Fungal keratitis is a severe infective corneal disease caused by pathogenic fungi, which has a high rate of blindness. The incidence of this disease has been constantly increasing, due to increased ocular trauma, especially agricultural trauma, extended contact lens usage, long-term antibiotic use, or excessive use of corticosteroids. The pathogens are varied due to different climates and environments, mainly including Aspergillus, Fusarium, Curvularia, Aspergillus, and Mycoloraloides. Among these, Fusarium and Aspergillus are the main pathogenic fungi. Host immune response to Aspergillus has a species specificity. Unlike most other Aspergillus species, which are in most cases nonpathogenic, A. fumigatus features an armory of virulence determinants to elicit a strong host immune response in general. Hosts recognized and eliminated these pathogenic microorganisms mainly through the immune system, which includes both innate immunity and adaptive immunity. The innate immunity is the first line of host defense to limit infection rapidly at an early stage. It can rapidly identify conserved structural motifs expressed by microbial pathogens or pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Then it produces cytokines and chemokines to recruit immune cells to the local tissue against invading pathogens. The PRRs participating in the immune response to fungi mainly include Toll-like receptors, C-type lectin-like receptors, and nucleotide-binding oligomerization domain-like receptors. Another class of PRRs includes the protease-activated receptors (PARs). PARs, a family of G-protein-coupled receptors, are activated by various proteinases, essentially of the serine proteinase family, via proteolytic cleavage. During inflammation, host- or fungal-derived proteinases are released into the extracellular environment. Certain extracellular proteinases can specifically cleave and trigger PARs. PARs are viewed as an integral component of the host antimicrobial alarm system capable of affecting host defense and immunity.

To date, four members of the PAR family have been cloned and characterized: PAR-1, PAR-2, PAR-3, and PAR-4. Among these, PAR-2 has been identified as a key mediator in multiple biological, inflammatory, and immune responses. PAR-2 is mainly expressed on epithelial cells, endothelial cells, neutrophils, macrophages, and dendritic cells, which indicates a role in protecting the host from external pathogenic microorganisms. The activation of PAR-2 also plays a key role in leukocyte motility, cytokine production, adhesion molecule expression, and a variety of other physiological or pathophysiological functions. In the immortalized human SZ95 sebaceous gland cell, PAR-2 agonist peptide (PAR-2 AP) and Propionibacterium acnes supernatant can elevate IL-8, TNF-α, and human-defensin-2 (hBD-2) production. PAR-2 small interfering RNA treatment can significantly suppress the proinflammatory cytokine production, which suggests PAR-2...
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mediates inflammation in response to P. acnes. Moreover, PAR-2 plays a crucial role in different animal models of intestinal diseases, focal segmental glomerulosclerosis, skin inflammatory diseases, myocarditis, and airway inflammation. Recent studies have shown that human corneal epithelial cells express PAR-2. In human corneal epithelial cells, inhibition of PAR-2 by a specific antagonist prevents proinflammatory cytokine production and reduces inflammation induced by Acanthamoeba plasminogen activator, a serine protease secreted by Acanthamoeba trophozoites, that is involved in the pathogenesis of Acanthamoeba keratitis, indicating that disruption of PAR-2 activity might have a major impact on preventing inflammatory responses in Acanthamoeba keratitis. During the immune response, host immune cells and fungi derived proteases to release into the extracellular environment. Certain proteases activated PAR-2 to elicit a variety of responses.

It is not known whether A. fumigatus can activate PAR-2 and then trigger inflammatory responses. The expression and function of PAR-2 during A. fumigatus keratitis are still unknown, which was investigated in the current study.

**Materials and Methods**

**Animals and Corneal Infection**

Female 8-week-old C57BL/6 mice were purchased from Changzhou Cavens Laboratory Animal Co., LTD. (Jiangsu, China). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized using 8% chloral hydrate and placed beneath a stereoscopic microscope (×40 magnification). The central corneal epithelium (2-mm diameter range) of the left eye was removed. A 5-μl aliquot (1 × 10^6 cells) of A. fumigatus strain 3.0772 (China General Microbiological Culture Collection Center, Beijing, China), was topically applied to the ocular surface. Then the left eyes of C57BL/6 mice (n = 8/group/time) were injected subconjunctivally with 5 μL PAR-2 antagonist (1.25 mmol/L; Tocris Bioscience, Bristol, UK) 1 day before infection. One day p.i., an additional 200 μg/100 μl was injected intraperitoneally (i.p.); controls were similarly injected with sterile water. Mice corneas were harvested for real-time RT-PCR at 12 hours and 1 and 2 days post infection (p.i.); for Western blot at 1, 2, and 3 days p.i. Eyeballs were removed at 5 days p.i. for immunofluorescent staining.

**PAR-2 Antagonist Treatment**

The left eyes of C57BL/6 mice (n = 6/group/time) were injected subconjunctivally with 5 μL PAR-2 antagonist (1.25 mmol/L; Tocris Bioscience, Bristol, UK) 1 day before infection. One day p.i., an additional 200 μg/100 μl was injected intraperitoneally (i.p.); controls were similarly injected with sterile water. Mice corneas were harvested for PCR at 1 day p.i.; for Western blot and ELISA at 2 days p.i.; for myeloperoxidase (MPO) assay at 3 days p.i. Eyeballs were removed at 5 days p.i. for immunofluorescent staining.

**Isolation of Polymorphonuclear Neutrophilic Leukocytes (PMNs)**

C57BL/6 mice were injected i.p. with 1 mL sterile 9% casein (Sigma, Shanghai, China). After 24 hours, similar injection was given again. Three hours after the second injection, mice were anesthetized using 8% chloral hydrate. Then, 10 mL Dulbecco’s modified Eagle’s medium (DMEM; Gibco, San Diego, CA, USA) with 5% fetal bovine serum (FBS; Gibco) was injected into the abdominal cavity; the liquid was sucked out and collected. After centrifugation at 300g for 10 minutes and 4°C, neutrophils were collected, purified using Percoll, and used for studies.

**PMN Culture and A. fumigatus Stimulation**

PMNs were cultured in DMEM with 10% FBS (Gibco) at 37°C, and 5% CO2. Near 80% confluence, the cells were cultured for 3 hours for cell attachment and incubated with A. fumigatus hyphae (to the final concentration of 5 × 10^6 CFU/mL) for 0, 12, 18, and 24 hours in six-well plates. Cells were used for Western blot. PAR-2 protein levels of PMN were detected by Western blot at 12, 18, and 24 hours' stimulation.

**PAR-2 Antagonist Treatment of PMNs**

PMNs were pretreated with or without PAR-2 antagonist (FSLLR-NH2, 100 μM; Tocris Bioscience, UK) for 1 hour and then incubated with A. fumigatus hyphae (final concentration of 5 × 10^6 CFU/mL) for 18 hours. PMNs without treatment with PAR-2 antagonist served as the normal control group. Cells were harvested to detected PAR-2 protein levels by Western blot at 18 hours and the supernatant was collected to evaluate the protein levels of IL-1β, TNF-α, IFN-γ, and macrophage inflammatory protein 2 (MIP-2) at 18 hours p.i.

**Real-Time RT-PCR**

C57BL/6 mice corneas were collected at the indicated times after treatments. Total corneal RNA was isolated by using RNAiso plus reagent and quantified by spectrophotometry. RNA (2 μg) was used for first-strand cDNA synthesis according to the protocol for a reverse transcription system. cDNA products were diluted 1.25 with diethylpyrocarbonate-treated water and a 2-μL cDNA aliquot was used for real-time RT-PCR (20 μL total reaction volume) according to the manufacturer’s instructions. Real-Time PCR Master Mix (TaKaRa, Dalian, China) was used for the PCR reaction with primer concentrations of 5 μM. All reactions were performed with the following cycling parameters: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and a final stage of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Relative transcription levels were calculated by using the relative standard curve method that compares the amount of target normalized to the housekeeping gene β-actin. Data are shown as the mean ± SEM for relative mRNA levels. The primers used in this study are listed in the Table.

**Western Blot Analysis**

Corneas and cells were ground and were lysed in radioimmunoprecipitation assay (Solabio, Beijing, China) lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (Solabio) for 2 hours, and then were centrifuged at 4°C, 16,000g for 10 minutes. Supernatant was collected and tested the protein concentration. After adding SDS sample buffer, and boiling, total protein was separated on 10% acrylamide SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Sorlabio). The membranes were blocked with Western blocking buffer (Beiyotime, Jiangsu, China) at 37°C for 2 hours, and then were incubated with a polyclonal antibody to β-actin (1:2000; Bios, Beijing, China), and primary antibody to PAR-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or primary antibody to p-ERK1/2 (1:1000; Elabscience, Wuhan, China) or primary antibody to ERK1/2 (1:1000; Elabscience) at 4°C overnight. After washing in PBS containing 0.5% Tween 20 (Bio-Rad, Hercules, CA, USA) three times, membranes were incubated with corresponding peroxidase-conjugated secondary antibodies (1:8000; Santa Cruz Biotechnology) at 37°C for 1...
hour. Then the blots were developed by using chemiluminescence (ECL, Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme-linked Immunosorbent Assay

All individual corneas taken for ELISA analysis were homogenized in 500 μL PBS with 0.1% Tween 20 with a protease inhibitor cocktail (Cwbiotech, Beijing, China) and centrifuged at 5000g for 10 minutes. Normal (uninfected) and infected corneas were removed at 2 days p.i. from C57BL/6 mice (n = 6/group/time) and 100 μL of each sample was assayed in duplicate for IL-1β, TNF-α, and IFN-γ protein; MIP-2 protein was tested with 50 μL of each sample in duplicate, according to the manufacturer’s instructions (Elabscience).

Quantitation of Corneal PMN

An MPO assay was used to quantitate PMN number. Previous studies have reported that mice A. fumigatus keratitis response is the most obvious at 3 days p.i. Therefore, corneas (n = 6/group/time) were removed at 3 days p.i., and homogenized in 1.0 mL second agent of MPO test kit (Njjcbio, Nanjing, China) according to the manufacturer’s instructions. Samples were freeze-thawed four times. After centrifugation and water bath, the change was immediately monitored in absorbance (460 nm). The slope of the line was determined for each sample and used to calculate units of MPO per cornea. One unit of MPO activity is equivalent to approximately 2 × 10^5 PMN.

Immunofluorescent Staining

Eyeballs were removed (n = 3/group/time) at 3 days p.i. from C57BL/6 mice, immersed in 0.01M PBS, embedded in optimum cutting temperature compound (SAKURA Tissue-Tek, Torrance, CA, USA), and frozen in liquid nitrogen. Ten-micrometer sections were cut, mounted to poly-L-lysine-coated glass slides, and stored at 37°C overnight. After a 5-minute fixation in acetone, slides were blocked with 0.01M PBS containing 10% blocking serum for 30 minutes at room temperature. To label PMNs, the sections were incubated at a 1:100 dilution of rat anti-mouse NIMP-R14 (Santa Cruz Biotechnology) at 4°C overnight. This was followed by FITC-conjugated goat anti-rat secondary antibody (1:50, 1 hour, room temperature, without light; Cwbiotech, Wuhan, China). Isotype IgG was used as the negative control. Finally, sections were visualized, and digital images captured with a Zeiss Axiocvert microscope at ×40 magnification.

Statistical Analysis

The difference in clinical score between two groups at each time was tested by the Mann-Whitney U test. An unpaired, two-tailed Student’s t-test was used to determine the statistical significance of the real-time RT-PCR, MPO, ELISA, and cell-sorting data. Data were considered significant at P < 0.05. All experiments were repeated once to ensure reproducibility and data from a representative experiment are shown as mean ± SEM.

RESULTS

PAR-2 Expression in Cornea of C57BL/6 Mice

To investigate whether PAR-2 is involved in mice A. fumigatus keratitis, we tested mRNA and protein levels of PAR-2 in normal uninfected and infected C57BL/6 corneas by real-time RT-PCR and Western blot. Results indicated that relative PAR-2 mRNA levels were upregulated in infected mice corneas at 12 hours, 1 day, 2 days than in normal uninfected corneas (Fig. 1A; P < 0.01, P < 0.01, P < 0.01, respectively). To confirm these data, PAR-2 protein was examined by Western blot. Western blot analysis also showed an obviously elevated PAR-2 level in infected mice corneas at 1, 2, and 3 days, compared with normal uninfected corneas (Fig. 1B). In addition, low protein level was detected in the normal corneas.

Effect of PAR-2 on Clinical Score

To determine effective concentration of FSLLRY-NH2, relative mRNA levels of PAR-2 were tested by real-time RT-PCR in normal control, infected, and 0.25, 0.5, and 1.25 mmol/L FSLLRY-NH2–treated corneas. Data showed that treatment with 0.5 mmol/L and 1.25 mmol/L FSLLRY-NH2 significantly inhibited A. fumigatus-induced upregulation of PAR-2 mRNA levels (Fig. 2A; P < 0.05, P < 0.001). Moreover, the latter inhibition effect is better. However, PAR-2 mRNA had no significant difference after treatment with 0.25 mmol/L FSLLRY-NH2. Therefore, in the next studies, 1.25 mmol/L was chosen as the application concentration. To confirm these data, PAR-2 protein was tested by Western blot; 1.25 mmol/L FSLLRY-NH2 markedly inhibited PAR-2 protein when compared with infected control (Fig. 2B).

To illustrate the disease response in FSLLRY-NH2–treated versus infected control mice, we used a slit lamp to take photographs at 3 days p.i. Clinical score was significantly lower in FSLLRY-NH2–treated (Fig. 2D) compared with infected control mice (Figs. 2C, 2E; P < 0.01).

Effect of PAR-2 on PMN Infiltration

FSLLRY-NH2 treatment significantly reduced MPO levels compared with infected control (Fig. 3A; P < 0.01). Immunostain-
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**Discussion**

PAR-2 plays a crucial role in immune responses. PAR-2 is a specific target to elicit protease-mediated inflammation. PAR-2 is mainly expressed in epithelial cells, endothelial cells, neutrophils, macrophages, and dendritic cells. Results presented in this study revealed that PAR-2 was expressed in normal and fungi-infected mouse corneas and that its expression was significantly upregulated after *A. fumigatus* infection. These data suggest a potential role for PAR-2 in mice *A. fumigatus* keratitis, and are consistent with results in a previous study from another laboratory showing that PAR-2 can promote the progression of canine inflammatory bowel disease (IBD) by modifying cytokines. This was further confirmed by a study in vitro, showing that relative PAR-2 mRNA and protein levels of PMNs were significantly enhanced after *A. fumigatus* stimulation than in normal controls. These findings are consistent with those previous studies showing that PAR-2 was markedly upregulated in human corneal epithelial (HCE) cells after *Acanthamoeba* plasminogen activator stimulation. These results indicated that PAR-2 may be involved in *A. fumigatus* keratitis.

Previous studies have reported that PAR-2 is involved in chronic rhinosinusitis (CRS). PAR-2 is significantly upregulated in CRS with asthma groups compared with the control group. PAR-2 might contribute to the inflammation characteristic of allergic diseases by upregulating cytokines. Duodenal PAR-2 expression levels were higher in dogs with IBD than in healthy control dogs. These results suggest that PAR-2 may contribute to the pathogenesis of canine IBD by inducing expression of inflammatory mediators. In a murine model of German cockroach (GC) feces (frass)-mediated allergic airway inflammation, it is shown that airway neutrophilia induced by a single exposure to GC frass is partially dependent on the activation of PAR-2. *A. fumigatus* expression was significantly upregulated after *A. fumigatus* keratitis, and are consistent with results in a previous study from another laboratory showing that PAR-2 was involved in canine IBD by inducing expression of cytokines.21 Duodenal PAR-2 expression levels were higher in dogs with IBD than in healthy control dogs. These results suggest that PAR-2 may contribute to the pathogenesis of canine IBD by inducing expression of inflammatory mediators. In a murine model of German cockroach (GC) feces (frass)-mediated allergic airway inflammation, it is shown that airway neutrophilia induced by a single exposure to GC frass is partially dependent on the activation of PAR-2.22 TNF-α expression substantially decreased in airway neutrophils from PAR-2-deficient mice, which suggested a role for PAR-2 in neutrophil-derived cytokine production. To further determine the role of PAR-2 in *A. fumigatus* keratitis, FSLR/LVNH2 was used to selectively inhibit PAR-2 and then further confirmed the importance of PAR-2 in *A. fumigatus* keratitis. Results indicated that FSLR/LVNH2 treatment significantly decreased mRNA levels of IL-1β, TNF-α, IFN-γ, and MIP-2 in comparison with infected control corneas. ELISA also showed that FSLR/LVNH2 markedly decreased IL-1β, TNF-α, IFN-γ, and MIP-2 protein levels compared with infected control mice. These results indicate that PAR-2 might be emerging as a member of innate immunity, which can potentiate the immune sensing of fungal PAMPs and enlarge the inflammatory response. On the other hand, excess activity of PAR-2 might result in damage to organs. We conclude that appropriate manipulation of the activity of PAR-2 would be beneficial for the control of inflammation-related diseases. Our findings are consistent with studies showing that PAR-2 AP induces proinflammatory cytokine expression in S929 seocytes, which can be attenuated by PAR-2 silencing.11 Our findings also are consistent with studies showing that PAR-2-activating peptide induced granulocyte-macrophage colony-stimulating factor, IL-6, and IL-8 production in airway epithelium, which were significantly inhibited by PAR-2 antagonist peptide. This protease-mediated activation of airway epithelium may be implicated in the development and exacerbation of airway inflammation.
FIGURE 2. Effects of FSLLRY-NH₂ treatment on clinical scores. After 0.5 mmol/L and 1.25 mmol/L FSLLRY-NH₂ treatment, relative mRNA levels of PAR-2 (A) were significantly decreased compared with infected control mice. No difference was detected between groups at 0.25 mmol/L FSLLRY-NH₂-treated and infected control mice. FSLLRY-NH₂ also significantly decreased PAR-2 protein (B) compared with infected control. Photographs were taken by a slit lamp at 3 days p.i. FSLLRY-NH₂ significantly decreased clinical score compared with infected control (C–E).

A

![Graph showing relative PAR-2 mRNA levels.](image)

B

![Western Blot showing PAR-2 and GAPDH expression.](image)

C

![Photograph showing clinical scores.](image)

D

![Photograph showing clinical scores.](image)

E

![Bar graph showing disease score.](image)
FIGURE 3. (A–D) Effects of FSLLRY-NH$_2$ on PMN number in corneas. FSLLRY-NH$_2$ significantly reduced MPO levels compared with infected control (B). Immunofluorescent staining demonstrated that FSLLRY-NH$_2$ markedly decreased PMN number compared with infected control (B–D).
FIGURE 4. Effects of FSLRRY-NH$_2$ treatment on IL-1β, TNF-α, IFN-γ, and MIP-2 in corneas. FSLRRY-NH$_2$ significantly decreased relative mRNA and protein levels of IL-1β (A, B), TNF-α (C, D), IFN-γ (E, F), and MIP-2 (G, H) compared with infected control.
FIGURE 5. Role of PAR-2 in PMNs. PAR-2 protein levels (A) were upregulated in the infected PMNs at 12, 18, and 24 hours p.i. compared with normal control cells, and PAR-2 protein levels were highest at 18 hours p.i. This upregulation can be inhibited by FSLLRY-NH$_2$ at 18 hours p.i. (B). FSLLRY-NH$_2$ significantly inhibited IL-1$\beta$ (C), TNF-$\alpha$ (D), IFN-$\gamma$ (E), MIP-2 (F), and p-ERK1/2 (G) protein expression compared with infected control cells at 18 hours p.i.
allergic disease.\textsuperscript{18} Pretreatment of HCE cells with FSLLRY-NH\textsubscript{2} significantly decreased proinflammatory cytokine expression induced by \textit{Acanthamoeba} plasminogen activator.\textsuperscript{20} A previous study has reported that PAR-2 also can regulate migration of neutrophils.\textsuperscript{21} PAR-2 AP increases [Ca\textsuperscript{2+}], and induces neutrophil morphological changes.\textsuperscript{24} In human neutrophils, PAR-2 AP or trypsin treatment enhanced neutrophil motility, suggesting a role for PAR-2 in the regulation of neutrophil migration. In accord with this, PAR-2 agonists enhanced adhesion molecule expression on neutrophils,\textsuperscript{25} which raises a possibility that extracellular PAR-2 may recruit immune cells to infection or injury sites. Our studies showed that FSLLRY-NH\textsubscript{2} treatment significantly reduced MPO levels and PMN number compared with infected control corneas. PMNs play an essential role for the initiation and execution of the inflammatory response to fungi, whereas excessive activity of PMNs results in damage to organs.\textsuperscript{24} These results provided corroborative data that reduction of PAR-2 was beneficial to disease outcome.

Therefore, to fully determine the role of PAR-2 in innate immunity to \textit{A. fumigatus} in cornea, a study in vitro was performed in PMNs. Results indicated that FSLLRY-NH\textsubscript{2} did inhibit IL-1\textbeta, TNF-\alpha, IFN-\gamma, and MIP-2 protein production induced by \textit{A. fumigatus}. In addition, results indicated that FSLLRY-NH\textsubscript{2} significantly inhibited p-ERK1/2 protein production induced by \textit{A. fumigatus}. It is recognized that ERK1/2 signal is one principal pathway of the cornea to regulate the cytokines and cells to participate in the inflammatory process.\textsuperscript{26–28} Collectively, these results indicated that \textit{A. fumigatus} might stimulate PMNs via the PAR-2–ERK1/2 pathway. Our findings are consistent with studies showing that FXa can induce cytokines, IL-6, IL-8, TNF-\alpha, and IFN-\gamma, in RAW 264.7 macrophages through the PAR-2–ERK1/2 pathway.\textsuperscript{29} These findings also are consistent with those previous studies showing that in protease-driven inflammation the activation of PAR-2 stimulated ERK1/2 phosphorylation.\textsuperscript{30}

In summary, the data presented herein indicated that PAR-2 was expressed in cornea. PAR-2 expression was significantly elevated by \textit{A. fumigatus}, which was significantly inhibited by FSLLRY-NH\textsubscript{2} in mice corneas. Inhibition of PAR-2 decreased the disease response of infected mice, PMN infiltration in corneas, and expression of IL-1\textbeta, TNF-\alpha, IFN-\gamma, and MIP-2. The in vitro cellular experiment showed that PAR-2 was detected in PMNs, and expression of PAR-2 increased after stimulation of \textit{A. fumigatus}, which was significantly inhibited by FSLLRY-NH\textsubscript{2}. In addition, we provided evidence that inhibition of PAR-2 decreased expression of IL-1\textbeta, TNF-\alpha, IFN-\gamma, and MIP-2 in PMNs. Moreover, \textit{A. fumigatus} activated ERK1/2 through PAR-2. These data suggest that PAR-2 plays a proinflammatory role during \textit{A. fumigatus} keratitis. PAR-2 may emerge as a novel therapeutic target for fungal keratitis. Further studies need to be done to investigate the pathway of how PAR-2 plays a role in the pathogenesis of fungal keratitis.

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**References**


