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Flagellin-induced expression of CXCL10 mediates direct fungal killing and recruitment of NK cells to the cornea in response to *Candida albicans* infection

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We previously showed that topical flagellin induces profound mucosal innate protection in the cornea against microbial infection, a response involving multiple genes and cell types. In this study, we used a *Candida albicans (CA)*-C57BL/6 mouse keratitis model to delineate the contribution of CXCL10- and CXCR3-expressing cells in flagellin-induced protection. Flagellin pretreatment markedly enhanced CXCL10 expression at 6 h post CA infection (hpi), but significantly dampened CXCL10 expression at 24 hpi. At the cellular level, CXCL10 was expressed in the epithelia at 6 hpi in flagellin-pretreated corneas, and concentrated at lesion sites 24 hpi. CXCR3-expressing cells were detected in great numbers at 24 hpi, organized within clusters at the lesion sites in CA-infected corneas. CXCL10 or CXCR3 neutralization increased keratitis severity and dampened flagellin-induced protection. CXCR3-positive cells were identified as NK cells, the depletion of which resulted in severe CA keratitis. Contributions from NK T-cells were excluded by finding no change in flagellin-induced protection in *Rag1* KO mice. Recombinant CXCL10 inhibited CA growth in vitro and accelerated fungal clearance and inflammation resolution in vivo. Taken together, our data indicate that epithelium-expressed CXCL10 plays a critical role in fungal clearance and that CXCR3-expressing NK cells contribute to CA eradication in mouse corneas.

Keywords: Antimicrobial peptides · CXCL10 · Fungal keratitis · Innate immunity · Natural killer cells

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Introduction

Candida albicans (CA) is a commensal fungus of the normal flora, yet may cause opportunistic infections of the cornea following

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trauma or surgery, during periods of immunosuppression such as prolonged corticosteroid use, and during topical anesthetic abuse [1–6]. Although rare, the incidence of fungal keratitis has increased in recent years, especially in contact lens wearers [7, 8]. To date, there is no clinically amenable measure to prevent fungal keratitis. Current practice in treating fungal keratitis involves the use of topical antifungal drops such as natamycin and amphotericin B. Topical antifungals can cause toxicity such as punctate keratitis, chemosis, and recurrent corneal epithelial erosions [9]. Hence, understanding the pathogenesis of fungal keratitis and host responses will aid in identifying new therapeutics to improve the prognosis of this condition.

Using various murine models of fungal keratitis, recent research indicates that innate immunity, primarily mediated by various TLRs and the MyD88 signaling pathway, plays a key role in the host response to fungal infection [10, 11]. We have developed a mouse experimental *CA* keratitis model [12, 13] to show that pre-exposure of the cornea to TLR5 ligand flagellin induces a strong innate defense and promotes robust resistance to infection in the cornea [14]. The activation of innate immunity after TLR5 stimulation is attributed to flagellin-induced reprogramming of gene expression in corneal epithelial cells (CECs) in response to pathogens, including diminished inflammatory response to *CA* and at the same time, enhanced production of antimicrobial peptides (AMPs) in a TLR5-dependent manner [14–17]. Our data suggested that topical applied flagellin functions as an immunostimulant to activate the innate defense apparatus, resulting in resistance to infectious keratitis [14, 18, 19]. More recently, we have shown that flagellin pretreatment followed by *Pseudomonas (P) aeruginosa* infection resulted in 890 genes upregulated and 37 genes downregulated [17]. At the cell levels, neutrophils were shown to be required for flagellin-induced protection against *P. aeruginosa* and *CA* [14, 19]. The depletion of neutrophils resulted in *P. aeruginosa* dissemination from the eye to the entire body and in death within 2 dpi and in corneal perforation in otherwise self-healing, *CA*-infected corneas of C57BL/6 mice [14, 19]. Others have shown the importance of macrophages in corneal defense against fungal and bacterial pathogens [20, 21]. Hence, flagellin-induced protection may have the participation of most, if not all, innate immune cell types as well as the participation of residential CECs and stromal fibroblasts. As flagellin, a virulent Gram-negative protein, is unlikely to be used clinically, identifying the downstream effectors that participate in flagellin-induced fungus killing is of great importance.

In an effort to study transcriptional regulation, we identified interferon regulatory factor 1 (IRF1) as one of the most highly upregulated transcription factors in response to *P. aeruginosa* infections in vitro and in vivo [22]. We showed that IRF1, by regulating CXCL10 expression, plays a role in corneal innate immune response. Moreover, we identified IFN-γ produced by NK cells as a key factor that augmented IRF1 and CXCL10 expression in flagellin-pretreated corneas [22]. In another study, we also reported great upregulation of CXCL10 in response to epithelial wounding in a DC-related manner in mouse CECs [17]. CXCL10 is a member of the IFN-inducible tripeptide motif Glu-Leu-Arg-negative (ELR$^-$) CXC chemokines [23]. This chemokine, along with CXCL9 and CXCL11, signals through a G-protein-coupled receptor, CXCR3 that is expressed primarily on activated T lymphocytes and NK cells, and functions primarily in the recruitment of these cells to the sites of infection and inflammation [24–26]. In addition to their roles in leukocyte recruitment, CXCR3 ligands exert direct antimicrobial effects that are comparable to the effects mediated by cationic AMPs, including defensins [27]. To date, whether CXCL10 participates in corneal innate defense against fungal infection remains to be determined.

In this study, we sought to better understand the molecular mechanisms underlying flagellin-induced protection and corneal innate immunity using an experimental *Candida* keratitis model. We showed that while CXCL10 was mostly expressed in CECs, especially at the early stage of infection, CXCR3 was expressed only in the infiltrating cells, most of which were NK1.1-positive. Neutralizing CXCL10, CXCR3 or depleting NK cells impaired innate defense against *CA* in the cornea. Our results reveal that the CXCL10-CXCR3 signaling pathway plays a role in mucosal innate immunity against fungal infection and that CXCL10 might be developed as an adjuvant treatment for microbial keratitis.

Results

Flagellin-induced protection against CA is T- and B-cell independent

As flagellin-induced protection can be detected as early as 6 hpi (where hpi is hours post *CA* infection) and was very apparent at 24 hpi, and the outcome of infectious keratitis was strongly related to the pathogen load at 1 dpi [14, 19, 28], we suspect that the protection is mostly related to innate immunity. To test this hypothesis, we used *Rag1* KO (*Rag1*KO mice that lack all T cells and B cells, in comparison with WT C57BL/6 mice, to determine the role of adaptive immunity in flagellin induced protection. We previously showed that 10^5 *CA (*human isolate strain SC5314) was sufficient to cause fungal keratitis in C57BL/6 mice [14]. Figure 1A shows a normal C57BL/6 mouse eye, and eyes infected with *CA* at 24 hpi, with or without flagellin pretreatment. The protective effects of flagellin pretreatment were evident by the presence of small areas of opacification in flagellin-pretreated eyes, whereas the control-infected cornea (PBS) was 50% covered with patches of opacification. The severity of keratitis under different experimental conditions was quantitated with clinical scoring. The *CA* keratitis in C57BL/6 mice was moderate at 1 dpi and exhibited mild eye diseases at 5 dpi. There were no differences in clinical scores, opacification of the cornea, PMN infiltration indicated by myeloperoxidase (MPO) enzyme activities, and the expression of proinflammatory cytokine CXCL2 between *Rag1*KO and C57BL/6 mice. The flagellin-pretreated corneas in these mice remained keratitis free at 5 dpi. Thus, we conclude that flagellin-induced

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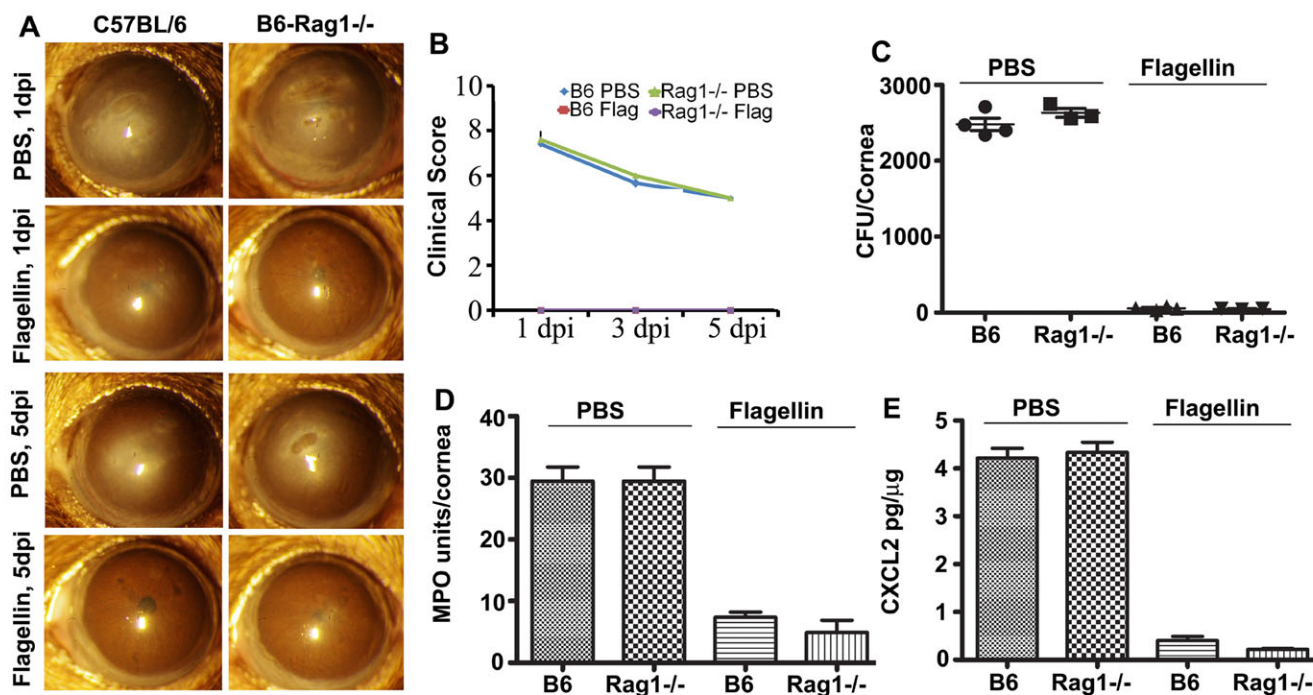

Figure 1. Flagellin induces mucosal protection against *C. Albicans* in an innate immunity-dependent manner. (A) The centers of C57BL/6 or *Rag1*$^{-/-}$ mouse corneas were gently scratched with 26-gauge needles (three scratches, each 1 mm long) followed by topical application of 500 ng flagellin in 5 μL PBS or 5 μL PBS alone to the injury sites. Corneas were then scratched again 24 h after pretreatment and inoculated with 1.0×10^5 CFU of CA. A naïve cornea (normal), CA-infected cornea with flagellin (flag), and the control (PBS) pretreated CA-infected cornea were photographed at 1 or 5 dpi with a slit lamp. One representative image for each condition ($n = 5$) is presented. (B) Disease severity was assessed in mice treated as in (A) and is represented by clinical scores performed at 1, 3, and 5 dpi. 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 (**$p < 0.01$, $n = 5$). (C) Four WT C57BL/6 and three Rag1$^{-/-}$ mice, treated as in (A), were euthanized at 1 dpi. The corneas were excised and subjected to fungal plate counting with the results presented as CFU CA per cornea. Each symbol represents an individual mouse and means ± SD are indicated. (D) Myeloperoxidase determination (units/cornea) and (E) CXCL10 ELISA were performed at 1 day postinfection in mice treated with or without flagellin. (D and E) Data are shown as means + SD of five samples and (B–E) are representative of two independent experiments.

protection does not require functional B or T cells, but rather primarily involves innate immune cells, including neutrophils [14, 19] and NK cells.

Flagellin pretreatment regulates *Cxcl10* and *Cxcr3* expression in corneas in response to CA infection

Our recent study of *P. aeruginosa* keratitis identified CXCL10 as a major chemokine induced by infection and augmented by flagellin pretreatment in an IFN-γ and IRF1-dependent manner [22]. To determine if CXCL0 also plays a role in corneal innate defense against fungal pathogens, we assessed the expression of *Cxcl10* and its receptor *Cxcr3* in response to fungal infection, with or without flagellin pretreatment (Fig. 2). In normal, homeostatic corneas (NL), no expression of *Cxcl10* and *Cxcr3* was detected. Flagellin-pretreated, but not PBS-instilled, corneas expressed detectable *Cxcl10* and *Cxcr3* at 24 h posttreatment, prior to pathogen inoculation. *CA* infection triggered the expression of both *Cxcl10* and *Cxcr3,* and flagellin pretreatment had disparate effects on the expression of *Cxcr3* (downregulated) and *Cxcl10* (upregulated) in C57BL/6 mouse corneas at 6 hpi. At 24 hpi, both *Cxcl10* and *Cxcr3* were more abundant in *C. albicans* infected corneas, but flagellin pretreatment dampened their expression (Fig. 2A). Robust upregulation of *Cxcl10*, but not other *Cxcr*3 ligands, *Cxcl9* and *Cxcl11*, and *Cxcr3* in response to infection, and their suppression by flagellin pretreatment, were also detected by real-time PCR (Fig. 2B). The dramatic downregulation of *Cxcl10* and *Cxcr3* in flagellin-pretreated corneas was consistent with a lack of any sign of infection and/or inflammation in the treated corneas at 1 dpi shown in Figure 1.

Flagellin pretreatment alters CXCL10-CXCR3 signaling by increasing the expression of CXCL10

We next investigated if *Cxcl10* and *Cxcr3* were expressed at the protein level and how they were distributed in *CA*-infected corneas, with or without flagellin pretreatment, using immunohistochemistry (Fig. 3). No CXCL10- or CXCR3-positive cells were detected in the normal, homeostatic corneas (Fig. 3A). Flagellin pretreatment through topical application to needle injury sites (Fig. 3C), but not injury itself (Fig. 3B), was sufficient to induce the expression of CXCL10 at the basal cell layer of the stratified

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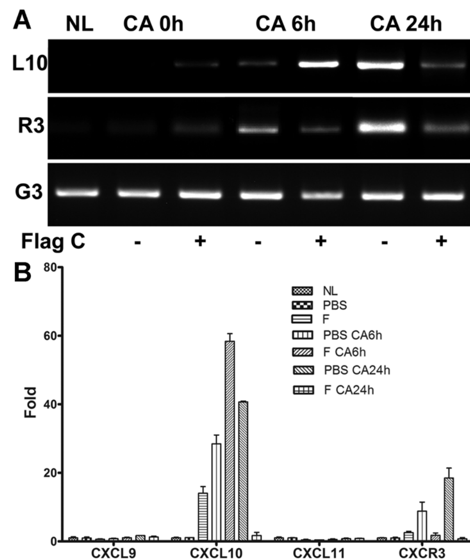

Figure 2. Topical flagellin affects expression of *Cxcl10* and *Cxcr3* in C57BL/6 mouse corneas. C57BL/6 mouse corneas were flagellin-pretreated and *CA*-inoculated as described in Figure 1. The naïve (NL) and *CA*-infected corneas with or without flagellin pretreatment for 24 h were excised at 0 (no fungal inoculation, 0 h), 6, and 24 hpi, and subjected to (A) RNA isolation and RT-PCR analysis for *Cxcl10* (L10) and *Cxcr3* (R3), with *Gapdh* (G3) as an internal control or (B) real-time PCR for *Cxcl9, Cxcl10, Cxcl11,* and *Cxcr3*. The fold increase over that of the naïve corneas (value 1) in (B) is the mean + SD of three samples for each condition with β-actin as the internal control for normalization. The results are representatives of two independent experiments.

epithelia and of CXCR3 underneath the epithelial layer, suggesting paracrine signaling. At 6 hpi, there was a clear increase of CXCL10 in all epithelial cell layers in *CA*-infected corneas with a few CXCR3-positive cells in the stroma (Fig. 3D). Consistent with the PCR results, flagellin pretreatment augmented *CA*-induced CXCL10 expression in all CECs, with a few cells strongly expressing CXCR3 in the stroma (Fig. 3E) at 6 hpi. At 24 hpi, epithelial lesions were detected in the infected corneas. In the control, PBS-treated corneas, there was a large lesion with reduced epithelial cell layers. The CECs surrounding the lesion expressed high levels of CXCL10, while CXCR3-positive cells, many of which were in clusters, were primarily detected in the stroma (Fig. 3F). In flagellin-pretreated corneas at 24 hpi, a portion of the epithelium became thicker, assumed to be the entering site of invading *CA*; there were strongly CXCL10-stained cells (CXCL10^{high}) in the epithelial layer, many of which were at the apical side of the thickening site, and in the stroma, mostly at the posterior side. A cluster of CXCR3-positive cells was inserted into the epithelium (Fig. 3G). In both *CA*-infected corneas with or without flagellin pretreatment at 24 hpi, CXCR3-positive cells were only found where epithelial lesions or abnormalities were detected. The overall staining intensity of CXCL10 and CXCR3 in flagellin-pretreated corneas (Fig. 3G) is much less than that in the control (Fig. 3F) at 24 hpi. The expression patterns of CXCL10 and CXCR3 detected by immunohistochemistry were consistent with that detected by real-time PCR (Fig. 2B).

CXCL3-positive cells around lesion sites are CXCL10-positive NK cells

We next investigated cell type(s) that express CXCL10 and CXCR3 in *CA*-infected corneas with or without flagellin pretreatment (Fig. 4). Our previous work revealed that neutrophils play a key role in eradicating invading pathogens, including *CA* [14, 19]. To assess the potential involvement of other innate immune cells, such as NK, macrophages, and DCs in fungal keratitis, we prescreened *CA*-infected corneal sections and found that among these cell types, NK1.1-positive cells exhibited a similar staining pattern of CXCL3-positive cells. As such, we used double immunofluorescence staining to determine if indeed those CXCL10- and/or CXCR3-positive cells were also NK1.1-positive in *CA*-infected corneas. In the control, *CA*-infected corneas at 24 hpi, CECs were CXCL10-positive whereas CXCL10^{high} cells were found to surround a small epithelial lesion site with a large cluster of CXCL10^{high} cells on top of the epithelium and in the stroma underneath the epithelial layer (arrows, Fig. 4A, i). NK1.1 staining was totally negative for CECs, while NK1.1-positive cells showed an identical localization of CXCL10^{high} cells in the epithelium as well as in the stroma (Fig. 4A, iii and v). In flagellin-pretreated corneas, CXCL10 was expressed in the epithelium and in some stroma cells (Fig. 4A, ii); the density of NK1.1-positive cells was low and located primarily at the posterior side of the stroma (Fig. 4A, iv); and the distribution of these NK1.1-positive cells matched well with the CXCL10^{high} cells (Fig. 4A, ii and vi). There were cells in the stroma that were CXCL10-positive (arrowheads), but NK1.1-negative.

In CXCR3 and NK1.1 double-stained sections, CXCR3-positive cells in the stroma and epithelium (arrowhead) were also NK1.1-positive (Fig. 4B, i, iii, and v). In flagellin-pretreated corneas, all CXCR3-positive cells were NK1.1-positive (Fig. 4B, ii, iv, and vi). It should be mentioned that NK1.1-positive cells were mostly located at or near lesion sites in *CA*-infected corneas. Taken together, since we excluded contamination with conventional T cells, including NK T-cells through the use of *Rag1*$^{-/-}$ mice [29, 30], we concluded that the infiltrated cells expressing CXCR3 are NK cells.

CXCL10-CXCR3-mediated signaling is required for fungal clearance in the cornea

Having shown that both CXCL10 and CXCR3 were expressed in *CA*-infected corneas at 24 hpi, we next investigated if CXCL10 functions as an AMP or as a signaling molecule that involves its receptor CXCR3 by Ab-mediated functional neutralization (Fig. 5). The CXCL10 or CXCR3 neutralizing antibodies, along with control rabbit IgG, were injected into subconjunctival spaces 4 h prior to flagellin pretreatment for 24 h, followed by *CA* inoculation. As shown in Figure 5, the corneas of the control (unspecific IgG injected, PBS instilled corneas) were partially covered with opacification at 1 dpi while flagellin pretreatment protected the C57BL/6 mouse corneas from *CA* infection (Fig. 5A). The CXCL10- or CXCR3-neutralized corneas had much more severe keratitis, with

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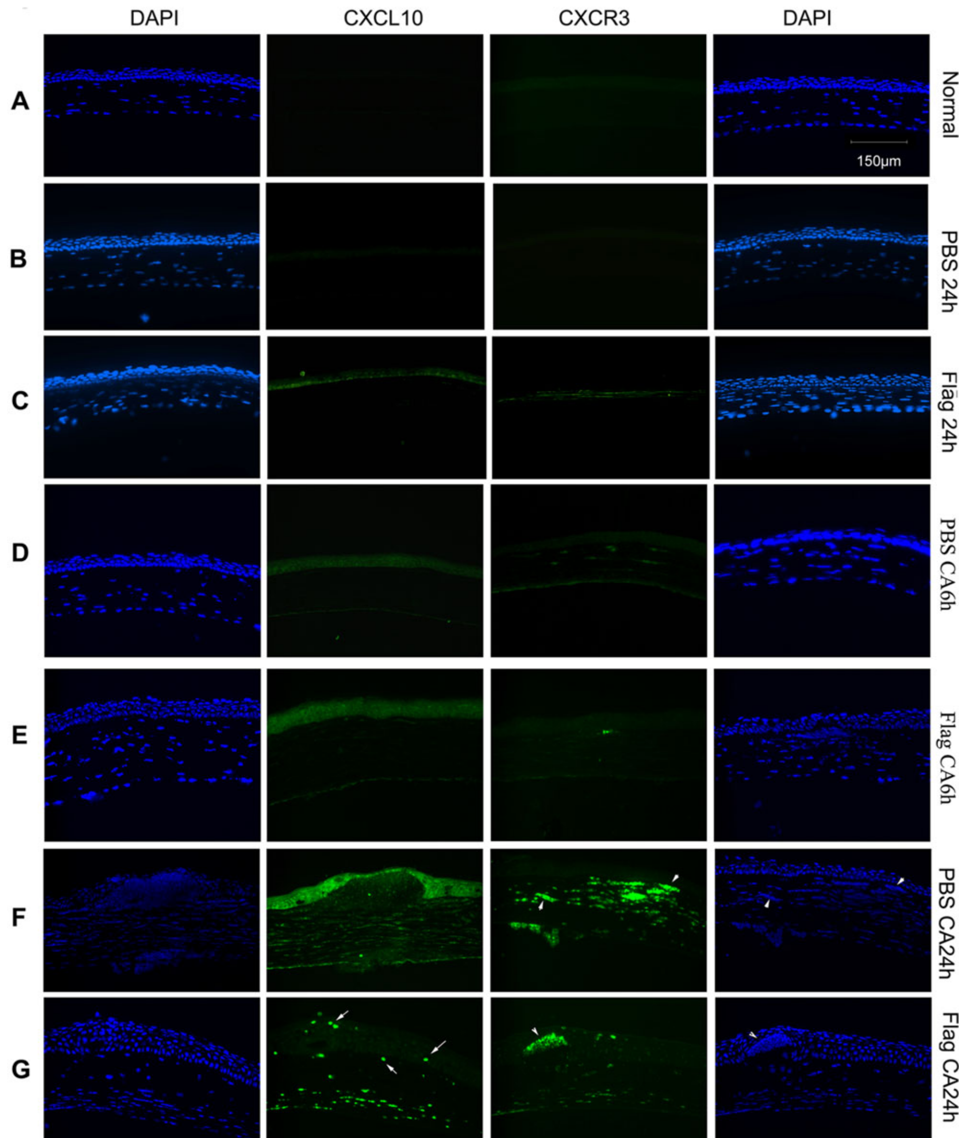

Figure 3. CXCL10 and CXCR3 expression and distribution in *CA*-infected corneas with or without flagellin pretreatment. C57BL/6 mouse corneas were pretreated with or without topical flagellin for 24 h, followed by CA inoculation as described in Figure 1. At 0 (no bacterial inoculation), 6, and 24 h postinoculation, corneas ($n = 3$ for each condition) were collected, embedded in Optimal Cutting Temperature (OCT), and sectioned. The sections were subjected to immunohistochemical analysis with rabbit anti-mouse CXCL10 (1:100), with DAPI staining for nuclei of the same section at left and CXCR3 (1:250) with DAPI staining at right. Arrowheads indicate positive cell clusters. Two independent experiments were performed, one representative image for each condition is presented.

the whole corneas covered with opacification of different intensity in the control mice. Moreover, there was also partial corneal melting (arrows, Fig. 5A) in CXCL10- or CXCR3-neutralized corneas, with clinical scores >10, indicating severe keratitis (Fig. 5B). While flagellin-induced protection was diminished when CXCL10 or CXCR3 were neutralized, the severity of keratitis was less in the flagellin-pretreated corneas compared with the counterparts of the control, PBS-treated ones, with clinical scores of approximately 6 (moderate keratitis). Consistent with the severity of keratitis, both CXCL10- and CXCR3-neutralized corneas had much greater numbers of *CA* compared with IgG-injected corneas. Surprisingly, neutralizing CXCR3 had a significantly higher fungal burden (Fig. 5C) while exhibiting similar effects on PMN infiltration (Fig. 5D) and CXCL2 expression (Fig. 5E), compared to CXCL10 neutralized.

NK cells are important for corneal innate defense against CA infection

Since NK cells expressed CXCR3, we next investigated the role of NK cells in fungal clearance and PMN infiltration by the subconjunctival injection of NK1.1-neutralizing Ab (Fig. 6). Corneas

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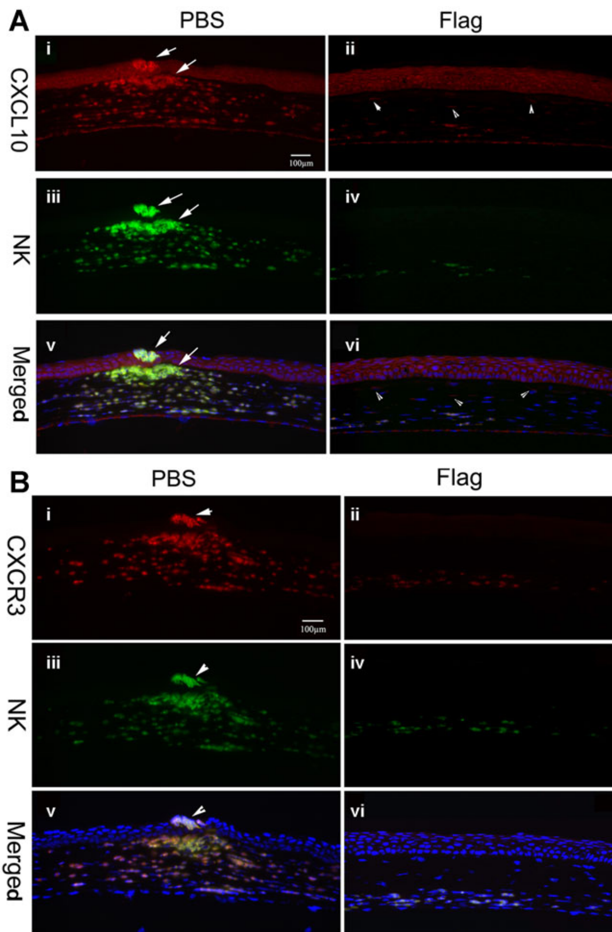

Figure 4. Cell-type determination of infiltrated cells expressing CXCL10 and CXCR3. C57BL/6 mouse corneas ($n = 3$ for each condition) were pretreated with (ii, iv, vi subpanels in (A) and (B)) or without (i, iii, v subpanels in (A) and (B)) topical flagellin for 24 h, followed by CA inoculation as described in Figure 1, then stained with (A) CXCL10 or (B) CXCR3 and co-stained with NK1.1 (green) antibodies. Arrowhead indicates co-localized cells. Three independent experiments were performed, one representative image for each condition is presented.

injected with NK1.1 had much more severe keratitis (Fig. 6A) at 1 dpi, with opacification covering the entire cornea, severe ocular surface irregularity, signs of inflammation in aqueous humor, and neovascularization. The much small size of NK1.1-treated eyes was indicative of the cornea near perforation. Figure 6B showed that the subconjunctival injection of NK1.1 greatly reduced the number of cells expressing CXCR3; the staining intensity of these CXCR3-positive infiltrated cells (Fig. 6B, i and ii) was also relatively lower compared with IgG-treated corneas, suggesting the reduction or depletion of NK cells. The NK1.1-treated cornea sections were stained with FITC-labeled NK1.1, four lightly stained cells were detected (arrows, Fig. 6B, iv) in NK1.1-treated corneas while large number of NK1.1-positive cells were observed in IgG-injected controls (Fig. 6B, iii). Thus, the subconjunctival injection of NK1.1 resulted in the depletion of NK cells. Functionally, NK-cell depletion resulted in great thinning of the cornea (ulceration, Fig. 6B, ii and iv), consistent with the average clinical score >10 (Fig. 6C), indicating severe keratitis. Plate counting revealed a small increase in fungal burden in NK-cell depleted corneas (1.37-fold) (Fig. 6D). PMN infiltration determined by MPO assay was also significantly higher in NK-depleted corneas (Fig. 6E). Taken together, NK-cell depletion resulted in severe fungal keratitis in C57BL/6 mouse corneas.

In vitro fungicidal activity of CXCL10

CXCR3 ligands have been shown to possess the ability to kill microorganisms, mostly bacteria [23, 31, 32]. To determine if CXCL10 can kill *CA*, as demonstrated for CCL20 [32], we performed in vitro fungus killing assay and found that under physiological condition (PBS), recombinant human CXCL10 killed CA in a concentration-dependent manner (Fig. 7).

Recombinant CXCL10 dampens CA keratitis

As flagellin-induced protection was associated with the endogenous expression of CXCL10, and human CXCL10 is capable of killing *CA* in vitro, we next investigated whether exogenous CXCL10 can be used to protect corneas from fungal keratitis. Two routes of application were used. In one group, CXCL10 (500 ng in 5 μL) was subconjunctivally injected into mice with PBS solution as the control 4 h prior to *CA* inoculation, followed by the topical application of 5 μL PBS with or without 100 ng CXCL10 at 4 hpi (Fig. 8). At 1 dpi, the PBS-treated eyes showed keratitis, whereas the CXCL10-treated corneas exhibited low levels of inflammation with a few small and faint areas of opacification (Fig. 8A). Moreover, exogenous CXCL10 significantly decreased the fungal burden (Fig. 8B), PMN infiltration (Fig. 8C), and CXCL2 expression (Fig. 8E) at 1 dpi.

In the second group, CXCL10 was dissolved in Soothe emollient eye drops for extended retention at the ocular surface; 5 μL containing 25 ng/μL CXCL10 was topically applied to the infected corneas starting at 6 hpi, five times for the first day and three times daily until day 7. Figure 9 shows the images of infected corneas at day 3, 5, and 7 after CXCL10 treatment (dpt). At 3 dpt (88 h postinfection), the control, Soothe instilled corneas, displayed dense opacification localized near the center of the cornea, while the CXCL10-treated cornea had less than half of the cornea covered with a small central ring of dense opacification. At day 5, there was improvement for both conditions. In Soothe-treated eyes, some peripheral areas were now transparent, but a central dense opaque area persisted, whereas the density of opacification in CXCL10-treated eyes was substantially decreased (Fig. 9A). At 7 dpt, the treated cornea was almost free of diseases, while the control eye remained in a state of moderate keratitis. Figure 9B shows the clinical scores of these two groups over 7 days ($n = 5$); in each day examined, CXCL10-treated corneas had significantly less keratitis than in eyes instilled with Soothe alone.

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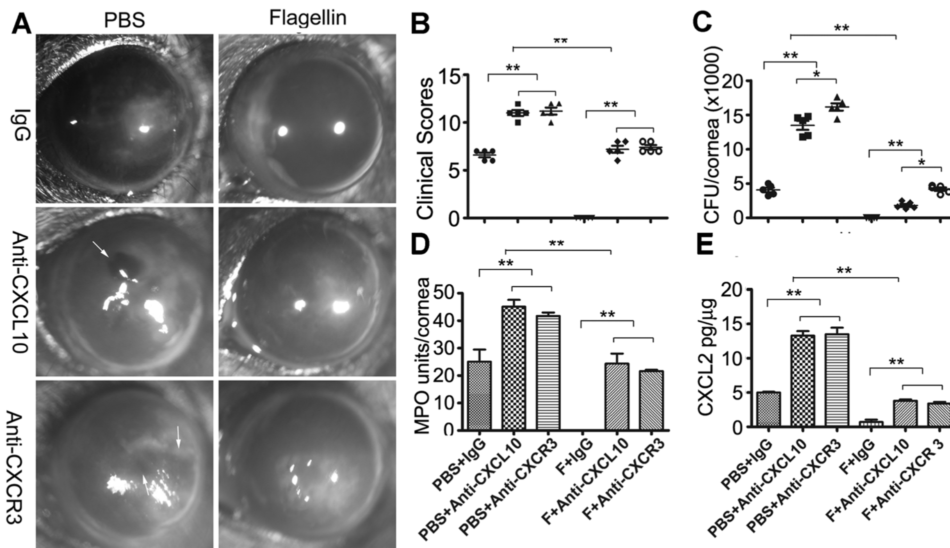

Figure 5. CA keratitis occurs upon local neutralization of CXCL10 or CXCR3. C57BL/6 mouse corneas were pretreated with flagellin or PBS at −24 h, as described in Figure 1. At −6 h, CXCL10- or CXCR3-neutralizing antibodies (2.5 μg/5 μL), with the same dosage of rabbit IgG as the control, were injected into the subconjunctival space, followed by CA inoculation at 0 h. Eyes were (A) photographed and (B) scored at 24 hpi. (C) Mice were then euthanized, the corneas were excised and subjected to fungal plate counting with the results presented as CFU (×1000 *CA* per cornea). In (B) and (C), each symbol represents an individual mouse and mean ± SD are indicated with horizontal lines. (D) MPO determination (units/cornea) and (E) CXCL2 ELISA were performed using corneal supernatants. Results in (D) and (E) show mean + SD and are representative of two independent experiments ($n = 5$ each) and indicated, $^{*}p < 0.05$, $^{**}p < 0.01$ (one-way ANOVA).

Discussion

Using flagellin as an immunostimulant to activate innate immunity and *CA* as a model pathogen, we report here that both CXCL10 and CXCR3 become upregulated in the cornea in response to *CA*. Flagellin pretreatment resulted in CXCL10 expression in basal epithelial cells and CXCR3 expression in the subbasal stroma in uninfected corneas, and in augmented CXCL10 expression and in the altered distribution of CXCR3-expressing cells in infected corneas. The highly elevated expression of CXCL10 by flagellin pretreatment may contribute to the elimination of invading pathogens within CXCL10-expressing epithelium,

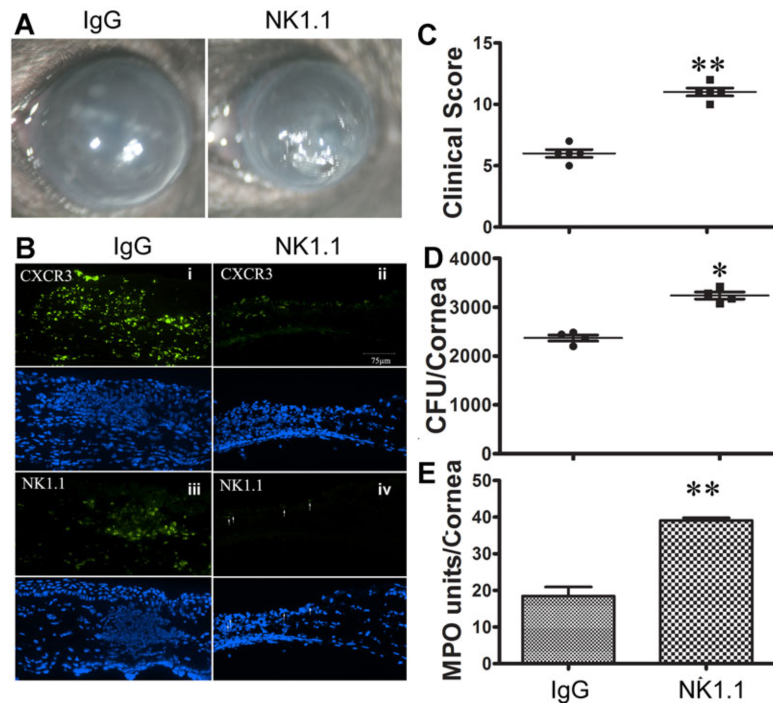

Figure 6. *CA* keratitis with local depletion of NK cells. Mouse corneas were subconjunctivally injected with NK1.1 (2.5 μg/5 μL) neutralizing antibodies, with the same dosage of rabbit IgG as the control, 6 h prior to CA inoculation. (A) Eyes were photographed with a dissection microscope equipped with a digit camera. (B) At 1 dpi, mice were than euthanized, the corneas were excised and subjected to cryostat sectioning and immunostaining with FITC-conjugated CXCR3 or NK1.1 antibodies, with DAPI for nuclear staining. (C) Clinical scoring was performed to quantitate disease severity. (D) Fungal plate counting was performed, with the results presented as CFU CA per cornea. Each symbol represents an individual mouse and mean ± SD are indicated. (E) MPO determination was performed (units/cornea) and data are shown as means + SD ($n = 5$, Student's t-test). Results are representative of two independent experiments.

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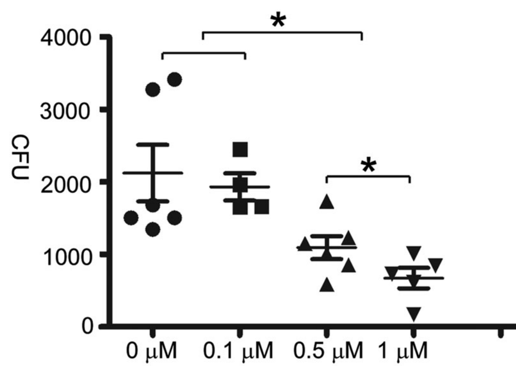

Figure 7. In vitro fungicidal activity of recombinant and epithelial-expressed Cxcl10.CA (10^4 CFU) in 400 μL PBS were incubated with recombinant human CXCL10 in different concentration for 2 h in 37°C. At the end of incubation, the samples were subjected to fungal culture and plate-colony counting. The results were presented as CFU per concentration. Each symbol represents an individual sample and mean ± SD are indicated. Results are representative of three independent experiments. $^*p < 0.05$ (one-way ANOVA).

resulting in enhanced innate immunity and the eradication of fungal keratitis. However, in untreated, naïve corneas, CXCL10-CXCR3 signaling and CXCL10-mediated immune cell infiltration are an important part of mucosal innate defense mechanisms against *CA* infection, since the depletion of either one resulted in more severe keratitis. Using double immunofluorescence staining, we identified two types of CXCL10-positive cells in *CA*-infected corneas: epithelial cells and cells with more abundant CXCL10 that infiltrated both the epithelial and stromal layers. These CXCL10^{high} cells are also CXCR3- and NK1.1-positive, indicating that they are NK cells as flagellin-induced protection was shown not to require any functional T cells (thus excluding NK T-cells involvement) or B cells. Depleting NK cells increases fungal keratitis severity. Flagellin pretreatment dampened the infiltration of NK cells and PMN [14], probably due to reduced pathogen burden (fourfold decrease at 6 hpi and eightfold decrease at 24 hpi [14]), which consequently reduced the need for infiltrated cells. Finally, we showed the therapeutic effects of recombinant CXCL10 in preventing corneal infection and in accelerating the resolution of *CA*-induced inflammation. Taken together, our study for the first time reveals that while epithelial CXCL10 is fungicidal, the CXCL10-CXCR3 signal pathway plays a role in the recruitment and activation of NK cells, which contribute to innate defense against *CA* keratitis.

Our previous studies unraveled a part of the underlying mechanisms for flagellin-induced protection, including the persistent induction of antimicrobial CRAMP (human LL-37 homolog) in corneal epithelia and the infiltration of a small number of neutrophils in the stroma prior to pathogen inoculation [14, 19, 33]. In this study, we demonstrated that flagellin exposure resulted in the induction of CXCL10 in basal epithelial cells and its receptor CXCR3 underneath the CXCL10-positive cells at the anterior side of the stroma. Interestingly, we observed that flagellin pretreatment enhanced the expression of CXCL10 at the early stage of infection (6 hpi) but suppressed it at a later stage (24 hpi). CXCL9, CXCL10, and CXCL11 are chemokines that are rapidly induced upon infection and upon TLR activation in epithelial cells, endothelial cells, and monocytes [34, 35]. In this study, we showed that among the three CXCR3 ligands, only CXCL10 is expressed in CECs in response to *CA* infection in C57BL/6 mice. While CXCL10 expression is augmented, CXCR3 expression was suppressed by flagellin pretreatment at 6 hpi. Hence, the flagellin-augmented expression of CXCL10, but not its receptor CXCR3, is likely the result of reprogramming rather than the additive effects of flagellin and *CA* challenge [17]. CXCL10 is a multifunctional chemokine, exerting direct antimicrobial effects against bacteria as effective as β-defensin [36, 37]. Whether CXCL10 has fungicidal activity has not been directly assessed, but it can be inferred from that of CL20 [32]. In this study, we demonstrate that

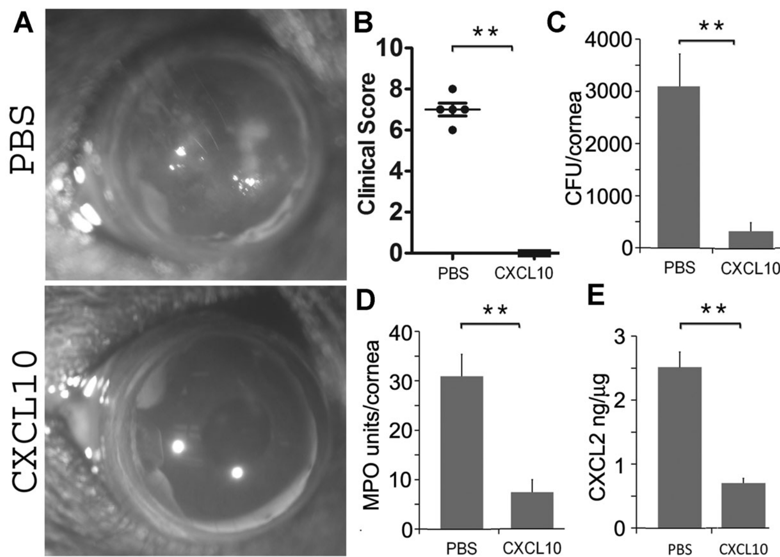

Figure 8. Effects of exogenous CXCL10 on *CA* keratitis. Mouse corneas were subconjunctivally injected with 5 μL PBS with or with 500 ng recombinant Cxcl10 4 h prior to *CA* inoculation. Eyes were (A) photographed at 24 hpi with a dissection microscope equipped with a digital camera and (B) scored. Photographs are representative of five corneas each in one of three independent experiments. Each symbol in (B) represents an individual mouse cornea and mean ± SD are shown. (C) Mice were then euthanized, the corneas were excised and subjected to fungal plate counting, with the results presented as CFU CA per cornea. (D) MPO determination (units/cornea); and (E) CXCL2 measurement (pg/μg cell extracts) were determined using ELISA. The results in (C–E) show means + SD ($n = 5$ each) and are representative of three independent experiments. $^{**}p < 0.01$ (Student's *t*-test).

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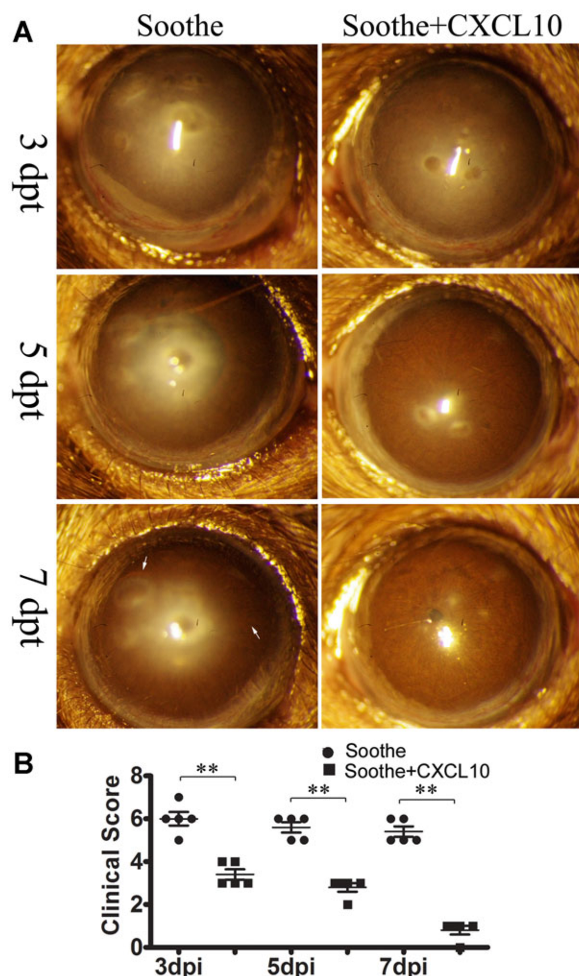

Figure 9. Effects of topical, postinfection-applied Cxcl10 on CA keratitis. Mouse corneas were inoculated with 1.0×10^5 CFU of CA at 0 h. Soothe eye drops with or without 100 ng in 5 μL CXCL10 were instilled five times at day 1 of the treatment (dpt) starting 6 hpi; in the following days, three times daily until 7 dpt. (A) The eyes were photographed at 3, 5, and 7 dpt with a slit lamp microscope. Photographs are a representative of five corneas. (B) Disease severity is represented by clinical scores, where each symbol represents an individual sample and horizontal lines represent the mean clinical score ± SD. A nonparametric Mann–Whitney *U*-test was performed to compare each CXCL10-treated to Soothe-instilled group. The results are representative of three independent experiments ($n = 5$ each). $^{**}p < 0.01$ (Student's *t*-test).

recombinant CXCL10 possesses moderate fungicidal activity under physiological conditions. As significant fungal clearance was observed at 6 hpi in flagellin-pretreated corneas [17] when the infiltration of immune cells, particularly CXCR3-positive cells, are sparse (Fig. 3). Hence, we suggest that although the in vitro fungicidal activity is moderate, the augmented expression of CXCL10 in the epithelium contributes to flagellin-induced innate protection by direct killing of the fungi at the epithelial layer. This was confirmed by subconjunctival injection of CXCL10, followed by a topical application that nearly abolished *CA* keratitis. Other AMPs shown to be involved include CRAMP (human LL-37) and β-defensins [14, 38, 39]. The elimination of invading pathogens at the early stages of infection induced by flagellin pretreatment is likely to result in less infiltration of professional immune cells, including PMN [14], NK cells, macrophages, resulting in reduced inflammation, and, as such, less or no tissue damage.

CXCL10 signals through CXCR3, and acts primarily in the recruitment of NK cells and plasmacytoid DCs to the sites of infection and inflammation [26, 36, 37, 40]. Several studies of HSV-1 keratitis revealed that the CXCL10 but not CXCL9 and CXCL11, CXCR3 signaling axis plays a critical protective role in the mouse model of viral keratitis [41, 42]. To determine if CXCL10 primarily works as a fungicidal molecule, or as a chemokine that acts through its engagement with CXCR3, we used anti-CXCL10 or anti-CXCR3 antibodies and found that neutralizing either CXCL10 or CXCL3 resulted in significantly higher fungal load and greater inflammation than the control. Surprisingly, neutralizing CXCR3 resulted in a greater increase in fungal burden in both the control and flagellin-pretreated corneas, suggesting that CXCL10-mediated signaling and/or recruitment are more important than its fungicidal activity at 1 dpi. Our results differ from what has been reported in a murine model of inhalational anthrax infection, where the neutralization of CXCL10, but not its receptor CXCR3, increased bacterial burden [36], suggesting that CXCL10 acts as an important AMP in the anthrax infection model. Importantly, neutralizing CXCL10 or CXCR3 impaired, but did not abolish, flagellin-induced protection, suggesting that CXCL10-independent effects of flagellin, including the augmented expression of other AMPs, such as CRAMP and defensins, and the presence of innate immune cells such as PMN at the time of pathogen–host interaction [14, 19]. The effects of neutralizing CXCR3 on the severity of *CA* keratitis is likely due to the effects of CXCL10-mediated recruitment of professional innate immune cells. Indeed, we observed greatly increased NK cells in *CA*-infected corneas 24 hpi. By this time, a large number of CXCR3-positive cells were detected in the control corneas, located mostly at the posterior side, while CXCL10-positive cells were found both in the epithelial layer and the stroma. As CECs are generally CXCR3-negative, the epithelial cell-derived CXCL10 is likely to recruit other immune cells from the limbal region rich in blood supply including macrophages and NK cells. As such, it is of great interest to identify CXCR3 and/or CXCL10 positive cells in *CA*-infected cells.

The staining pattern of NK1.1 is similar to CXCR3 in *CA*-infected corneas, suggesting that the infiltrated CXCR3-positive and/or $CXCL10^{high}$ cells are indeed NK or NK T cells. Our study using Rag1KO mice ruled out the involvement of NK T cells. Hence we concluded that the CXCR3-positive cells in *CA*-infected cornea are indeed NK cells. Interestingly, the NK1.1-positive cells are also CXCL10-positive, suggesting a possible autocrine signaling in the recruited NK cells. Alternatively, CXCL10 expressed by the epithelium binds to CXCR3 on the surface of the NK cells, resulting in $CXCL10^{high}$ cells. In the *CA*-infected cornea, the cells expressing abundant CXCL10 and CXCR3 were found to form densely packed clusters on the top of and underneath the thin epithelial layer in a lesion (infection) site. These cells are also positively stained with NK1.1. We postulate that NK cells surround invading *CA* to form densely packed clusters in which *CA* were eradicated. This is consistent with a recent report showing human

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NK cells are capable of ingesting *CA*, resulting in their activation and in killing these microbes by secreting perforin [43]. Moreover, the activated NK cells may also be indirectly involved in fungal clearance by modulating neutrophil antifungal activity [43]. It is interesting to note that when NK cells are neutralized, there are more neutrophils present and severe tissue damage (Fig. 6), similar to that observed in dextran sodium sulfate induced colitis in mice [44]. One potential role of NK cells is to regulate neutrophil function in a context-dependent manner: NK cells are activated upon encountering *CA* and activate neutrophils [43]; when *CA* are being eliminated by NK-cell released perforin [43] and/or by NK-activated neutrophils, NK cells may induce neutrophil apoptosis [45]. In addition, NK cells may also participate in tissue repair in the cornea as demonstrated recently in the epithelial wound model [46, 47]. Consistent with NK cells as a critical component of innate immunity, our NK-cell depletion study revealed severe keratitis in the absence of NK cells, including corneal ulcers and potential fungal dissemination to the anterior chamber. The other known effectors cells are neutrophils, which we have shown to be critical for flagellin-induced protection [14, 19]. Recent studies showed that a unique population of BM neutrophils produce and respond to IL-17 [48, 49]. These neutrophils can be induced to produce ROS in response to IL-17, resulting in an increase in fungal killing in vitro and in a model of *Aspergillus*-induced keratitis. These studies suggest a role of IL-17 in corneal innate defense [48, 49]. The mechanisms underlying NK recruitment, activation, fungicidal activity, and their interaction with other immune, particularly neutrophils, and residential cells in response to infection are under investigation in our laboratories.

Finally, we tested the therapeutic potential of CXCL10 and demonstrated that the administration of this chemokine prior to pathogen inoculation significantly alleviated *CA* keratitis. More importantly, the topical application of CXCL10 at 6 hpi was still effective in accelerating the resolution of infection-associated inflammation and in inhibiting infection and inflammation induced hem- and lymphangiogenesis (Gao and Yu, unpublished results). Hence, CXCL10 has the potential to be used as an adjunctive therapy to reduce microbial keratitis-induced tissue damage.

Materials and methods

Fungi

CA strain SC5314, a clinical isolate capable of producing experimental keratomycosis, was cultured on YPD agar (Sigma) for 3 days at 25°C. Colonies were harvested after 3 days of inoculation and diluted in sterile PBS to yield 2×10^5 CFU/μL based on the OD at 600 nm, using a predetermined OD_{600} conversion factor of 1 OD = 3×10^7 CFU/mL [15].

Animals

WT C57BL/6 mice (8 weeks of age; 20–24 g weight) were used. C57BL/6.129S7-Rag1tm1Mom/J (Rag1$^{-/-}$, 8 weeks females with C57BL/6 females as the control) were purchased from the Jackson Lab. Animals were treated in compliance with the ARVO Statement on 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 preparation, pretreatment, and infection procedure

Flagellin was prepared from *P. aeruginosa* strain PA01 as described earlier [16, 50]. The bioactivity of each preparation was compared with previous samples for their ability to induce tolerance in cultured human CECs [51]. For flagellin pretreatment, mice (n = 5/group/treatment) were anesthetized with ketamine/xylazine and placed beneath a stereoscopic microscope at a magnification of 40×, and the corneas were scratched gently with three 1 mm incisions using a sterile 26-gauge needle. Purified flagellin (500 ng in 5 μL of PBS) or PBS for the control was applied to the injured corneas of the same mice (one serves as the control for the other; the data were presented as the number of eyes). After 24 h, corneas were excised and processed for CXCL10 and CXCR3 expression. For corneal infection, mice were anesthetized, the pretreated corneas were rescratched 24 h post-flagellin application, and a 5 μL suspension containing a 1×10^5 CFU of *CA* strain SC5314 was applied to the surface of the scarified cornea. The anesthetized mice were kept on a fixed position with the instilled solution remaining on the ocular surface until they awakened (usually >20 min).

Clinical scoring

For the assessment of clinical scores, the mice were color-coded and examined by two independent observers daily, one with the knowledge of codes and the other in a blind fashion, and photographed at 1, 3, 5, or 7 days after infection (dpi). If there was a conflict in clinical scoring, a third person, usually the corresponding author, would perform scoring from photographs. Ocular disease was graded in clinical scores ranging from 0 to 12, according to the scoring system developed by Wu et al. [52]. A grade of 0–4 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. At the indicated time, all infected corneas were photographed with a slide lamp or a dissection microscope to illustrate the disease progression.

Fungal load determination, cytokine ELISA, and MPO measurement

We used our previously modified methods that allowed all three assays (fungal load, MPO determination, and cytokine ELISA measurement) to be performed with a single mouse cornea. Briefly, the corneas were excised from the enucleated eyes, minced, and

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homogenized in 100 μL PBS. The homogenates were divided into two parts, one for plate fungal counting and the other for cytokine ELISA and MPO measurements [14]. In each experiment, the controls without flagellin pretreatment were expected to have 2000–5000 CFU recovered, while flagellin pretreatment eradicated invading pathogens (<10 CFU/cornea) at 1 dpi.

RT-PCR and real-time PCR

Mouse cornea RNA was extracted using RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. cDNA was generated with an oligo(dT) primer (Invitrogen) followed by analysis using real-time PCR with the Power SYBR Green PCR Master Mix (AB Applied Biosystems, University Park, IL) based on the expression of β-actin. Generated cDNA was amplified by PCR by using primers for mouse Cxcl10, Cxcr3, and β-actin. The PCR products and the internal control β-actin were subjected to electrophoresis on 1% agarose gels containing ethidium bromide. Stained gels were captured by using a digital camera. The following primer pairs were used: 5′-GACGGCCAGGTCATCACTATTG-3′, 5′-AGGAAGGCTGGAAAAGAGCC-3′ for β-actin, 5′-CCATCAGCA CCATGAACCCAAGT-3′, 5′-CACTCCAGTTAAGGAGC-3′ for Cxcl10, 5′-GATCAAACCTGCCTAGATCC-3′, 5′-GGCTGTGTAGA ACACAGAGT-3′ for Cxcl9, 5′-ATTACCAGGCTGCAGAACTTT-3′, 5′-TCTTCCTAGATGTTCGTGTGC-3′ for Cxcl11, and 5′-AGAATC ATCCTGGTCTGAGACAA-3′, 5′-AAGATAGGGCATGGCAGCTA-3′ for Cxcr3.

Immunohistochemistry

At the indicated times, the corneas were excised from sacrificed mice and were frozen in Optimal Cutting Temperature (OCT) compound; posterior parts along with the lens were removed after the global parts were just frozen. The corneas were cut into 6 μm thick sections by cryostat sectioning and the sections were mounted to polylysine-coated glass slides. After a 10 min fixation in 4% paraformaldehyde, the sections were blocked with 10 mM PBS, containing 2% BSA, for 1 h at room temperature. Sections were then incubated with primary antibodies: rabbit anti-mouse CXCL10 (Peprotech; 1:100), CXCR3 (Abnova, 1:250), followed by the secondary Ab, Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Inc), with or without FITC-labeled anti-mouse NK1.1 (eBiosciences; 1:100). Slides were mounted with DAPI mounting media. Controls were similarly treated, but the primary Ab was replaced with nonspecific rabbit IgG.

CXCL10, CXCR3, and NK1.1 neutralization

Mice were anesthetized as prescribed and rabbit anti-murine CXCL10 or CXCR3, anti-mouse NK1.1 (clone PK136) (2.5 μg/5 μL, Abnova) were injected subconjunctivally, 2.5 μg/5μL, 6 h before *CA* infection.

In vitro assay of antifungal activity of recombinant CXCL10

For recombinant CXCL10, 400 μL PBS with different concentrations of recombinant mouse CXCL10 were incubated with 1×10^4 CFU *CA* at 37°C for 2 h. Serial dilutions of each reaction mixture were made to inoculate YPD agar plates. Samples (100 μL) were spread evenly over the surface of the plates with sterile glass spreaders. After incubation at 25°C for 72 h, the number of colonies was counted. Experiments were repeated once.

To determine whether CECs expressing CXCL10 possessed fungicidal activity, HUCL cells (a human CEC line [53]) were cultured on 12-well tissue culture plates (Costar), and then were transfected with 1.5×10^{11} CFU AAV2 (recombinant adeno-associated virus vector-2) with inserted DNA that encoded the human CXCL10 with green fluorescence protein as the control (a gift from Dr. Xiao Xiao, University of North Carolina at Chapel Hill). The transfected cells expressed high levels of the target genes at day 3. At day 3 post-AAV2 infection, cells were cultured in 1 mL fresh defined keratinocyte—a serum-free medium (Invitrogen-Life Technologies, Carlsbad, CA)—for 24 h and the culture media were then collected and used for in vitro assay of antifungal activities as described for recombinant CXCL10.

Statistical analysis

Data were presented as the means ± SD. Statistical differences among three or more groups were identified using one-way ANOVA. Differences were considered statistically significant at $p < 0.05$. Between two groups, 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 to determine statistical significance for clinical scores. Experiments were repeated at least twice to ensure reproducibility.

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References

1 **Thomas, P. A.**, Fungal infections of the cornea. *Eye (Lond.)* 2003. **17**: 852–862.

2 **Thomas, P. A. and Geraldine, P.**, Infectious keratitis. *Curr. Opin. Infect. Dis.* 2007. **20**: 129–141.

3 **Jackson, B. E., Wilhelmus, K. R. and Mitchell, B. M.**, Genetically regulated filamentation contributes to *Candida albicans* virulence during corneal infection. *Microb. Pathog.* 2007. **42**: 88–93.

4 **Galarreta, D. J., Tuft, S. J., Ramsay, A. and Dart, J. K.**, Fungal keratitis in London: microbiological and clinical evaluation. *Cornea* 2007. **26**: 1082–1086.

5 **Ritterband, D. C., Seedor, J. A., Shah, M. K., Koplin, R. S. and McCormick, S. A.**, Fungal keratitis at the New York eye and ear infirmary. *Cornea* 2006. **25**: 264–267.

6 **Chern, K. C., Meisler, D. M., Wilhelmus, K. R., Jones, D. B., Stern, G. A. and Lowder, C. Y.**, Corneal anesthetic abuse and *Candida* keratitis. *Ophthalmology* 1996. **103**: 37–40.

7 **Gorscak, J. J., Ayres, B. D., Bhagat, N., Hammersmith, K. M., Rapuano, C. J., Cohen, E. J., Burday, M. et al.**, An outbreak of Fusarium keratitis associated with contact lens use in the northeastern United States. *Cornea* 2007. **26**: 1187–1194.

8 **Patel, A. and Hammersmith, K.**, Contact lens-related microbial keratitis: recent outbreaks. *Curr. Opin. Ophthalmol.* 2008. **19**: 302–306.

9 **FlorCruz, N. V., Peczon, I. V. and Evans, J. R.**, Medical interventions for fungal keratitis. *Cochrane Database Syst. Rev.* 2012. **2**: CD004241.

10 **Tarabishy, A. B., Aldabagh, B., Sun, Y., Imamura, Y., Mukherjee, P. K., Lass, J. H., Ghannoum, M. A. et al.**, MyD88 regulation of Fusarium keratitis is dependent on TLR4 and IL-1R1 but not TLR2. *J. Immunol.* 2008. **181**: 593–600.

11 **Yuan, X. and Wilhelmus, K. R.**, Toll-like receptors involved in the pathogenesis of experimental *Candida albicans* keratitis. *Invest. Ophthalmol. Vis. Sci.* 2010. **51**: 2094–2100.

12 **Jackson, B. E., Wilhelmus, K. R. and Mitchell, B. M.**, Genetically regulated filamentation contributes to *Candida albicans* virulence during corneal infection. *Microb. Pathog.* 2007. **42**: 88–93.

13 **Yuan, X., Mitchell, B. M. and Wilhelmus, K. R.**, Gene profiling and signaling pathways of *Candida albicans* keratitis. *Mol. Vis.* 2008. **14**: 1792–1798.

14 **Gao, N., Kumar, A., Guo, H., Wu, X., Wheater, M. and Yu, F. S.**, Topical flagellin-mediated innate defense against *Candida albicans* keratitis. *Invest. Ophthalmol. Vis. Sci.* 2011. **52**: 3074–3082.

15 **Kumar, A., Yin, J., Zhang, J. and Yu, F. S.**, Modulation of corneal epithelial innate immune response to pseudomonas infection by flagellin pretreatment. *Invest. Ophthalmol. Vis. Sci.* 2007. **48**: 4664–4670.

16 **Gao, N., Kumar, A., Jyot, J. and Yu, F. S.**, Flagellin-induced corneal antimicrobial peptide production and wound repair involve a novel NF-kappaB-independent and EGFR-dependent pathway. *PLoS One* 2010. **5**: e9351.

17 **Gao, N., Sang Yoon, G., Liu, X., Mi, X., Chen, W., Standiford, T. J. and Yu, F. S.**, Genome-wide transcriptional analysis of differentially expressed genes in flagellin-pretreated mouse corneal epithelial cells in response to Pseudomonas aeruginosa: involvement of S100A8/A9. *Mucosal Immunol.* 2013. **6**: 993–1005.

18 **Oppenheim, J. J., Tewary, P., de la Rosa, G. and Yang, D.**, Alarmins initiate host defense. *Adv. Exp. Med. Biol.* 2007. **601**: 185–194.

19 **Kumar, A., Gao, N., Standiford, T. J., Gallo, R. L. and Yu, F. S.**, Topical flagellin protects the injured corneas from *Pseudomonas aeruginosa* infection. *Microbes Infect.* 2010. **12**: 978–989.

20 **Leal, S. M., Jr., Cowden, S., Hsia, Y. C., Ghannoum, M. A., Momany, M. and Pearlman, E.**, Distinct roles for dectin-1 and TLR4 in the pathogenesis of *Aspergillus fumigatus* keratitis. *PLoS Pathog.* 2010. **6**: e1000976.

21 **Sun, Y., Karmakar, M., Roy, S., Ramadan, R. T., Williams, S. R., Howell, S., Shive, C. L. 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.

22 **Yoon, G. S., Dong, C., Gao, N., Kumar, A., Standiford, T. J. and Yu, F. S.**, Interferon regulatory factor-1 in flagellin-induced reprogramming: protective role of CXCL10 in cornea innate defense against *Pseudomonas* infection. *Invest. Ophthalmol. Vis. Sci.* 2013. **54**: 7510–7521.

23 **Cole, A. M., Ganz, T., Liese, A. M., Burdick, M. D., Liu, L. and Strieter, R. M.**, Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J. Immunol.* 2001. **167**: 623–627.

24 **Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A. E. et al.**, The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* 1998. **101**: 746–754.

25 **Cao, W. and Liu, Y. J.**, Innate immune functions of plasmacytoid dendritic cells. *Curr. Opin. Immunol.* 2007. **19**: 24–30.

26 **Mohan, K., Cordeiro, E., Vaci, M., McMaster, C. and Issekutz, T. B.**, CXCR3 is required for migration to dermal inflammation by normal and in vivo activated T cells: differential requirements by CD4 and CD8 memory subsets. *Eur. J. Immunol.* 2005. **35**: 1702–1711.

27 **Cole, A. M., Ganz, T., Liese, A. M., Burdick, M. D., Liu, L. and Strieter, R. M.**, Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J. Immunol.* 2001. **167**: 623–627.

28 **Yoneyama, M., Suhara, W. and Fujita, T.**, Control of IRF-3 activation by phosphorylation. *J. Interferon Cytokine Res.* 2002. **22**: 73–76.

29 **Bedoui, S., Kupz, A., Wijburg, O. L., Walduck, A. K., Rescigno, M. and Strugnell, R. A.**, Different bacterial pathogens, different strategies, yet the aim is the same: evasion of intestinal dendritic cell recognition. *J. Immunol.* 2010. **184**: 2237–2242.

30 **Zhou, X. F., Yu, J., Chang, M., Zhang, M., Zhou, D., Cammas, F. and Sun, S. C.**, TRIM28 mediates chromatin modifications at the TCRalpha enhancer and regulates the development of T and natural killer T cells. *Proc. Natl. Acad. Sci. USA* 2012. **109**: 20083–20088.

31 **Crawford, M. A., Lowe, D. E., Fisher, D. J., Stibitz, S., Plaut, R. D., Beaber, J. W., Zemansky, J. et al.**, Identification of the bacterial protein FtsX as a unique target of chemokine-mediated antimicrobial activity against Bacillus anthracis. *Proc. Natl. Acad. Sci. USA* 2011. **108**: 17159–17164.

32 **Yang, D., Chen, Q., Hoover, D. M., Staley, P., Tucker, K. D., Lubkowski, J. and Oppenheim, J. J.**, Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J. Leukoc. Biol.* 2003. **74**: 448–455.

33 **Yoon, G. S., Dong, C., Gao, N., Kumar, A., Standiford, T. J. and Yu, F. S.**, Interferon regulatory factor-1 in flagellin-induced reprogramming: potential protective role of CXCL10 in cornea innate defense against *Pseudomonas aeruginosa* infection. *Invest. Ophthalmol. Vis. Sci.* 2013. **54**: 7510–7521.

34 **Muller, M., Carter, S., Hofer, M. J. and Campbell, I. L.**, Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity—a tale of conflict and conundrum. *Neuropathol. Appl. Neurobiol.* 2010. **36**: 368–387.

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35 **Groom, J. R. and Luster, A. D.**, CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol. Cell Biol.* 2011. **89**: 207–215.

36 **Crawford, M. A., Burdick, M. D., Glomski, I. J., Boyer, A. E., Barr, J. R., Mehrad, B., Strieter, R. M. et al.**, Interferon-inducible CXC chemokines directly contribute to host defense against inhalational anthrax in a murine model of infection. *PLoS Pathog.* 2010. **6**: e1001199.

37 **Crawford, M. A., Zhu, Y., Green, C. S., Burdick, M. D., Sanz, P., Alem, F., O'Brien, A. D. et al.**, Antimicrobial effects of interferon-inducible CXC chemokines against *Bacillus anthracis* spores and bacilli. *Infect. Immun.* 2009. **77**: 1664–1678.

38 **Kolar, S. S., Baidouri, H., Hanlon, S. and McDermott, A. M.**, Protective role of murine beta-defensins 3 and 4 and cathelin-related antimicrobial peptide in Fusarium solani keratitis. *Infect. Immun.* 2013. **81**: 2669–2677.

39 **Yuan, X., Hua, X. and Wilhelmus, K. R.**, The corneal expression of antimicrobial peptides during experimental fungal keratitis. *Curr. Eye Res.* 2010. **35**: 872–879.

40 **Colonna, M., Trinchieri, G. and Liu, Y. J.**, Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 2004. **5**: 1219–1226.

41 **Carr, D. J., Wuest, T. and Ash, J.**, An increase in herpes simplex virus type 1 in the anterior segment of the eye is linked to a deficiency in NK cell infiltration in mice deficient in CXCR3. *J. Interferon Cytokine Res.* 2008. **28**: 245–251.

42 **Wuest, T. R. and Carr, D. J.**, Dysregulation of CXCR3 signaling due to CXCL10 deficiency impairs the antiviral response to herpes simplex virus 1 infection. *J. Immunol.* 2008. **181**: 7985–7993.

43 **Voigt, J., Hunniger, K., Bouzani, M., Jacobsen, I. D., Barz, D., Hube, B., Loffler, J. and Kurzai, O.**, Human NK cells act as phagocytes against *Candida albicans* and mount an inflammatory response which modulates neutrophil antifungal activity. *J. Infect. Dis.* 2014. **209**: 616–626.

44 **Hall, L. J., Murphy, C. T., Quinlan, A., Hurley, G., Shanahan, F., Nally, K. and Melgar, S.**, Natural killer cells protect mice from DSS-induced colitis by regulating neutrophil function via the NKG2A receptor. *Mucosal Immunol.* 2013. **6**: 1016–1026.

45 **Thoren, F. B., Riise, R. E., Ousback, J., Della Chiesa, M., Alsterholm, M., Marcenaro, E., Pesce, S. et al.**, Human NK cells induce neutrophil apoptosis via an NKp46- and Fas-dependent mechanism. *J. Immunol.* 2012. **188**: 1668–1674.

46 **Li, Z., Burns, A. R., Rumbaut, R. E. and Smith, C. W.**, Gamma delta T cells are necessary for platelet and neutrophil accumulation in limbal vessels and efficient epithelial repair after corneal abrasion. *Am. J. Pathol.* 2007. **171**: 838–845.

47 **Liu, Q., Smith, C. W., Zhang, W., Burns, A. R. and Li, Z.**, NK cells modulate the inflammatory response to corneal epithelial abrasion and thereby support wound healing. *Am. J. Pathol.* 2012. **181**: 452–462.

48 **Taylor, P. R., Leal, S. M., Jr., Sun, Y. and Pearlman, E.**, Aspergillus and Fusarium corneal infections are regulated by Th17 cells and IL-17-producing neutrophils. *J. Immunol.* 2014. **192**: 3319–3327.

49 **Taylor, P. R., Roy, S., Leal, S. M., Jr., Sun, Y., Howell, S. J., Cobb, B. A., Li, X. et al.**, Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORgammat and dectin-2. *Nat. Immunol.* 2014. **15**: 143–151.

50 **Zhang, J., Xu, K., Ambati, B. and Yu, F. S.**, Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest. Ophthalmol. Vis. Sci.* 2003. **44**: 4247–4254.

51 **Kumar, A., Hazlett, L. D. and Yu, F. S.**, Flagellin suppresses the inflammatory response and enhances bacterial clearance in a murine model of *Pseudomonas aeruginosa* keratitis. *Infect. Immun.* 2008. **76**: 89–96.

52 **Wu, T. G., Wilhelmus, K. R. and Mitchell, B. M.**, Experimental keratomycosis in a mouse model. *Invest. Ophthalmol. Vis. Sci.* 2003. **44**: 210–216.

53 **Rheinwald, J. G., Hahn, W. C., Ramsey, M. R., Wu, J. Y., Guo, Z., Tsao, H., De Luca, M. et al.**, A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Mol. Cell. Biol.* 2002. **22**: 5157–5172.

Abbreviations: **AMP**: antimicrobial peptide · **CA**: *Candida albicans* · **CEC**: corneal epithelial cell · **IRF1**: interferon regulatory factor 1 · **MPO**: myeloperoxidase

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