The NLRP3 Inflammasome Is Required for Protection Against Pseudomonas Keratitis

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Purpose. The current study was designed to examine the role of the NLRP3 inflammasome pathway in the clearance of *Pseudomonas aeruginosa* (PA) infection in mouse corneas.

Methods. Corneas of wild type and NLRP3$^{-/-}$ mice were infected with PA. The severity of bacterial keratitis was graded on days 1 and 3 post-infection by slit lamp, and then corneas were harvested for: (i) bacterial enumeration, (ii) immune cell analysis by flow cytometry, (iii) immunoblotting analysis of cleaved caspase-1 and IL-1β, and (iv) IL-1β quantification by ELISA. In parallel experiments, severity of keratitis was examined in the wild-type mice receiving a subconjunctival injection of a highly selective NLRP3 inhibitor immediately prior to infection.

Results. Compared to wild type mice, NLRP3$^{-/-}$ mice exhibited more severe infection, as indicated by an increase in opacity score and an increase in bacterial load. The hallmark of inflammasome assembly is the activation of proinflammatory caspase-1 and IL-1β by cleavage of their precursors, pro-caspase-1 and pro-IL-1β, respectively. Accordingly, increased severity of infection in the NLRP3$^{-/-}$ mice was associated with reduced levels of cleaved forms of caspase-1 and IL-1β and reduced IL-1β^{+} neutrophil infiltration in infected corneas. Likewise, corneas of mice receiving subconjunctival injections of NLRP3 inhibitor exhibited increased bacterial load, and reduced IL-1β expression.

Conclusions. Activation of NLRP3 pathway is required for the clearance of PA infection in mouse corneas.

Keywords: NLRP3, inflammasome, pseudomonas keratitis

With approximately 30,000 cases annually in the United States, microbial keratitis is a common cause of vision loss. Intact corneas are naturally resistant to infection due to antimicrobial components in tear film and physical barrier generated by tight junctions in epithelial cells. However, minor trauma to the corneal epithelium caused by contact lens wear or other noxious agents and exposure to contaminated solutions, including lens care products are two major predisposing factors[1–5] that facilitate bacterial penetration into the corneal stroma, where they precipitously replicate. A gram-negative bacterium, *Pseudomonas aeruginosa* (PA), has been identified as the most frequently isolated pathogen in all culture-positive cases of contact lens-related microbial infection of the cornea. Once inside the stroma, PA produces a fulminating, highly destructive, sight-threatening corneal infection. One of the serious consequences of PA corneal infection is blindness resulting from persistent inflammatory reaction in the stroma, leading to corneal ulceration with subsequent corneal scarring, and, in some cases, perforation. The current therapy includes antibiotic treatment, which reduces the bacterial burden; despite this, tissue damage occurs as a result of an uncontrolled inflammation.

The innate immune system is the first line of defense against pathogens and is initiated by pattern recognition receptors (PRRs), which respond to invading microbes.[6–10] Previous studies using a mouse model of PA keratitis have revealed a critical role of IL-1β in immune cell recruitment and subsequently bacterial clearance. In this regard, studies in the Hazlett Lab[11,12] have shown that combined subconjunctival and intraperitoneal injection of IL-1β polyclonal antibody significantly reduces: (i) the severity of corneal disease, (ii) PMN infiltration, and (iii) bacterial load in the infected mouse corneas, thereby suggesting that decreasing levels of IL-1β in the corneas improves disease outcome. Pearlman et al.[13,14] have shown that IL-1β-deficient mice as well as IL-1R1-deficient mice have significantly higher bacterial burden that is associated with delayed neutrophil recruitment to the corneas. It is thought that whereas IL-1β is required for bacterial clearance, high levels of IL-1β are detrimental for the timely resolution of the disease.

In the classical immune response to bacterial infection, generation of mature IL-1β is a two-step process. The first step is the induction of pro-IL-1β expression, which in the context of bacterial infections, is generally achieved by TLR-mediated activation of NF-κB pathway that results in the induction of pro-IL-1β as well as certain components of the inflammasomes, such as NLRP3 (signal 1). A second signal then triggers the assembly of the large multimolecular inflammasome complex leading to the cleavage of caspase 1. Active (cleaved) caspase-1 cleaves pro-IL-1β and pro-IL-18 to generate mature cytokines which are secreted from the cell to mediate downstream inflammatory effects that clear the infection.[15–17] Several distinct inflammasomes have been identified each activated by unique activators. Studies aimed at the characterization of the role of inflammasomes in providing protection from PA keratitis have shown that in the BALB/c mouse, which is resistant to PA keratitis, NLRC4 inflammasome-mediated activation of caspase-1 and IL-1β production is required for bacterial resistance.[18] In in vitro studies, Deng et al.[19] have demonstrated that in human macrophages, PA infection induces the assembly of the NLRP3 inflammasome leading to secretion of caspase-1 and IL-β that triggers autophagy to escape intracellular killing. Studies aimed at characterization of the role of caspases in the pathogenesis of PA keratitis have shown that: (i) Wedelolactone, a medicinal plant-derived coumestan, ameliorates PA-induced inflammation and corneal injury by suppressing caspase-4/5/11/GSDMD-mediated non-canonical pyroptosis,[20] (ii) TREM2 promotes host resistance against PA keratitis by inhibiting caspase-1-dependent pyroptosis,[21] and (iii) caspase-1 inhibitor reduces severity of PA keratitis in C57BL/6 mice by downregulation of IL-1β.[22] In addition, studies with caspase-1 knockout (ICE$^{-/-}$) mice have shown that endogenous absence of caspase-1 in ICE$^{-/-}$ mice produce significantly reduced disease that is associated with significantly lower levels of IL-1β protein when compared to C57BL/6 wild type (WT) mice.[23] Overall, these data suggest that capsase-1 is a major enzyme involved in the processing of IL-1β in the infected eye. In contrast, Karmakar et al.[13] have shown that in the C57BL/6 mouse, which is susceptible to PA keratitis, the production of active IL-1β is mediated by neutrophil elastase and is independent of caspase-1/inflammasome pathway. Thus, at present, whether NLRP3 inflammasome pathway plays a role in the regulation of innate immune response in PA keratitis is an open question. In the present study, using NLRP3$^{-/-}$ mice and a highly selective NLRP3 inhibitor, we demonstrate that in C57BL/6 mice, activation of NLRP3 pathway is required for the clearance of PA corneal infection.

Materials and Methods

Mice

In the current study, 7 to 8 weeks old WT and NLRP3$^{-/-}$ mice (C57BL/6, mixed gender; Jackson Laboratory) were used. All experimental procedures were in complete agreement with the AAALAC guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Infection

Corneas of WT and NLRP3$^{-/-}$ mice were infected under deep anesthesia induced by IP injection of ketamine and xylazine. Central corneas of mice were scarified with 3 parallel 1-mm incisions using a 26-gauge needle, and a 5-µl drop containing 100 CFU/eye of PA strain 6077 was applied to the eyes. The eyes were examined on day 1 post-infection (p.i.) for the development of corneal keratitis and opacity using the scoring system, graded from 0 to 4, as previously described[24]: 0 = eye macroscopically identical to the uninfected control eye; 1 = partial corneal opacity covering the pupil; 2 = dense corneal opacity covering the pupil; 3 = dense opacity covering the entire anterior segment; and 4 = perforation of the cornea, phthisis bulbi (shrinkage of the globe post-inflammatory disease). Corneal thickness was measured by optical coherence tomography (OCT) using Bioptigen Envisu C2300 (spectral domain ophthalmic imaging system; Bioptigen Envisu, Durham, NC, USA) equipped with a 12-mm telecentric OCT cornea lens designed for imaging mouse eyes. To quantify bacterial load, each cornea was homogenized in 250 µl of tryptic soy agar with 0.05% Tween 20 (TSBT). A 10 µl of the corneal homogenate was serially diluted in TSBT. Selected dilutions were plated in triplicate on Pseudomonas isolation agar plates (Becton-Dickinson, Franklin Lakes, NJ, USA). Plates were incubated overnight at 37°C and the number of viable colonies were manually counted. To examine the effect of NLRP3 and caspase-1 inhibitors on the outcome of keratitis, immediately prior to scarification, WT mice received a subconjunctival injection of NLRP3 inhibitor (MCC950; AdipoGen, San Diego, CA, USA; 300 µM in 10 µl PBS) or caspase-1 inhibitor (VX765; AdipoGen, San Diego, CA, USA; in 10 ul PBS containing 50% DMSO) using a 32-gauge syringe (Hamilton, Reno, NV, USA). Control mice were injected with 10 µl vehicle (NLRP3 inhibitor group: PBS; caspase-1 inhibitor group: PBS containing 50% DMSO). Both the inhibitors used in the current study are highly specific, nontoxic, and have been rigorously validated for in vivo use in animals and human clinical trials.[25–28]

Western Blot Analysis

Protein extracts of normal and infected corneas of WT, NLRP3$^{-/-}$, and NLRP3 inhibitor-treated mice were prepared in a radioimmunoprecipitation (RIPA) buffer supplemented with a protease inhibitor cocktail (Complete tablets; Roche Applied Science, Mannheim, Germany) and 2% SDS. For preparation of tissue lysate, four to six corneas were pooled and considered as one biological replica. At least three biological replicas were used for each condition. Aliquots of lysates containing 10 to 30 µg of protein were subjected to electrophoresis in 4% to 15% gradient or 12% SDS-PAGE gels (Bio-Rad, Hercules, CA, USA), and transferred to a nitrocellulose membrane. The blots were blocked with Odyssey blocking buffer (OBB; Li-Cor Biosciences, Lincoln, NE, USA) and incubated with mouse anti-capsapse-1 (clone caspase-1, 1:1000; AdipoGen, San Diego, CA, USA) and goat anti-IL-1β (clone AF-401-NA, 1:1000; R&D Systems, Minneapolis, MN, USA) primary antibodies in OBB (4°C, overnight). The secondary antibodies used were anti-goat IgG IRDye 800CW and anti-mouse IgG IRDye 680LT (Li-Cor) diluted in OBB (1:10,000, 25°C, 45 minutes). Blots were then scanned with the Odyssey Infrared Imaging System using Image Studio version 3.0 software (Li-Cor). After image acquisition, the blots were stripped using the NewBlot nitrocellulose stripping buffer (Li-Cor) and re-probed with mouse anti-β-actin (clone AC-15, 1:10,000; Santa Cruz Biotechnology, Dallas, TX, USA) as primary antibody, and anti-mouse IgG IRDye 680LT (Li-Cor) as secondary antibody.

Enzyme-Linked Immunosorbent Assay

Normal and infected corneas of WT, NLRP3$^{-/-}$, or NLRP3 inhibitor-treated mice were harvested, and homogenized in 500 µl PBS containing 1% BSA and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and centrifuged at 12,000 xg for 5 minutes. A 100 µl aliquot of supernatant was assayed in triplicate by ELISA to quantify IL-1β using Mouse IL-1β Duoset kit (R&D Systems, Minneapolis, MN, USA) designed to measure both natural and recombinant IL-1β.

Detection of IL-1β^+ Immune Cells in Infected Corneas and Flow Cytometry

PA-infected corneas were harvested from different groups of mice at indicated time points. Eight to 10 corneas per group were excised, pooled group wise, and digested with 60 U/mL Liberase (Liberase TL; Roche Diagnostics, Indianapolis, IN, USA) for 35 minutes at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer, single-cell suspensions were made in complete RPMI 1640 medium and processed for flow cytometry to quantify IL-1β^+ neutrophils (Ly6G positive) and macrophages (F4/80 positive). Briefly, cells were blocked with an unconjugated anti-CD32/CD16 (clone 2.4G2) mAb for 30 minutes in staining buffer (2% FBS, 5 mM EDTA in PBS) and then incubated for 30 minutes at 4°C with a cocktail of: CD45 (Clone 30-F11), CD11b-PerCP (clone M170), and Ly6G (clone 1A8) or F4/80 (clone 30F11). Then cells were washed 3 times using PBS, and incubated with APC-fixable viability dye (1/1000) for 30 minutes at 4°C. To determine IL-1β^+ immune cells, fixed cells were permeabilized with Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's recommendations. After permeabilization, cells were incubated with IL-1β antibody (clone NJTEN3, specific for mature IL-1β; Thermo Fisher) in permeabilization buffer for another 30 minutes at room temperature, samples were washed and acquired with FACS LSRII (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Caspase-1 activity was detected in the neutrophils and macrophages from infected corneas using a FAM-FLICA Caspase-1 assay kit (Immunochemistry Technologies, Bloomington, MN, USA) per manufacturer's instructions. Briefly, single cell suspensions of infected corneas from different groups of mice were incubated with FAM-FLICA caspase-1 at 37°C, 5% CO_2 for 45 minutes. At the end of the incubation period, cells were washed twice with 1X wash buffer provided in the kit followed by blocking of the Fc receptors with anti-mouse CD16/32 antibody on ice. Cell surface staining for CD45, CD11b, Ly6G, and F4/80 molecules was carried out and samples were immediately acquired using a LSR II flow cytometry. Data were analyzed using FlowJo software and presented as mean fluorescence intensity (MFI) ± SEM.

Statistical Analysis

Differences between the two groups were compared using the 2-tailed unpaired *t*-tests in the Prism software (GraphPad software Inc., La Jolla, CA, USA). Data in graphs represent mean ± SEM. The *P* values less than 0.05 were considered significant.

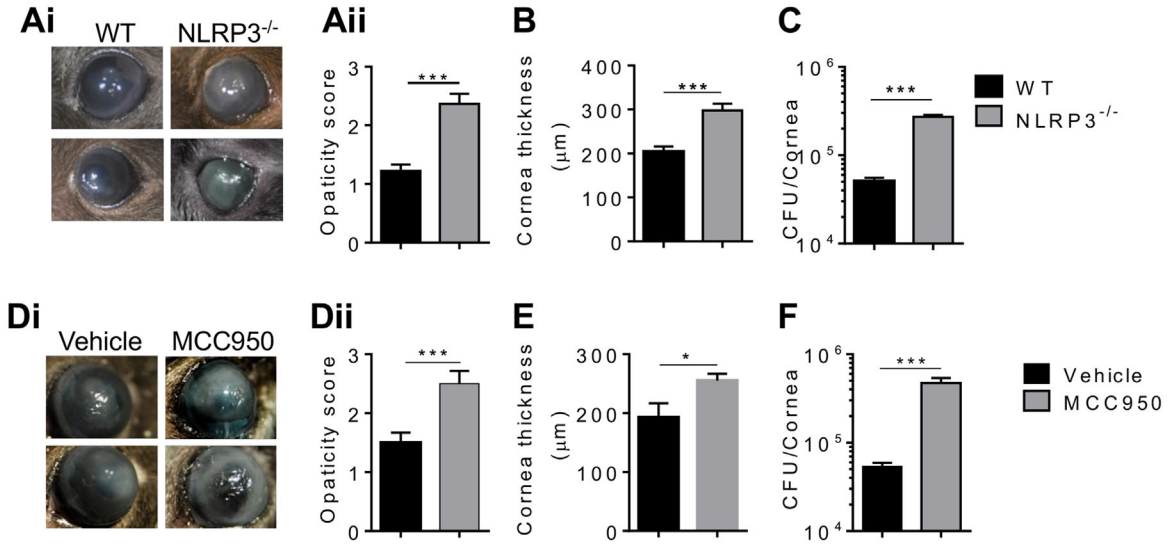

Figure 1. Activation of NLRP3 pathway is required for clearance of *P. aeruginosa* infection. (**A**, **B**, **C**) NLRP3$^{-/-}$ mice develop more severe keratitis: The central corneas of wild-type and NLRP3$^{-/-}$ mice were scarified with 3 parallel 1-mm incisions using a 26-gauge needle, and a 5-µl drop of bacteria (1×10^2 CFU, PA strain 6077) was applied to the cornea. On day 1 p.i., the severity of bacterial keratitis was graded using a scoring system ranging from 0 to 4 as discribed in Methods, corneal thickness was measured by OCT, and then corneas were harvested for bacterial enumeration. (**Ai**) Represntative images showing corneal opacity; (**Aii**) opacity score; **B** corneal thickness, **C** bacterial load. (**D**, **E**, **F**) Mice treated with NLRP3 inhibitor develop more severe keratitis. Immediately prioir to infection, C57BL/6 mice received a subconjunctival injection of NLRP3 inhibitor (MCC950, Adipogene) or vehicle (PBS) and on day 1 p.i., severity of infection was graded as decribed above. **Di** Representative images showing corneal opacity; **Dii** opacity score; **E** corneal thickness; **F** bacterial load. Bacterial load data are reported as log10 CFU/cornea ± SEM. Data show mean values ± SEM. Note that corneal opacity, thickness, and bacterial load are all higher in NLRP3$^{-/-}$ mice and NLRP3 inhibitor-treated mice compared to the respective control mice. Combined results of 3 separate experiments are shown; 8 to 10 corneas per group were used in each experiment. Statistical levels of significance were analyzed by the student *t*-test. *$P < 0.05$, ***$P < 0.001$.

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Results

NLPR3 Signaling Pathway is Required for Clearance of PA Infection

Three distinct criteria, corneal opacity, corneal thickness, and bacterial load, were used to assess the severity of infection. Corneal opacity, thickness, and bacterial load were all higher in NLRP3$^{-/-}$ mice compared to the WT mice (Figs. 1A–1C). To further confirm our observations, we next examined the effect of a highly selective NLRP3 inhibitor on the severity of corneal infection in the WT mice. Consistent with the observations made in the NLRP3$^{-/-}$ mice, WT mice treated with the NLRP3 inhibitor exhibited more severe keratitis compared to the vehicle-treated mice. Degree of corneal opacity, thickness, and bacterial load were all higher in the NLRP3 inhibitor-treated mice compared to the vehicle-treated mice (Figs. 1D–1F). Similar results were obtained regardless of whether infected corneas were collected on day 1 (see Fig. 1) or day 3 p.i. (Supplementary Fig. S1). In addition, similar results were obtained when mice were treated with subconjunctival injections of caspase-1 inhibitor instead of NLRP3 inhibitor (Supplementary Fig. S2).

NLRP3 Deletion Impairs Activation of IL-1β-Caspase-1 Pathway in Infected Corneas

To further confirm that the NLRP3 pathway is functional in cornea, PA-infected corneas of WT and NLRP3$^{-/-}$ mice were harvested on day 1 p.i. and corneal lysates were analyzed for the presence of cleaved/mature form of caspase-1 and IL-1β by Western blot analysis. Increased severity of infection in NLRP3$^{-/-}$ mice as well as an NLRP3 inhibitor-treated mice was associated with a decrease in the cleaved form of caspase-1 as well as IL-1β as detected by a shift in molecular weight (Figs. 2A, 2C). Levels of total IL-1β as determined by ELISA were also significantly reduced in the corneas of infected NLRP3$^{-/-}$ mice (Fig. 2B) as well as the inhibitor-treated mice (Fig. 2D).

NLRP3 Deletion Decreases Infiltration of IL-1β^{+} Neutrophils and Macrophages

To determine whether NLRP3 deletion, reduces the infiltration of activated immune cells, PA-infected corneas of WT and NLRP3$^{-/-}$ mice were processed for FACS analysis

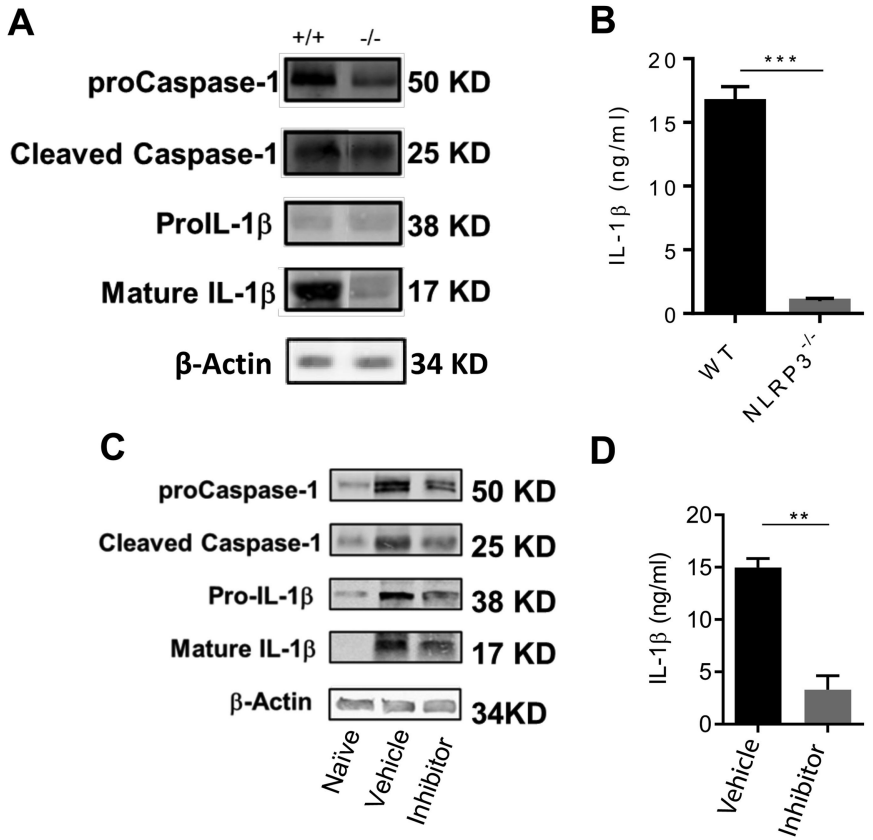

Figure 2. NLRP3 deletion impairs IL-1β-Caspase-1 pathway activation in infected corneas. (**A, B**) Infected corneas of NLRP3$^{-/-}$ and wild-type mice (6 mice/group) were harvested on day 1 p.i., pooled groupwise (6 corneas/group), and were processed either for Western blot analysis to detect pro- and cleaved caspase-1 and IL-1β, or ELISA to quantify total IL-β. **A** A representative Western blot showing that expression levels of activated caspase-1 and IL-1β are markedly reduced in corneas of NLRP3$^{-/-}$ mice, and **B** quantification of total IL-1β by ELISA. (**C, D**) Immediately prioir to infection, C57BL/6 mice received a subconjuctival injection of NLRP3 inhibitor (MCC950, Adipogene) or vehicle (PBS), and on day 1 p.i., corneas were harvested (6 eyes/group) and processed for Western blot analysis and ELISA. **C** A representative Western blot showing that expression levels of activated caspase-1 and IL-1β are markedly reduced in corneas of NLRP3 inhibitor-treated mice. **D** Quantification of total IL-1β by ELISA. **A**, **C** Blots are representative of three independent experiments. **B**, **D** Data are plotted as mean ± SEM. Combined results of three independent experiemnts are shown (6 pooled corneas/experiment). Statistical levels of significance were analyzed by the student *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$.

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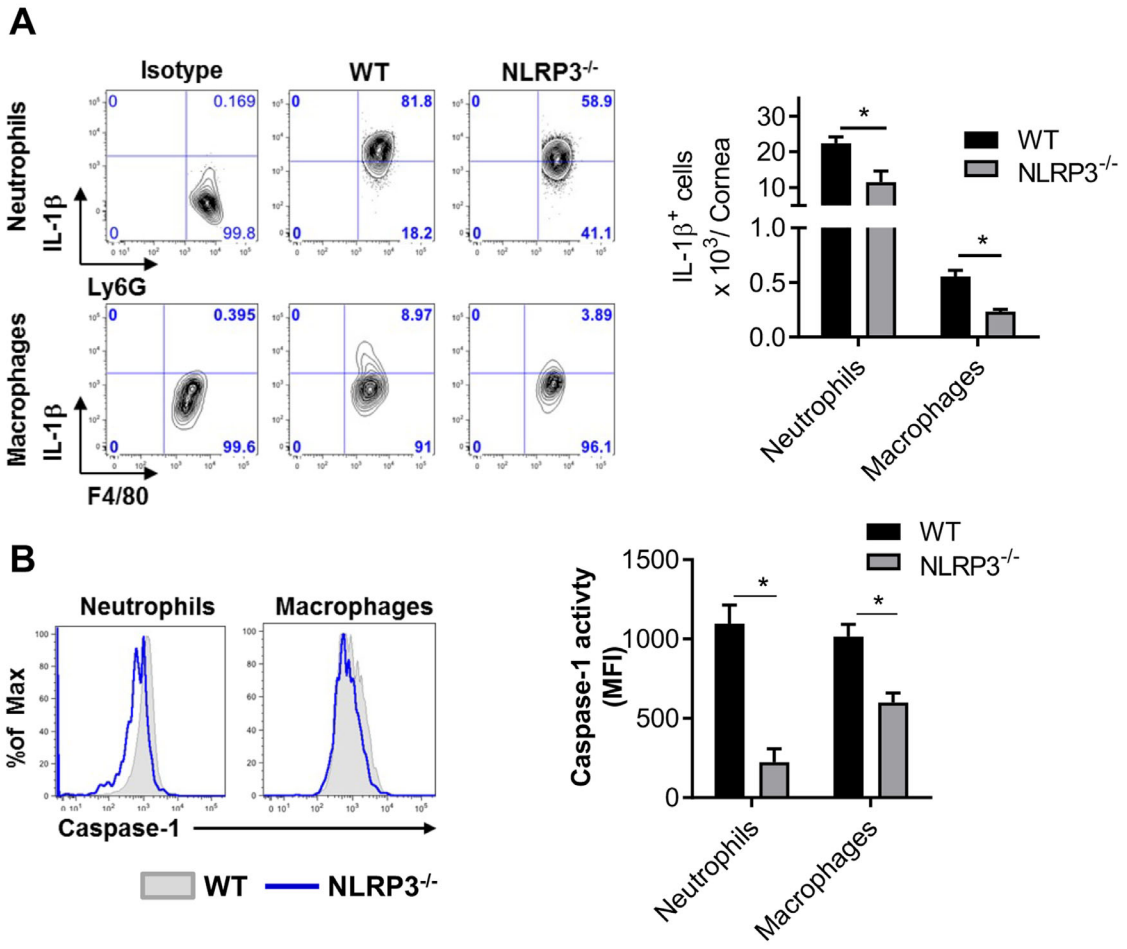

Figure 3. NLRP3 deletion decreases infiltration of activated immune cells. PA-infected corneas of wild-type and NLRP3$^{-/-}$ mice harvested on day 1 p.i., were processed to prepare single cell suspenssion for FACS anaylsis for quantitifaction of activated IL-1β^{+} CD45^{+} CD11b^{+} Ly6G^{+} neutrophils, and CD45^{+} CD11b^{+} 4/80^{+} macrophages, and quantitifaction of caspase-1 activity. (**A**) *Left*: A representative FACS plot showing IL-1β^{+} neutrophils and macrophages; *right*: quantification of IL-1β^{+} neutrophils and macrophages. (**B**) Caspase-1 activty in nuetrophils and macrophages was measured by FAM-FLICA caspase-1 assaay kit. *Left*: FACS histogram showing caspase-1 expression intensity on neutrophils and macorphages; *right*: quantification of caspase-1 using mean fluorescence intensity. Data show mean ± SEM. Combined results of 3 separate experiments are shown; 10 to 12 pooled corneas per group were used in each experiment. Statistical levels of significance were analyzed by the student *t*-test. *$P < 0.05$. Note that infiltration of neutrophils and macrophages expressing IL-1β as well as caspase activity of the infiltrated cells is significantly reduced in the infected corneas of the NLRP3$^{-/-}$ mice compared to the wild type mice.

to quantify CD45^{+} CD11b^{+} Ly6G^{+} IL-1β^{+} neutrophils and CD45^{+} CD11b^{+} F4/80^{+} IL-1β^{+} macrophages. Infiltration of neutrophils as well as macrophages expressing IL-1β was substantially reduced in the corneas of infected NLRP3$^{-/-}$ mice compared to the WT mice (Fig. 3A). Likewise, caspase activity of the infiltrated neutrophils and macrophages was significantly reduced in the infected corneas of the NLRP3$^{-/-}$ mice (Fig. 3B).

Discussion

The present study was designed to investigate the immunomodulatory role of NLRP3 in PA keratitis. We demonstrate here that the NLRP3 inflammasome pathway plays a critical role in clearance of PA infection in C57BL/6 mice. First, based on multiple criteria, including corneal opacity, corneal thickness, and bacterial load, we have established that the severity of PA keratitis is substantially higher in NLRP3$^{-/-}$ mice compared to that of WT mice. Second, severity of PA keratitis was also markedly higher in the WT mice treated with a subconjunctival injection of NLRP3 inhibitor immediately prior to infection. Similarly, the severity of PA infection was higher in the corneas treated with subconjunctival injections of a caspase-1 inhibitor compared to the corresponding WT mice treated with vehicle alone.

The hallmark of inflammasome assembly is the activation of proinflammatory caspase-1 and IL-1β by cleavage of their precursors, pro-caspase-1 and pro-IL-1β, respectively. Accordingly, we found that the increased severity of PA keratitis in NLRP3$^{-/-}$ mice as well as the NLRP3-inhibitor-treated WT mice was associated with a decrease in the cleaved form of caspase-1 as well as IL-1β. Finally, we demonstrated that: (i) infiltration of neutrophils as well as macrophages expressing IL-1β was substantially reduced in the corneas of infected NLRP3$^{-/-}$ mice compared to those of WT mice and (ii) caspase activity of the infiltrated

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neutrophils and macrophages was significantly reduced in the infected corneas of the NLRP3$^{-/-}$ mice. Together, the above data lead us to conclude that NLRP3 inflammasome pathway plays a critical role in the clearance of PA infection in the cornea. Our findings are consistent with the reports suggesting the role of caspase-1 in the resolution of PA keratitis,[22,23] but different from that of Karmakar et al.[13] reporting that in C57BL/6 mouse model of PA keratitis, the production of active IL1β from neutrophils is caspase-1 independent. Differences between our findings and those of Karmakar et al.[13] can be due to a variety of reasons including: (i) use of different strains of PA; (ii) differences in the commensal burden might account for different results in the studies carried out using the mice housed in different facilities[29]; and (iii) different cell types (e.g. epithelial cells vs neutrophils and macrophages) may use distinct mechanisms to generate mature IL-1β. As described earlier, in in vitro studies, Deng et al.[19] have demonstrated that in human macrophages, PA infection induces the assembly/activation of the NLRP3 inflammasome that triggers autophagy to escape intracellular killing. Significance of this finding remains to be determined in in vivo settings. We note, however, that in our study, activation of NLRP3 inflammasome reduced the severity of keratitis. This observation is contrary to what would be expected had the bacteria escaped the killing by autophagy or any other mechanism.

PA and *Staphylococcus aureus* (SA) are the two major bacterial pathogens responsible for most of the bacterial keratitis cases. It will be interesting to determine the role of NLRP3 inflammasome in the clearance of SA keratitis. Based on the nonocular studies showing that *SA*-induced IL-1β secretion, also requires NLRP3 inflammasome activation,[30,31] it is tempting to speculate that findings reported in the current study may also be relevant to SA keratitis. Several studies have investigated the role of NLRP3 inflammasome pathway in the noninfectious inflammatory disorders of the cornea. These studies have shown that: (i) inhibiting NLRP3 inflammasome: (a) remarkably prolongs the survival of corneal allografts[32,33] and (b) improves corneal wound healing after alkali burn,[34] and that (ii) the advanced glycation end products (AGEs)/ROS/NLRP3 inflammasome axis contributes to delayed diabetic corneal wound healing and nerve regeneration.[35]

Overall, our findings that NLRP3 inflammasome pathway is required for the clearance of infection in PA keratitis has broad implications for developing novel therapeutic strategies for the treatment of complications of infection resulting from hyperactive immune response. At present, treatment of PA keratitis is a major clinical problem. As described earlier, PA keratitis is notorious for causing rapidly fulminant disease often associated with corneal melting and permanent vision loss. Although the PMN-predominant innate response is no doubt needed for bacterial eradication, it is the persistent overactive innate response, the principal cause for the acute manifestations of PA keratitis. Aberrant activation of the NLRP3 inflammasome in various diseases including diabetes, atherosclerosis, metabolic syndrome, cardiovascular, and neurodegenerative diseases has provided impetus for the development of many high quality NLRP3 inhibitors which are currently undergoing clinical trials.[26,27] It is our hope that some of these inhibitors may be helpful in treating acute manifestations of PA keratitis that continues even after the pathogen has cleared.

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References

1. Green M, Apel A, Stapleton F. Risk factors and causative organisms in microbial keratitis. *Cornea*. 2008;27:22–27.
2. Robertson DM, Petroll WM, Jester JV, Cavanagh HD. Current concepts: contact lens related Pseudomonas keratitis. *Cont Lens Anterior Eye*. 2007;30:94–107.
3. Suzuki T, Inoue H. Mechanisms Underlying Contact Lens-Related Keratitis Caused by Pseudomonas aeruginosa. *Eye Contact Lens*. 2022;48:134–137.
4. Vijay AK, Willcox M, Zhu H, Stapleton F. Contact lens storage case hygiene practice and storage case contamination. *Eye Contact Lens*. 2015;41:91–97.
5. Willcox MD. Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. *Optom Vis Sci*. 2007;84:273–278.
6. Pandey RK, Yu FS, Kumar A. Targeting toll-like receptor signaling as a novel approach to prevent ocular infectious diseases. *Indian J Med Res*. 2013;138:609–619.
7. Roy S, Karmakar M, Pearlman E. CD14 mediates Toll-like receptor 4 (TLR4) endocytosis and spleen tyrosine kinase (Syk) and interferon regulatory transcription factor 3 (IRF3) activation in epithelial cells and impairs neutrophil infiltration and Pseudomonas aeruginosa killing in vivo. *J Biol Chem*. 2014;289:1174–1182.
8. Rathinam VA, Fitzgerald KA. Inflammasome Complexes: Emerging Mechanisms and Effector Functions. *Cell*. 2016;165:792–800.
9. Sahoo M, Ceballos-Olvera I, del Barrio L, Re F. Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections. *Scientific World Journal*. 2011;11:2037–2050.
10. Storek KM, Monack DM. Bacterial recognition pathways that lead to inflammasome activation. *Immunol Rev*. 2015;265:112–129.
11. Hazlett LD. Inflammatory response to Pseudomonas aeruginosa keratitis. *Ocul Surf*. 2005;3:S139–S141.
12. Rudner XL, Kernacki KA, Barrett RP, Hazlett LD. Prolonged elevation of IL-1 in Pseudomonas aeruginosa ocular infection regulates macrophage-inflammatory protein-2 production, polymorphonuclear neutrophil persistence, and corneal perforation. *J Immunol*. 2000;164:6576–6582.
13. Karmakar M, Sun Y, Hise AG, Rietsch A, Pearlman E. Cutting edge: IL-1beta processing during Pseudomonas aeruginosa infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1. *J Immunol*. 2012;189:4231–4235.
14. 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.
15. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol*. 2009;27:519–550.
16. Wonnenberg B, Bischoff M, Beisswenger C, et al. The role of IL-1beta in Pseudomonas aeruginosa in lung infection. *Cell Tissue Res*. 2016;364:225–229.

17. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol*. 2011;29:707–735.
18. McClellan SA, Jerome A, Suvas S, Hazlett LD. NLRC4 regulates caspase-1 and IL-1beta production in a CD11blowLy6Glow population of cells required for resistance to Pseudomonas aeruginosa keratitis. *PLoS One*. 2017;12:e0185718.
19. Deng Q, Wang Y, Zhang Y, et al. Pseudomonas aeruginosa Triggers Macrophage Autophagy To Escape Intracellular Killing by Activation of the NLRP3 Inflammasome. *Infect Immun*. 2016;84:56–66.
20. Xu S, Liu X, Liu X, et al. Wedelolactone ameliorates Pseudomonas aeruginosa-induced inflammation and corneal injury by suppressing caspase-4/5/11/GSDMD-mediated non-canonical pyroptosis. *Exp Eye Res*. 2021;211:108750.
21. Qu W, Wang Y, Wu Y, et al. Triggering Receptors Expressed on Myeloid Cells 2 Promotes Corneal Resistance Against Pseudomonas aeruginosa by Inhibiting Caspase-1-Dependent Pyroptosis. *Front Immunol*. 2018;9:1121.
22. Thakur A, Barrett RP, Hobden JA, Hazlett LD. Caspase-1 inhibitor reduces severity of pseudomonas aeruginosa keratitis in mice. *Invest Ophthalmol Vis Sci*. 2004;45:3177–3184.
23. Thakur A, Barrett RP, McClellan S, Hazlett LD. Regulation of Pseudomonas aeruginosa corneal infection in IL-1 beta converting enzyme (ICE, caspase-1) deficient mice. *Curr Eye Res*. 2004;29:225–233.
24. Berk RS, Hazlett LD, Beisel KW. Genetic studies on resistant and susceptibility genes controlling the mouse cornea to infection with Pseudomonas aeruginosa. *Antibiot Chemother (1971)*. 1987;39:83–91.
25. Chen J, Chen YQ, Shi YJ, et al. VX-765 reduces neuroinflammation after spinal cord injury in mice. *Neural Regen Res*. 2021;16:1836–1847.
26. Coll RC, Robertson AA, Chae JJ, et al. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med*. 2015;21:248–255.
27. Perera AP, Fernando R, Shinde T, et al. MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice. *Sci Rep*. 2018;8:8618.
28. Wen S, Deng F, Li L, Xu L, Li X, Fan Q. VX-765 ameliorates renal injury and fibrosis in diabetes by regulating caspase-1-mediated pyroptosis and inflammation. *J Diabetes Investig*. 2022;13:22–33.
29. Kugadas A, Christiansen SH, Sankaranarayanan S, et al. Impact of Microbiota on Resistance to Ocular Pseudomonas aeruginosa-Induced Keratitis. *PLoS Pathog*. 2016;12:e1005855.
30. Craven RR, Gao X, Allen IC, et al. Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One*. 2009;4:e7446.
31. Mariathasan S, Weiss DS, Newton K, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. 2006;440:228–232.
32. Wei C, Ma L, Xiang D, et al. Enhanced autophagy alleviated corneal allograft rejection via inhibiting NLRP3 inflammasome activity. *Am J Transplant*. 2022;22:1362–1371.
33. Zhu Z, Peng R, Shen H, et al. Treatment With Melatonin After Corneal Graft Attenuates Rejection. *Front Pharmacol*. 2021;12:778892.
34. Bian F, Xiao Y, Zaheer M, et al. Inhibition of NLRP3 Inflammasome Pathway by Butyrate Improves Corneal Wound Healing in Corneal Alkali Burn. *Int J Mol Sci*. 2017;18(3):562.
35. Wan L, Bai X, Zhou Q, et al. The advanced glycation end-products (AGEs)/ROS/NLRP3 inflammasome axis contributes to delayed diabetic corneal wound healing and nerve regeneration. *Int J Biol Sci*. 2022;18:809–825.

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