Topical Flagellin-Mediated Innate Defense against *Candida albicans* Keratitis

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PURPOSE. This study was conducted to investigate whether flagellin, the sole ligand of Toll-like receptor-5 (TLR5), induces an innate defense that is sufficient to protect injured corneas from *Candida albicans*.

METHODS. Scarified corneas of adult B6, *TLR5*^{-/-}, *Camp*^{-/-} (cathelicidin-related antimicrobial peptide), or PMN-depleted mice were pretreated with *Pseudomonas aeruginosa* flagellin or a mutant and then were inoculated with *C. albicans*. The corneas were compared for disease progression, cytokine and *Camp* expression, and PMN infiltration before and after *C. albicans* infection. Disease progress was recorded by digital photography and clinical scoring, cytokine levels were determined by ELISA, the levels of *Camp* gene product were assessed by Western blot, and PMN infiltration was measured by MPO determination and immunohistochemistry.

RESULTS. Topical application of flagellin induced profound protection against *Candida* keratitis in a TLR5-dependent manner. The improved disease outcome including reduced tissue inflammation and rapid functional recovery can be attributed to a marked decrease in fungal burden at the early stage of *C. albicans* infection in flagellin-exposed B6 mouse corneas. Although both PMN infiltration and *Camp* upregulation contributed to corneal innate defense against fungal infection, *Camp* ablation totally, and PMN depletion partially, abrogated flagellin-induced fungal clearance in B6 mouse corneas.

CONCLUSIONS. Flagellin induces a strong innate defense and promotes robust resistance to *C. albicans* infection in the cornea. Topical flagellin or its mimetic may become a new prophylactic agent for preventing contact lens or trauma/injury-associated microbial keratitis. (*Invest Ophthalmol Vis Sci*. 2011;52:3074–3082) DOI:10.1167/iov.10-5928

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Corneal innate immune responses are key mediators of the host's defense against microbial infection, including fungal keratitis. Fungal infections of the cornea are frequently caused by species of *Fusarium*, *Aspergillus*, *Curvularia*, and *Candida*, and trauma is the most important predisposing cause.^{1,2} *Candida albicans* is a commensal fungus of the normal flora, yet it causes opportunistic infection of the cornea after trauma or surgery, during periods of immunosuppression including prolonged corticosteroid use, and during topical anesthetic abuse.^{3–6} While keratitis caused by filamentous fungi (*Fusarium* and *Aspergillus*) is more prevalent in the tropics, *Candida* accounts for proportionately more fungal corneal isolates at temperate latitudes.⁷ To date, there has been no clinically applicable measure for preventing fungal keratitis.⁸ Hence, there is an urgent need for the development of a prophylactic strategy for prevention of fungal keratitis in populations at high risk, in light of an increasing incidence of fungal keratitis and of a *Fusarium* keratitis epidemic associated with contact lens solutions.^{9,10}

Several murine models of fungal keratitis have been reported, including intrastromal corneal injection of *Fusarium oxysporum* and *C. albicans*,^{8,11} topical inoculation of injured corneas,³ and contact lens with *Fusarium* biofilm.¹² Using these models, it was revealed that innate immunity, primarily mediated by various Toll-like receptors (TLRs) and the MyD88 signaling pathway play a key role in the host response to fungal infection.^{13,14} However, there appears to be a discrepancy regarding which TLR(s) is involved in the innate defense of the cornea. Whereas deletion of TLR2 or -4 was shown to have no effect on overall disease progression, they were shown to have a role in controlling growth and replication of *Candida*¹⁴ and *Fusarium*,¹³ respectively. Hence, TLR-mediated innate defense and immunity are important for fungal clearance in the cornea.

Recently, we discovered that pre-exposure of the lung and injured corneas to the TLR5 ligand flagellin protects these tissues from *Pseudomonas* infection in B6 mice.^{15,16} This treatment diminished the inflammatory response to *Pseudomonas* infection and at the same time enhanced the production of antimicrobial peptides (AMPs) and cytoprotective mediators in a TLR-dependent manner.^{17,18} We reasoned that, although TLR5 is not a known fungus-recognizing receptor, the protective mechanisms induced after its activation by flagellin may function effectively in controlling fungal infection. We report for the first time that the topical application of flagellin on wounded cornea is effective in preventing the development of fungal keratitis and that the antimicrobial peptide CRAMP (the gene product of *Camp*) is an important contributing factor in the innate defense of the cornea.

MATERIALS AND METHODS

Fungi

C. albicans strain SC5314, a clinical isolate capable of producing experimental keratomycosis, was cultured on YPD agar (Sigma-Aldrich,

St. Louis, MO) for 3 days at 25°C. Colonies were harvested after 3 days of inoculation and diluted in sterile phosphate-buffered saline (PBS) to yield 2×10^5 colony-forming units (CFU)/ μ L based on the optical density (OD) at 600 nm, using a predetermined OD₆₀₀ conversion factor of 1 OD = 3×10^7 CFU/mL.

Animals

Wild-type (WT) C57BL6 (B6) mice (8 weeks of age; 20–24 g weight) and *TLR5*^{-/-} mouse breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). *Camp*^{-/-} (CRAMP-deficient) breeding pairs on a B6 background¹⁹ were obtained from Robert L. Gallo (University of California San Diego). Both *TLR5*^{-/-} and *Camp*^{-/-} were bred in-house, and their pups were subjected to genotyping before use. The 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.

Flagellin Pretreatment and Infection Procedure

Flagellin was prepared from *P. aeruginosa* strain PA01 or PAK, as described earlier.^{18,20} For flagellin pretreatment, mice ($n = 5$ /group/treatment) were anesthetized with ketamine and xylazine and placed beneath a stereoscopic microscope at a magnification of 40 $\times$, and the cornea of the left eye was scratched with three 1-mm incisions made with a sterile 26-gauge needle. Purified flagellin (500 ng in 5 μ L of PBS) or PBS as the control was applied to the injured corneas. For corneal infection, the mice were anesthetized, the pretreated corneas were rescratched 6 to 72 hours after the application of flagellin; and a 5- μ L suspension containing 1×10^4 to 10^6 CFU of *C. albicans* strain SC5314 was applied to the surface of the scarified cornea.

Clinical Examination

For the assessment of clinical scores, the mice were color-coded and examined by two independent observers daily and photographed at 6 hours and 1, 3, or 5 days after infection (dpi). Ocular disease was graded in clinical scores ranging from 0 to 12, according to the scoring system developed by Wu et al.²¹ A grade of 0 to 4 was assigned to each of the following three criteria—area of opacity, density of opacity, and surface irregularity—resulting in a potential total score of 12. A total score of 5 or less indicated mild eye disease, 6 to 9 signaled moderate disease, and 9 showed severe disease. At 1, 3, or 5 dpi, all infected corneas were photographed with a dissection microscope to illustrate the disease progression.

Fungal Load Determination, Cytokine ELISA, and MPO Measurement

We modified our previous published methods to allow all three assays (fungal load, myeloperoxidase [MPO] determination, and cytokine ELISA measurement) to be performed on a single mouse cornea. The corneas were excised from the enucleated eyes, minced, and homogenized in 100 μ L PBS with a tissue grinder (Micro Dounce; Bellco Glass Corp., Vineland, NJ). The homogenates were divided into two samples. The first was subjected to the counting of fungal colonies. Aliquots (100 μ L) of serial dilutions were plated onto YPD agar plates in triplicate. The plates were incubated 3 days at 25°C, and the fungal colonies were counted. The results were expressed as the mean number of CFU/cornea $\pm$ SEM.

The second homogenate sample was mixed with 5 μ L of 1% SDS and 10% Triton X-100 with a 200- μ L pipette. For the MPO assay, 30 μ L of homogenate was mixed with 270 μ L of hexadecyltrimethylammonium 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 16,000g for 20 minutes. Twenty microliters of the supernatant was mixed with 180 μ L of 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 in a well

of a 96-well plate. The change in absorbance at 460 nm was monitored continuously for 5 minutes with a microplate reader (Synergy2; BioTek). The results were expressed in units of MPO activity/cornea. One unit of MPO activity corresponded to approximately 2.0×10^5 polymorphonuclear leucocytes (PMNs).²² For ELISA, protein concentration was first determined using a protein assay kit (Micro BCA; Pierce, Rockford, IL). One microgram of total protein was used to perform ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN).

Western Blot Analysis of CRAMP

For Western blot, flagellin-treated corneas were excised, minced, and homogenized in 200 μ L RIPA buffer, and the protein concentration was determined with the protein assay kit. A total of 30 μ g protein was separated with 5% to 15% gradient SDS-PAGE, transferred to pore size 0.2 μ m nitrocellulose (Bio-Rad, Hercules, CA), and stained with 2% Ponceau S. The membrane was then cut into strips, according to molecular mass, by using prestained protein molecular size markers. The top part, containing proteins >30 kDa, was stained with actin (1:100 dilution; Sigma-Aldrich) for equal protein loading and the bottom part was stained with purified rabbit anti-mouse CRAMP IgG (10 ng/mL), kindly provided by Robert Gallo. The membrane strips were then incubated with HRP-conjugated donkey anti-rabbit IgG (1:5000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA), and the bands were visualized with ECL (SuperSignal; Thermo Scientific, Pittsburgh, PA) on film (Kodak, Rochester, NY).

PMN Depletion

Mice were made neutropenic by IP injection of 100 μ g of anti-mouse Gr-1 (RB6-8C5 mAb; R&D Systems) in 0.2 mL PBS 24 hours before flagellin pretreatment and then were inoculated with *C. albicans*. The control mice received the same dose of rat IgG. To assess the efficacy of the RB6-8C5 antibody, we wounded the corneas of neutropenic and control mice by needle scratching and treated them by topical application of flagellin or PBS. After 6 hours of flagellin treatment, the eyes were either snap frozen for staining with antibody (NIMP-R14, 1:100 dilution; Abcam, Cambridge, MA) specific for mouse PMNs or inoculated with 1×10^4 CFU of *C. albicans*.

Immunohistochemistry

Mouse eyes were enucleated and embedded in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Six-micrometer-thick sections were cut and mounted to polylysine-coated glass slides. After a 10-minute fixation in acetone, the slides were blocked with 10 mM sodium phosphate buffer containing 2% BSA for 1 hour at room temperature. Sections were then incubated with primary antibody (NIMP-R14, 1:100) specific for mouse PMNs. This was followed by a secondary antibody, FITC-conjugated goat anti-rat IgG (1:100; Jackson ImmunoResearch Laboratories), and the slides were mounted with DAPI mounting medium. Control slides were similarly treated, but the primary antibody was replaced with rat IgG.

Statistical Analysis

An unpaired, two-tailed Student's *t*-test was used to determine statistical significance for data from fungal counts, cytokine ELISA, and the MPO assay. A nonparametric Mann-Whitney U test was performed for clinical scores. Mean differences were considered significant at $P < 0.05$. Experiments were repeated at least twice, to ensure reproducibility.

RESULTS

Topical Application of Flagellin Attenuates Clinical Symptoms of *C. albicans* Keratitis in the B6 Mouse

We modified the mouse model of experimental *C. albicans* keratitis developed by the Wilhelmus group (Jackson et al.³

and Wu et al.²¹), using immunocompetent B6 mice. We made three parallel incisions (1 mm each) on the central cornea and applied a 5 μ L inoculum of *C. albicans* containing 10^5 to 10^6 colony-forming units (CFU), in our preliminary studies, to the scarified corneas. After inoculation, the animals were maintained in the same position until they recovered from the anesthesia (usually >20 minutes). We found that 10^5 CFU of a human isolate strain (SC5314) was sufficient to cause fungal keratitis in more than 90% of the corneas; the dosage used was 10 times less than that reported by Yuan et al.²³ for immunocompetent BALB/c mice.

We recently found that topical flagellin prevents *Pseudomonas* keratitis.²⁴ To determine whether topical application of flagellin induces protective mechanisms against fungal infection, we applied 500 ng of flagellin or PBS topically (in a 5 μ L volume) to needle-injured corneas before *Candida* inoculation. The control corneas (PBS pretreated) were partially covered with a dense opacity over the pupil at 1 dpi, and a moderate keratitis (score ~6) remained at 3 and 5 dpi (Fig. 1A). Pre-exposure to flagellin for 24 hours protected corneas from *Candida*; the infected corneas had only slight opacity (score 2) at 1 dpi, little or no sign of inflammation at 3 dpi, and no sign of inflammation or infection at 5 dpi (score 0; Fig. 1B). Different pre-exposure times were also tested, and the protective

FIGURE 1. Topical application of flagellin was effective in inducing protection against *C. albicans* keratitis. The center of B6 mouse corneas were gently scratched with 26-gauge needles (1-mm long, three lines) followed by topical application of flagellin (500 ng) or PBS to the injury sites. The corneas were scratched again 24 hours after pretreatment and inoculated with 1.0×10^5 CFU of *C. albicans*. The infected corneas were photographed at 1, 3, and 5 dpi and representative micrographs from each group are shown (A). Disease severity is represented by clinical scores, where the horizontal line represents the mean clinical score. A nonparametric Mann-Whitney U test was performed to compare each flagellin pretreatment to the PBS group (** $P < 0.01$, $n = 5$) (B). Results are representative of six independent experiments.

FIGURE 2. Flagellin pretreatment increased fungal clearance in the B6 mouse cornea. B6 mouse corneas were pretreated with topically applied flagellin or PBS for 24 hours, as described in Figure 1. At the indicated time after *C. albicans* inoculation, the eyes were enucleated and subjected to fungal culture by colony counting. The results are presented as the number of CFU per cornea. A nonparametric Mann-Whitney U test was performed to compare each flagellin pretreatment to the PBS group (* $P < 0.05$, ** $P < 0.01$, $n = 5$). Results are representative of three independent experiments.

effect was observed as early as 6 hours, was much reduced by 48 hours, and had diminished by 72 hours (data not shown).

Rapid and total elimination of invading pathogens is essential for host defense against infection. To determine whether flagellin pretreatment enhances fungal clearance, we assessed fungal burden in infected B6 mouse corneas (Fig. 2). At 6 hours pi, a significant amount of fungi remained in the control corneas, and a threefold reduction was observed in the flagellin-pretreated eyes. The total CFU in the corneas declined further at 1 and 3 dpi in the control corneas. In the flagellin-pretreated corneas, the fungal colony number was dramatically reduced by 1 dpi and was essentially undetectable at 3 dpi. At all time points, the fungal load was significantly lower in the flagellin-pretreated than in the PBS-pretreated corneas of the B6 mice.

Topical Flagellin Suppressed *C. albicans*-Induced Inflammation in the B6 Mouse Cornea

We next determined the production of inflammatory cytokines and the infiltration of PMNs in the cornea in response to *Candida* infection at different time points. At 6 hours pi, low levels of IL-1 β and MIP-2 were detected, and no significant difference was observed between PBS- and flagellin-pretreated corneas (Figs. 3A, 3B). The levels of both cytokines were greatly increased in PBS-pretreated corneas but significantly decreased in flagellin-pretreated corneas at 1 dpi compared with 6 hours pi. At 3 dpi, both cytokines in PBS-pretreated corneas rapidly declined from 1 dpi, but remained at significantly higher levels than in the flagellin-pretreated corneas where both cytokines were almost undetectable. Similar patterns were also observed for PMN infiltration measured by the MPO assay, with great differences between flagellin- and PBS-pretreated corneas at 1 dpi (Fig. 3C). It is interesting to note that while cytokine levels were decreased, the MPO activity was significantly increased from 6 hours to 24 hours after infection in flagellin-pretreated corneas.

FIGURE 3. Flagellin pretreatment dampened cytokine production and PMN infiltration in *C. albicans*-infected corneas of B6 mice. B6 mouse corneas were pretreated with topically applied flagellin or PBS for 24 hours, as described in Figure 1, followed by *C. albicans* inoculation. The eyes were enucleated at 6 hours or 1 or 3 dpi for IL-1 β (A), MIP-2 (B) ELISA, and MPO determination (units/cornea) (C), respectively. The results are representative of three independent experiments, and the indicated *P* values were generated using the Student's *t*-test. **P* < 0.05; ***P* < 0.01, *n* = 5.

Flagellin-Induced Protection Requires TLR5

To define whether TLR5 is required for flagellin-induced protection, *TLR5*^{-/-} mice (B6 background) were used for experimental *Candida* keratitis. Intriguingly, the TLR5 knockouts developed severe keratitis (Fig. 4A) with average clinical score ~8 at 1 dpi and 11 at 3 dpi (Fig. 4B) compared with the WT B6 mice with clinical score ~7 at 1 dpi (Fig. 1A) and 6 at 3 dpi (Fig. 1B). At 3 dpi, multiple staphylomas were present in the corneas of the *TLR5*^{-/-} mice (Fig. 4A). Fungal burden was also higher in both flagellin- and PBS-pretreated corneas of the *TLR5*^{-/-} mice compared with that of the WT controls (Fig. 2). Most important, flagellin pretreatment did not provide significant protection against *Candida* infection in the corneas of the *TLR5*^{-/-} mice (Fig. 4A) with no significant differences observed for clinical score (Fig. 4B), fungal burden (Fig. 4C), or PMN infiltration (Fig. 4D) between flagellin-pretreated and control corneas.

The involvement of TLR5 was further investigated with a *P. aeruginosa* flagellin mutant (L94A) in the TLR5-binding domain.^{18,25} The L94A flagellin had reduced binding affinity for TLR5,²⁵ but not for Ipaf, an intracellular flagellin receptor.²⁶ We recently showed that L94A purified from PAK fails to stimulate the activation of NF- κ B and the expression of proinflammatory cytokines. Pretreatment of cultured human corneal epithelial cells did not dampen the inflammatory response caused by bacteria.¹⁸ As shown in Figure 5, while flagellin purified from WT PAK strain (type A), similar to that of PA10 (type B),²⁷ induced profound protection against *C. albicans*, L94A was ineffective in reducing clinical scores (Fig. 5B) and fungal burden (Fig. 5C) of WT B6 mouse corneas.

Flagellin Induces *Camp* Expression in the Cornea

Having observed that flagellin pretreatment for 6 and 24, but not 48, hours profoundly protected the cornea from the development of *Candida* keratitis in B6 mice, we next investigated the effects of topical flagellin on PMN infiltration and *Camp* expression in the cornea at these time points. Figure 6A shows PMN staining of injured mouse cornea treated with flagellin or PBS at various time points. A few NIMP-R14-positive cells were observed in needle-injured, PBS-treated corneas at 24 hours. In contrast, topical flagellin resulted in the presence of many NIMP-R14-positive cells at 6 hours. The density of NIMP-R14-

FIGURE 4. TLR5 ablation obliterated flagellin-induced protection in B6 mice. *TLR5*^{-/-} mice were pretreated with topical flagellin (500 ng) or PBS. After 24 pretreatment, the cornea was scarified and inoculated with 1.0×10^5 CFU of *C. albicans*. The infected corneas were photographed (A), and disease severity was scored from 0 to 12, with the results represented as individual and mean clinical scores (B). At 3 dpi, the corneas were excised and subjected to fungal counting (C) with the results presented as CFU ($\times 1000$) *C. albicans* per cornea and to MPO determination (units/cornea) (D). The results are representative of two independent experiments.

FIGURE 5. Flagellin mutated in the TLR5-binding region failed to induce protection against *C. albicans* infection in B6 mice. B6 mice were pretreated topically with either PAK flagellin or L94A mutant flagellin (500 ng) for 24 hours, and the cornea was inoculated with 1.0×10^5 CFU of *C. albicans*. The infected corneas were photographed (A) and the disease severity was scored from 0 to 12 with the results represented as individual and mean clinical scores (B). At 3 dpi, the corneas were excised and subjected to fungal counting, and the results are presented as CFU *C. albicans* ($\times 1000/\text{cornea}$) (C). The results are representative of two independent experiments.

positive cells markedly increased at 24 hours and declined to an undetectable level at 48 hours after flagellin challenge of the injured corneas.

Figure 6B shows the expression of *Camp*. No CRAMP was detected by Western blot in the control uninjured corneas, whereas injury alone (PBS-treated corneas) appeared to induce its expression as a very faint band that appeared at 6 hours after injured corneas. CRAMP reactivity was markedly increased in the corneas at 6 hours and 24 hours and became undetectable 48 hours after flagellin challenge. Thus, AMP expression and PMN recruitment correlated with innate protection against *Candida* keratitis in flagellin-pretreated corneas of the B6 mice.

Topical Flagellin Increases Fungal Clearance in PMN-Depleted Corneas

To define the role of PMN in flagellin-induced protection, mice rendered neutropenic by RB6-8C5 monoclonal antibody were used for a protection study. Unlike in the cornea model of *P. aeruginosa* infection,²⁴ PMN-depleted mice survived at 2 dpi, and there was no detectable fungal dissemination into the system (data not shown). Although the control antibody had no detectable effects on *C. albicans* keratitis, the disease was much more severe in the RB6-8C5-treated mice (Fig. 7A). At 1 dpi, there were apparent surface irregularities in the control, but not in the flagellin-pretreated corneas. At 2 dpi, both the PBS- and flagellin-pretreated corneas had severe keratitis and signs of perforation. The fungal load in PMN-depleted mice was several times higher than that in the controls (Fig. 2). Of interest, in the absence of PMN, flagellin pretreatment still resulted in a fivefold reduction of fungal loads, compared with that in PBS-pretreated corneas (Fig. 7B).

Antimicrobial CRAMP Is a Determinant Factor for Susceptibility to and Flagellin-Induced Protection against *Candida* in B6 Mice

To determine the role of CRAMP in fungal keratitis, we used *Camp*^{-/-} mice with a B6 background¹⁹ and first investigated the effects of CRAMP deficiency on the susceptibility of B6 mice to *Candida* (Fig. 8). Two dosages of *Candida* were used to infect the anesthetized, needle-injured corneas of the WT and *Camp*^{-/-} mice. *Candida* at 10^4 CFU elicited a mild inflammatory response at 1 dpi (score <6) that was rapidly

FIGURE 6. Flagellin pretreatment induced PMN infiltration and CRAMP production in the injured B6 mouse corneas. B6 mouse corneas were needle wounded and topically challenged with flagellin (flagellin or F) or PBS (PBS or P). At the indicated time points, the corneas were either OCT snap frozen, followed by sectioning and immunostaining with the antibody NIMP-R14, to detect PMNs (A), or homogenized, followed by Western blot analysis of CRAMP (B). For immunohistochemistry (A), the photographs were taken at the center of the cornea with DAPI as the counterstain for nuclei. For Western blot (B), two corneas were pooled, and two samples were used for each condition with immunoreactivity of β-actin used as the internal control for protein loading.

FIGURE 7. Flagellin pretreatment did not protect PMN-depleted mice against *C. albicans* infection. B6 mice were made neutropenic by intraperitoneal injection of the mAb RB6-8C5, and the controls were injected with the IgG isotype. The control and neutropenic B6 mouse corneas were pretreated with topical flagellin for 24 hours and infected with 1.0×10^5 CFU of *C. albicans*. (A) Micrographs of infected corneas were performed at 1 and 2 dpi. (B) Fungal load was determined at 2 dpi and the indicated *P* values were generated with Student's *t*-test, ***P* < 0.01, *n* = 5. The results are representative of two separate experiments.

resolved in the WT B6 mice, but caused severe and progressive keratitis in the *Camp*^{-/-} mice (Fig. 8A). When 10^5 CFU *Candida* was used, disease was more severe in the *Camp*^{-/-} knockout mice (clinical score, ≥ 10) than in WT (Fig. 8B).

We next investigated the role of CRAMP in flagellin-induced protection against *Candida* in *Camp* knockout mice (Fig. 9). While flagellin pretreatment protected the corneas of WT mice from *Candida*, it exhibited almost no effects on *Camp*^{-/-} mice compared with those pretreated with PBS. Corneas remained highly inflamed with multiple staphyloma at 3 dpi (Fig. 9A), and high clinical scores (Fig. 9B) were observed compared with WT (Fig. 1B). Fungal cultures revealed that a substantial amount of CFU were found in both PBS- and flagellin-pretreated corneas of *Camp*^{-/-} mice (Fig. 9C), with no significant differences among the two groups. The PMN activities were also high in both groups at 3 dpi (Fig. 9D).

DISCUSSION

In earlier work, we showed that pre-exposure of B6 mouse cornea and lung to flagellin, the natural ligand of TLR5 and the structural component of bacterial flagella, protects the tissues from *P. aeruginosa* keratitis^{15,17} and pneumonia,¹⁶ respectively. In the present study, we extended this observation and showed that topical flagellin enhanced innate immunity and

protected injured corneas from *C. albicans* keratitis in a TLR5-dependent manner. For the first time, we report that flagellin significantly increased pathogen clearance at an early stage of infection (6 hours pi) and that both types A and B of flagellin²⁷ were effective in inducing protective mechanisms in the cornea. Using PMN depletion and *Camp*^{-/-} mice, we further showed that flagellin-induced corneal innate immunity against fungal infection involves PMN infiltration and antimicrobial gene expression. Taken together, our studies suggest that topical flagellin elicits innate defense mechanisms that are capable of eliminating a variety of keratitis-causing pathogens, highlighting the benefits of using TLR agonists as the basis for developing new prophylactics for preventing contact lens-, trauma-, or ocular surgery-associated microbial keratitis.

It has been known for decades that pre-exposure of tissues to a TLR ligand such as endotoxin (TLR4 ligand) triggers hyporesponsiveness to a second stimulation of the same TLR ligand or of the pathogen bearing the ligand, a phenomenon termed tolerance that was thought to be important in regulating the host innate immune response.^{28,29} When the flagellin-exposed corneas were challenged with pathogen recognizable by TLR5 such as *P. aeruginosa*, the infection-associated inflammation was dampened, presumably through the tolerance effect. This raised an important question regarding the specificity

FIGURE 8. *Camp*^{-/-} mouse corneas were more susceptible than the wild type to *C. albicans*. *Camp*^{-/-} or WT (B6) mice were scarified and infected with either 1.0×10^4 (A) or 1.0×10^5 (B) CFU of *C. albicans*. The disease severity was scored from 0 to 12 at 1, 3, and 5 dpi and represented as mean clinical scores. A nonparametric Mann-Whitney U test was performed to compare each flagellin pretreatment to the PBS group (**P* < 0.05, ***P* < 0.01, *n* = 5). The results are representative of three independent experiments.

FIGURE 9. Topical flagellin was ineffective in protecting the cornea from *C. albicans* in *Camp*^{-/-} mouse cornea. *Camp*^{-/-} mice were pretreated with a topical application of flagellin (500 ng) or PBS. After 24 pretreatment, the cornea was inoculated with 1.0×10^5 CFU of *C. albicans*. The infected corneas were photographed at 1 and 3 dpi (A), and disease severity was scored from 0 to 12 and represented as the mean clinical score (B). The results are representative of three independent experiments. At 3 dpi, the corneas were excised and subjected to fungal counting (C), with the results presented as CFU ($\times 1000$) *C. albicans* per cornea, and to MPO determination (units/cornea) (D). No statistically significant differences were observed in the analyses in (B), (C), and (D).

of flagellin-induced protection against corneal infection—specifically, whether it was pathogen (TLR) specific. To that end, we adapted a recently reported mouse model of *C. albicans* keratitis^{3,14,21,23,30} and observed a great increase in fungal clearance in flagellin-treated B6 mouse corneas compared with that in the controls. The increase was observed as early as 6 hours and resulted in total elimination of invading pathogens at 3 dpi. In a preliminary study, we also observed protective effects of topical flagellin applied concomitantly and 24 hours before *Aspergillus fumigatus* infection.¹² In the experimental *Candida* keratitis model, we showed that pre-exposure of injured corneas to flagellin dampened fungal infection-associated inflammation. We suspect that the low levels of proinflammatory cytokines and PMN infiltration in flagellin-pretreated corneas during *C. albicans* infection were probably the result of much reduced fungal burden. To our knowledge, this is the first report that manipulation of innate immunity reduces or prevents fungal infection of an injured cornea. Because *C. albicans* is unlikely to be recognized by TLR5, our results also suggested that flagellin-induced protection against keratitis-causing pathogens in the cornea may not be directly related to TLR activation-mediated hyporesponsiveness (tolerance), but rather through flagellin-elicited, strong, innate defense and protective mechanisms, enabling rapid pathogen clearance and functional recovery. Indeed, our preliminary data using two dosages of Pam3Cys (TLR2 ligand) revealed that although Pam3Cys pretreatment reduced keratitis severity, it did not result in the eradication of pathogens at 3 dpi (data not shown).

In addition to TLR5, flagellin can also be recognized by intracellular receptor Ipaf/CARD12.^{31–33} Activation of Ipaf also restricted intracellular flagellated bacterial growth in vitro.^{34,35} To assess whether flagellin-induced protection is TLR5 dependent, we used TLR5 knockout mice and showed that flagellin failed to induce any protection against *C. albicans* in TLR5 deficient mice. Intriguingly, *C. albicans* keratitis appeared to be more severe in *TLR5*^{-/-} than in WT B6 mice. Although *TLR5*^{-/-} mice have been shown to be more susceptible to *Escherichia coli* urinary tract infection³⁶ and to acute *P. aeruginosa* lung infection,³⁷ Sun et al.³⁸ observed a similar pathologic presence of *P. aeruginosa* keratitis in B6 wt and *TLR5*^{-/-} mice. Our study is the first to show an increase in the severity of fungal infection in *TLR5* knockout mice. It is not clear whether this is because of the known metabolic syndrome³⁹ or other defects in the innate immunity of the knockouts. We also used a TLR5 binding mutant, L944A, which has

been shown ineffective in stimulating TLR5 activation, to pretreat cornea and found that this mutant failed to induce protection against *C. albicans*. Based on these studies, we conclude that initial TLR5 activation is necessary for flagellin to activate the innate defense apparatus that can kill a broad spectrum of pathogens.

Our study further confirms the results of previous studies of mouse *P. aeruginosa* keratitis²⁴ and pneumonia,¹⁶ which showed that flagellin-induced PMN infiltration and AMP expression are two important elements of the protective mechanisms against microbial infection. Using antibody-mediated PMN depletion, we showed more severe keratitis and corneal perforation in *C. albicans*-infected corneas of neutropenic mice. Unlike *P. aeruginosa* infection in which PMN depletion resulted in bacterial dissemination to other organs within 36 hours,²⁴ fungal infection was restricted to the eye. A comparison of flagellin's effects on pathogen clearance in PMN-depleted mice revealed that flagellin-induced PMN infiltration may play a more important role in bacterial clearance than in fungal clearance; flagellin decreased the *P. aeruginosa* load twofold,²⁴ but reduced the *C. albicans* load fivefold in PMN-depleted mice (Fig. 7). Furthermore, PMNs appeared to play a more prominent role in eliminating invading pathogens in the cornea than in the lung, where intranasally administered, flagellin-induced protection did not require PMN infiltration.¹⁶ Hence, flagellin-induced PMN infiltration may function in a tissue or pathogen-dependent manner.

In this study, we demonstrated that CRAMP levels were increased in flagellin-exposed B6 mouse corneas at 6 and 24 hours. Yuan et al.⁴⁰ recently reported that the levels of CRAMP increased rapidly within the inflamed murine corneal stroma after the initiation of fungal keratitis and proposed that the molecule plays a role in host responses that follow corneal trauma and infection. Our knockout mouse study revealed that *Camp* ablation resulted in the loss of flagellin-mediated protection, suggesting that this phenomenon is partially mediated by CRAMP. While both human cathelicidin LL-37 and mouse CRAMP were shown to kill *C. albicans* in vitro, CRAMP-deficient mice were similarly susceptible to *C. albicans* in an intradermal infection model.⁴¹ Hence, cathelicidin may play a more important role in the innate defense against fungal infection in the cornea than in the skin, each of which expresses a different spectrum of AMPs.^{40,42–44} Alternatively, cathelicidin may function more effectively at the mucosal surface, where it works in synergy with other AMPs secreted from epithelial cells in killing the invading pathogen.⁴² Finally, there was a

major difference in the pathogen clearance in *P. aeruginosa* and *C. albicans* keratitis induced by topical flagellin in the absence of CRAMP. Although flagellin induced significantly increased bacterial clearance,²⁴ it exhibited no effect in *C. albicans* clearance in *Camp*^{-/-} mice (Fig. 9). Hence, the presence of CRAMP in flagellin-pretreated cornea may contribute to fungal clearance at the early stage of infection, whereas both PMN and CRAMP are required for bacterial clearance. The difference in pathogen clearance in *Camp*^{-/-} mice may also be related to whether CRAMP is secreted mainly from epithelial cells or intracellular in PMNs.⁴⁵ Consistent with this postulation, it has been shown that, although LL-37 can kill *C. albicans* in vitro, the fungicidal activities against *C. albicans* in blood-killing assays or in an intradermal infection model of the WT and *Camp*^{-/-} mice were the same.⁴¹ Further studies aimed at understanding the mechanism underlying cathelicidin-mediated pathogen killing at the ocular surface are warranted.

In summary, our results show that topical flagellin can induce innate protection against fungal infection. Thus, flagellin or its derivative can be exploited as a prophylactic modality to prevent infectious keratitis. This novel treatment should benefit patients undergoing ocular surgery and, potentially, contact lens wearers.

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References

1. Thomas PA. Fungal infections of the cornea. *Eye (Lond)*. 2003;17:852–862.
2. Thomas PA, Geraldine P. Infectious keratitis. *Curr Opin Infect Dis*. 2007;20:129–141.
3. Jackson BE, Wilhelmus KR, Mitchell BM. Genetically regulated filamentation contributes to *Candida albicans* virulence during corneal infection. *Microb Pathog*. 2007;42:88–93.
4. Galarreta DJ, Tuft SJ, Ramsay A, Dart JK. Fungal keratitis in London: microbiological and clinical evaluation. *Cornea*. 2007;26:1082–1086.
5. Ritterband DC, Seedor JA, Shah MK, Koplin RS, McCormick SA. Fungal keratitis at the New York Eye and Ear Infirmary. *Cornea*. 2006;25:264–267.
6. Chern KC, Meisler DM, Wilhelmus KR, Jones DB, Stern GA, Lowder CY. Corneal anesthetic abuse and *Candida* keratitis. *Ophthalmology*. 1996;103:37–40.
7. Leck AK, Thomas PA, Hagan M, et al. Aetiology of suppurative corneal ulcers in Ghana and south India, and epidemiology of fungal keratitis. *Br J Ophthalmol*. 2002;86:1211–1215.
8. Zhang H, Chen H, Niu J, Wang Y, Xie L. Role of adaptive immunity in the pathogenesis of *Candida albicans* keratitis. *Invest Ophthalmol Vis Sci*. 2009;50:2653–2659.
9. Gorscak JJ, Ayres BD, Bhagat N, et al. An outbreak of *Fusarium* keratitis associated with contact lens use in the northeastern United States. *Cornea*. 2007;26:1187–1194.
10. Patel A, Hammersmith K. Contact lens-related microbial keratitis: recent outbreaks. *Curr Opin Ophthalmol*. 2008;19:302–306.
11. Zhong W, Yin H, Xie L. Expression and potential role of major inflammatory cytokines in experimental keratomycosis. *Mol Vis*. 2009;15:1303–1311.
12. Sun Y, Chandra J, Mukherjee P, Szczotka-Flynn L, Ghannoum MA, Pearlman E. A murine model of contact lens-associated *Fusarium* keratitis. *Invest Ophthalmol Vis Sci*. 2010;51:1511–1516.
13. Tarabishy AB, Aldabagh B, Sun Y, et al. MyD88 regulation of *Fusarium* keratitis is dependent on TLR4 and IL-1R1 but not TLR2. *J Immunol*. 2008;181:593–600.
14. Yuan X, Wilhelmus KR. Toll-like receptors involved in the pathogenesis of experimental *Candida albicans* keratitis. *Invest Ophthalmol Vis Sci*. 2010;51:2094–2100.
15. Kumar A, Hazlett LD, Yu FS. Flagellin suppresses the inflammatory response and enhances bacterial clearance in a murine model of *Pseudomonas aeruginosa* keratitis. *Infect Immun*. 2008;76:89–96.
16. Yu FS, Cornicelli MD, Kovach MA, et al. Flagellin stimulates protective lung mucosal immunity: role of cathelicidin-related antimicrobial peptide. *J Immunol*. 2010;185:1142–1149.
17. Kumar A, Yin J, Zhang J, Yu FS. Modulation of corneal epithelial innate immune response to *Pseudomonas* infection by flagellin pretreatment. *Invest Ophthalmol Vis Sci*. 2007;48:4664–4670.
18. Gao N, Kumar A, Jyot J, Yu FS. Flagellin-induced corneal antimicrobial peptide production and wound repair involve a novel NF-kappaB-independent and EGFR-dependent pathway. *PLoS One*. 2010;5:e9351.
19. Buchau AS, Morizane S, Trowbridge J, et al. The host defense peptide cathelicidin is required for NK cell-mediated suppression of tumor growth. *J Immunol*. 2010;184:369–378.
20. Zhang J, Xu K, Ambati B, Yu FS. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest Ophthalmol Vis Sci*. 2003;44:4247–4254.
21. Wu TG, Wilhelmus KR, Mitchell BM. Experimental keratomycosis in a mouse model. *Invest Ophthalmol Vis Sci*. 2003;44:210–216.
22. Williams RN, Paterson CA, Eakins KE, Bhattacharjee P. Quantification of ocular inflammation: evaluation of polymorphonuclear leukocyte infiltration by measuring myeloperoxidase activity. *Curr Eye Res*. 1982;2:465–470.
23. Yuan X, Mitchell BM, Wilhelmus KR. Gene profiling and signaling pathways of *Candida albicans* keratitis. *Mol Vis*. 2008;14:1792–1798.
24. Kumar A, Gao N, Standiford TJ, Gallo RL, Yu FS. Topical flagellin protects the injured corneas from *Pseudomonas aeruginosa* infection. *Microbes Infect*. 2010;12:978–989.
25. Verma A, Arora SK, Kuravi SK, Ramphal R. Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response. *Infect Immun*. 2005;73:8237–8246.
26. Franchi L, Stoolman J, Kanneganti TD, Verma A, Ramphal R, Nunez G. Critical role for Ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation. *Eur J Immunol*. 2007;37:3030–3039.
27. Campodonico VL, Llosa NJ, Grout M, Doring G, Maira-Litran T, Pier GB. Evaluation of flagella and flagellin of *Pseudomonas aeruginosa* as vaccines. *Infect Immun*. 2010;78:746–755.
28. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol*. 2009;30:475–487.
29. Fan H, Cook JA. Molecular mechanisms of endotoxin tolerance. *J Endotoxin Res*. 2004;10:71–84.
30. Yuan X, Mitchell BM, Wilhelmus KR. Expression of matrix metalloproteinases during experimental *Candida albicans* keratitis. *Invest Ophthalmol Vis Sci*. 2009;50:737–742.
31. Miao EA, Alpuche-Aranda CM, Dors M, et al. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol*. 2006;7:569–575.
32. Franchi L, Amer A, Body-Malapel M, et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol*. 2006;7:576–582.
33. Miao EA, Ernst RK, Dors M, Mao DP, Aderem A. *Pseudomonas aeruginosa* activates caspase 1 through Ipaf. *Proc Natl Acad Sci U S A*. 2008;105:2562–2567.
34. Akhter A, Gavrillin MA, Frantz L, et al. Caspase-7 activation by the Nlr4/Ipaf inflammasome restricts *Legionella pneumophila* infection. *PLoS Pathog*. 2009;5:e1000361.
35. Abdelaziz DH, Amr K, Amer AO. Nlr4/Ipaf/CLAN/CARD12: more than a flagellin sensor. *Int J Biochem Cell Biol*. 2010;42:789–791.
36. Andersen-Nissen E, Hawn TR, Smith KD, et al. Cutting edge: Tlr5^{-/-} mice are more susceptible to *Escherichia coli* urinary tract infection. *J Immunol*. 2007;178:4717–4720.
37. Morris AE, Liggitt HD, Hawn TR, Skerrett SJ. Role of Toll-like receptor 5 in the innate immune response to acute *P. aeruginosa* pneumonia. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L1112–L1119.

38. Sun Y, Karmakar M, Roy S, et al. TLR4 and TLR5 on corneal macrophages regulate *Pseudomonas aeruginosa* keratitis by signaling through MyD88-dependent and -independent pathways. *J Immunol.* 2010;185:4272–4283.
39. Vijay-Kumar M, Aitken JD, Carvalho FA, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science.* 2010;328:228–231.
40. Yuan X, Hua X, Wilhelmus KR. The corneal expression of antimicrobial peptides during experimental fungal keratitis. *Curr Eye Res.* 2010;35:872–879.
41. Lopez-Garcia B, Lee PH, Yamasaki K, Gallo RL. Anti-fungal activity of cathelicidins and their potential role in *Candida albicans* skin infection. *J Invest Dermatol.* 2005;125:108–115.
42. McDermott AM. The role of antimicrobial peptides at the ocular surface. *Ophthalmic Res.* 2009;41:60–75.
43. Schaubert J, Gallo RL. Antimicrobial peptides and the skin immune defense system. *J Allergy Clin Immunol.* 2008;122:261–266.
44. Metz-Boutigue MH, Shooshtarizadeh P, Prevost G, Haikel Y, Chich JF. Antimicrobial peptides present in mammalian skin and gut are multifunctional defence molecules. *Curr Pharm Des.* 2010;16:1024–1039.
45. Sorensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood.* 1997;90:2796–2803.