, such as contact lens wearers.

In summary, we have identified an anti-inflammatory mechanism induced by prolonged activation of TLR5 in cultured HCECs. Our findings suggest that manipulating TLR signaling and tolerance using their ligands can be used to dampen inflammation and control infection of the cornea.

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Figure 1.

Flagellin-elicited NF- κ B activation in flagellin-pretreated HCECs: dose and time-dependent course studies. HUCL cells were incubated with various concentrations of flagellin for 24 hours: (A) with 50 ng/mL flagellin before a second 1-hour incubation with various concentrations of flagellin; (B) or with 50 ng/mL flagellin for various periods before a second 1-hour incubation with 100 ng/mL flagellin, with unstimulated cells as the control. (C) Total cell lysate was prepared after the second flagellin challenge and analyzed for phospho-I κ B- α (I κ B- α) and degradation (I κ B- α) by immunoblot analysis. NF- κ B p65 DNA-binding activity was detected by ELISA ($n = 3$) and colorimetry. Results are representative of those in three independent experiments. 1^o, primary treatment; 2^o, secondary treatment.

Figure 2. Flagellin-stimulated I κ B- α phosphorylation and degradation and IL-8 secretion in HCECs. HUCL cells were stimulated with different concentrations of flagellin as indicated for 1 hour, and the cell lysates were subjected to SDS-PAGE, followed by phospho-I κ B α (P-I κ B α) and I κ B α immunoblot analysis (**A**). IL-8 secretion from supernatant of cultured HUCL cells challenged with 10, 50, and 250 ng/mL flagellin for 12 hours was analyzed by ELISA (**B**). The amount of cytokines was normalized with protein concentration of cell lysate (ng/mg cell lysate). The data shown are representative of triplicate experiments.

Figure 3.

Activation of MAPK signaling was inhibited in flagellin-tolerized cells. HUCL cells were either pretreated with 50 ng/mL flagellin or left untreated for 24 hours and then challenged with 250 ng/mL flagellin for the indicated times. Total cell lysates were blotted with antibodies specific for phospho-p38, -JNK, and -IκB-α. The cellular levels of these proteins were assessed in parallel with Western blot analysis. Flagellin-pretreated cells showed diminished MAPK signaling compared with untreated naive cells. The data shown are representative of duplicate experiments.

Figure 4.

Expression of TLR5 in unstimulated and flagellin-tolerized HCECs. (A) HUCL cells were stimulated with flagellin (50 ng/mL) and, at the indicated times, were lysed for TLR5 detection by Western blot analysis. (B) HUCL cells were cultured with 50 ng/mL for 24 hours and were then stained with anti-TLR5 antibody or isotype control IgG and analyzed by flow cytometry. Cell surface expression of TLR5 was determined by flow cytometry. Control cells remained unstimulated. Data are representative of results in two independent experiments.

Figure 5.

Flagellin-induced tolerance to a second flagellin or PA01 challenge in the production of TNF- α and IL-8. Primary HCECs (passage 3) or HUCL cells were cultured with or without 50 ng/mL flagellin for 24 hours. After being washed twice with PBS, the cells were stimulated with 250 ng/mL flagellin (A, C) or live PA01 (B, D) for 4 hours. TNF- α (A, B) and IL-8 (C, D) secretion into culture supernatants was assayed by ELISA. Data represent the mean $\pm$ SD of results in five independent experiments. * $P < 0.001$. 1^o, primary treatment; 2^o, secondary treatment.

Figure 6.

Effect of flagellin pretreatment on PA01-mediated gene expression in HCECs. HUCL cells were cultured in KBM in the absence (Medium) or presence (Flag) of 50 ng/mL flagellin for 24 hours and then challenged with *P. aeruginosa* (multiplicity of infection, 100). At the indicated times, the cells were processed for semiquantitative RT-PCR, to assess mRNA expression of IL-8, TNF- α , hBD2, and LL-37, with GAPDH as the internal control (A). The secretion of antimicrobial peptides into the culture media was assessed by slot-blot analysis (B). Data are representative of results in two independent experiments.