**Aspergillus fumigatus** Increased PAR-2 Expression and Elevated Proinflammatory Cytokines Expression Through the Pathway of PAR-2/ERK1/2 in Cornea

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**PURPOSE.** To determine the role of protease-activated receptor-2 (PAR-2) in cornea infected by *Aspergillus fumigatus*.

**METHODS.** PAR-2 was tested in normal and infected corneas of C57BL/6 mice. Mice corneas were infected with *A. fumigatus* with or without pretreatment of PAR-2 antagonist (FSLLRY-NH2). Polymorphonuclear neutrophilic leukocytes (PMNs) were stimulated with 75% ethanol-killed *A. fumigatus* with or without pretreatment of FSLLRY-NH2. Disease severity was documented by clinical score and photographs with a slit lamp. PCR, Western blot, and ELISA tested expression of PAR-2, IL-1β, TNF-α, IFN-γ, MIP-2, and p-ERK1/2. PMN infiltration was assessed by myeloperoxidase assay and immunofluorescent staining.

**RESULTS.** PAR-2 expression was significantly elevated by *A. fumigatus*, whereas the upregulation was significantly inhibited by FSLLRY-NH2 in mice cornea. FSLLRY-NH2 decreased disease response, PMN infiltration, and proinflammatory cytokine expression compared with infected control. In PMNs, PAR-2 expression was also significantly increased by *A. fumigatus*, which was significantly inhibited by FSLLRY-NH2. FSLLRY-NH2 significantly inhibited proinflammatory cytokine protein expression, as compared with that in infected control cells, which may be modified by p-ERK1/2.

**CONCLUSIONS.** These data provide evidence that *A. fumigatus* increased PAR-2 expression and elevated disease, PMN infiltration, and proinflammatory cytokine expression through PAR-2, which may be modified by p-ERK1/2.

Keywords: PAR-2, *Aspergillus fumigatus*, keratitis, mice

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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 *Fusarium*, *Curvularia*, *Aspergillus*, and *Mycotorulaoides*. Among these, *Fusarium* and *Aspergillus* are the main pathogenic fungi. Host immune response to *Aspergillus* has a species specificity. Unlike most *Aspergillus* species, which are in most cases nonpathogenic, *A. fumigatus* features an array 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...
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 of 1 × 10^6 colony-forming units (CFU)/mL of *A. fumigatus*, strain 3.0772 (China General Microbiological Culture Collection Center, Beijing, China), was topically applied to the ocular surface. Then the ocular surface was covered with a soft contact lens and the eyelids were sutured. Mice corneas were harvested for real-time RT-PCR at 1, 2, and 3 days post infection (p.i.); for Western blot and ELISA at 2 days p.i.; for myeloperoxidase (MPO) assay at 3 days p.i.; for Western blot at 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 (FSLLRNY-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 macrophase 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 RNeasy 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 radioimmuno precipitation assay (Solarbio, Beijing, China) lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Solarbio) for 2 hours, and then were centrifuged at 4°C, 16,000 g 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 (Solarbio). The membranes were blocked with Western blocking buffer (Boyetime, 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 0.05% 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.
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-NH\(_2\), 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-NH\(_2\)-treated corneas. Data showed that treatment with 0.5 mmol/L and 1.25 mmol/L FSLLRY-NH\(_2\) 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-NH\(_2\). 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-NH\(_2\) markedly inhibited PAR-2 protein when compared with infected control (Fig. 2B).

To illustrate the dose response in FSLLRY-NH\(_2\)-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-NH\(_2\)-treated (Fig. 2D) compared with infected control mice (Figs. 2C, 2E; \( P < 0.01 \)).

### Effect of PAR-2 on PMN Infiltration

FSLLRY-NH\(_2\) treatment significantly reduced MPO levels compared with infected control (Fig. 3A; \( P < 0.01 \)).
were markedly decreased compared with infected control at 2 1 P 4A; (green) number was markedly decreased in FSLLRY-NH2–
treated versus infected control corneas at 3 days p.i. (Figs. 3B, 3C). After FSLLRY-NH2 treatment, PMN significantly elevated after infection when compared with untreated staining demonstrated that PMN (green) number was significant-
ty increased in the infected corneas than in normal corneas at 1, 2, and 3 days p.i. (Fig. 5D; <P 0.001). 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 inflam-
mation, it is shown that airway neutrophilia induced by a single exposure to GC frass is partially dependent on the activation of PAR-2. TFN-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, FSLRNY-NH2 was used to selectively inhibit PAR-2 and then further confirmed the importance of PAR-2 in A. fumigatus keratitis. Results indicated that FSLRNY-NH2 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 FSLRNY-NH2 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 SZ95 sebocytes, which can be attenuated by PAR-2 silencing. 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

**FIGURE 1.** Expression of PAR-2 in normal corneas and A. fumigatus-infected corneas. Relative mRNA levels of PAR-2 (A) were significantly increased in the infected corneas than in normal controls at 12 hours, and 1 and 2 days p.i. Protein levels of PAR-2 (B) were significantly increased in the infected corneas than in normal corneas at 1, 2, and 3 days p.i. Superficial staining was used for mouse cornea section to detect whether PMN number was changed after FSLRNY-NH2 treatment. Immunostaining demonstrated that PMN (green) number was significantly elevated after infection when compared with untreated control (Figs. 3B, 3C). After FSLRNY-NH2 treatment, PMN (green) number was markedly decreased in FSLRNY-NH2-treated versus infected control corneas at 3 days p.i. (Figs. 3C, 3D).

**Effect of PAR-2 on Cytokines in Corneas Infected With A. fumigatus**

FSLRNY-NH2 significantly reduced mRNA levels of IL-1β (Fig. 4A; P < 0.001), TNF-α (Fig. 4C; P < 0.05), IFN-γ (Fig. 4E; P < 0.01), and MIP-2 (Fig. 4G; P < 0.001) induced by A. fumigatus. Moreover, ELISA showed that after FSLRNY-NH2 treatment, IL-1β (Fig. 4B; P < 0.001), TNF-α (Fig. 4D; P < 0.001), IFN-γ (Fig. 4F; P < 0.05), and MIP-2 (Fig. 4H; P < 0.001) protein levels also were markedly decreased compared with infected control at 2 days p.i.

**Role of PAR-2 in PMNs**

PAR-2 protein levels were upregulated in the stimulated PMNs at 12, 18, and 24 hours compared with normal control cells (Fig. 5A). Moreover, this upregulation can be inhibited by FSLRNY-NH2 (Fig. 5B).

Next, we sought to determine in PMNs whether the expression of cytokines induced by A. fumigatus hyphae depended on PAR-2 expression. ELISA data showed that FSLRNY-NH2 did inhibit IL-1β (Fig. 5C; P < 0.001), TNF-α (Fig. 5D; P < 0.001), IFN-γ (Fig. 5E; P < 0.001), and MIP-2 production (Fig. 5F; P < 0.001) compared with infected control cells.

**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.

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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).
FIGURE 3. (A–D) Effects of FSLLRNYH$_2$ on PMN number in corneas. FSLLRNYH$_2$ significantly reduced MPO levels compared with infected control (B). Immunofluorescent staining demonstrated that FSLLRNYH$_2$ markedly decreased PMN number compared with infected control (B–D).
FIGURE 4. Effects of FSLRRY-NH\textsubscript{2} treatment on IL-1\textbeta, TNF-\alpha, IFN-\gamma, and MIP-2 in corneas. FSLRRY-NH\textsubscript{2} significantly decreased relative mRNA and protein levels of IL-1\textbeta (A, B), TNF-\alpha (C, D), IFN-\gamma (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.18 Pretreatment of HCE cells with FSLRNY-NH₂ significantly decreased proinflammatory cytokine expression induced by Acanthamoeba plasminogen activator.20

A previous study has reported that PAR-2 also can regulate migration of neutrophils.21 PAR-2 AP increases [Ca²⁺], and induces neutrophil morphological changes.22 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,23 which raises a possibility that extracellular PAR-2 may recruit immune cells to infection or injury sites. Our studies showed that FSLRNY-NH₂ 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.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 A. fumigatus in cornea, a study in vitro was performed in PMNs. Results indicated that FSLRNY-NH₂ did inhibit IL-1β, TNF-α, IFN-γ, and MIP-2 protein production induced by A. fumigatus. In addition, results indicated that FSLRNY-NH₂ significantly inhibited p-ERK1/2 protein production induced by 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.26–28 Collectively, these results indicated that 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-α, and IFN-γ, in RAW 264.7 macrophages through the PAR-2-ERK1/2 pathway.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.30

In summary, the data presented herein indicated that PAR-2 was expressed in cornea. PAR-2 expression was significantly elevated by A. fumigatus, which was significantly inhibited by FSLRNY-NH₂ in mice corneas. Inhibition of PAR-2 decreased the disease response of infected mice, PMN infiltration in corneas, and expression of IL-1β, TNF-α, IFN-γ, 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 A. fumigatus, which was significantly inhibited by FSLRNY-NH₂. In addition, we provided evidence that inhibition of PAR-2 decreased expression of IL-1β, TNF-α, IFN-γ, and MIP-2 in PMNs. Moreover, A. fumigatus activated ERK1/2 through PAR-2. These data suggest that PAR-2 plays a proinflammatory role during A. fumigatus keratitis. PAR-2 may emerge as a novel therapeutic target to 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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