·Basic Research·

TREM-1 expression in rat corneal epithelium with *Aspergillus fumigatus* infection

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Abstract

• AIM: To investigate the expression of triggering receptor expressed on myeloid cells-1 (TREM-1) in the aberrant inflammation within the corneal epithelium at early period of fungal infection.

• METHODS: A total of 65 Wistar rats were randomly divided into control group, sham group and fungal keratitis (FK) group, in which the cornea was infected by *Aspergillus fumigatus* (*A. fumigatus*). After executed randomly at 8, 16, 24, 48 and 72h after experimental model being established, the severity of keratomycosis in rats was scored visually with the aid of a dissecting microscope and slit lamp. Then corneas in three groups were collected to assess the expression of TREM-1 through quantitative reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence technique and Western blot analysis. The correlation between FK inflammation and expression of TREM-1 was also analyzed.

• RESULTS: Corneal inflammation scores increased with time after fungal infection ($F=49.74$, $P=0.000$). The inflammation scores in FK group were obviously higher than those in sham group on the whole ($F=137.78$, $P=0.000$). Levels of TREM-1 in the infected rat corneal epithelium had elevated at 8h and peaked at 48h ($P<0.001$, compared with control group). Western blot analysis also showed an obviously elevated TREM-1 level in rat corneal epithelium at 24h and 48h after fungal infection. Immunofluorescence technique showed that TREM-1 mainly existed in corneal epithelium and infected corneal stoma of rat. TREM-1 protein expression was enhanced after fungal infection. Moreover, severity of FK inflammation was significantly related to TREM-1 expression in FK ($r=0.942$, $P=0.000$).

• CONCLUSION: TREM-1 may contribute to amplify the inflammation in the cornea infected with *A. fumigatus* and play critical roles in the battle against *A. fumigatus* in the innate immune responses.

• **KEYWORDS:** fungal keratitis; triggering receptor expressed on myeloid cells-1; *Aspergillus fumigatus*; corneal epithelium

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INTRODUCTION

Fungal keratitis (FK) is an opportunistic infection of the cornea that is caused by pathogenic fungi and has a high blindness rate. The incidence of FK has been increasing in recent years due to eye injury, long-term antibiotic or corticosteroid use, and decreased body immunity[1]. There are two main pathogenic fungi, *Fusarium* and *Aspergillus*[2]. After being invaded by a fungus, the corneal epithelium identifies fungus and toxins, initiates immune responses, and secretes cytokines to mediate inflammatory cells to the site of the infection. A large number of cells, cytokines, and chemokines are involved in the removal of fungus and the injury and repair of the cornea[3-5].

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily that is constitutively expressed by monocytes and polymorphonuclear leucocytes (PMNs)[6]. Bacterial or fungal infections can cause upregulation of membrane-bound TREM-1, rendering it a useful early inflammatory biomarker for systemic infection[7]. Although the endogenous TREM-1 ligand remains unknown, it is activated upon bacterial recognition by host cells, triggering a number of intracellular signaling events that result in enhanced production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)[6,8,9]. It is also reported in *Aspergillus*-containing granulomas and the lungs of *Aspergillus fumigatus* (*A. fumigatus*)-sensitized mice suggesting that this receptor might be involved in a host's antifungal responses[10].

Furthermore, the role of TREM-1 as an amplifier of the inflammatory response has also been confirmed in a mouse model of septic shock in which blocking signaling through

Table 1 Visual scoring system for rat fungal keratitis models

Criteria	Grade 1	Grade 2	Grade 3	Grade 4
Area of corneal opacity	1%-25%	26%-50%	51%-75%	76%-100%
Density of corneal opacity	Slight cloudiness, outline of iris and pupil discernable	Cloudy, but outline of iris and pupil remain visible	Cloudy, opacity not uniform	Uniform opacity
Suface regulatity	Slight surface irregularity	Rough surface, some swelling	Significant swelling, crater or serious descemetocele formation	Perforation or descemetocele

TREM-1 partially protected animals from death [9,11]. In this study, we sought to investigate the expression and role of TREM-1 in corneas infected by *A. fumigatus* to explore a novel therapeutic strategy for fungal keratitis.

MATERIALS AND METHODS

Materials RNAiso Plus and reverse transcription-polymerase chain reaction (RT-PCR) kits and SYBR Premix Ex Taq™ (Tli RNaseH Plus) were purchased from TaKaRa (Dalian, Liaonig Province, China); polyclonal rabbit anti- rat TREM-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); goat anti-rabbit IgG-FITC was obtained from ZSGB-BIO (Beijing, China); propidium iodide (PI) was purchased from Solarbio (Beijing, China); bicinchoninic acid assay and ECL Western Blotting Detection Reagent were purchased from Beyotime (Shanghai, China); and phenylmethylsulfonyl fluoride (PMSF) and cell lysis buffer (RIPA) were purchased from Solarbio (Beijing, China).

Animals Wistar rats (both male and female, weighing 200-300 g) were purchased from the Qingdao Institute of Drug Control (Qingdao, Shandong Province, China). Those with corneal disease were excluded after slit-lamp examination. The remains were allowed to acclimatize to the laboratory conditions for 1wk. The animals were fasted for 12h before experiments but allowed free access to water. All experimental procedures were performed in accordance with the guide lines provided in the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of *Aspergillus fumigatus* The standard *A. fumigatus* strain was purchased from China General Microbiological Culture Collection Center (CGMCC) and grown in Sabouroud medium at 28℃ for 5-7d. The fungal conidia were inoculated to liquid medium at 37℃ for 3-4d. We collected the hyphae after centrifugal and grinded them to the size of 20 μm-40 μm fragment. Then the fungal conidia were collected to be inactivated in 6h by treatment with 75% ethanol, washed three times in sterile phosphate buffer saline (PBS).

Induction of Fungal Keratitis Sixty-five Wistar rats were randomly divided into 3 groups: 10 for control group (corneas were collected without any scrape or other treatment), 20 for sham group (rats were the completed models but not inoculated with *A. fumigatus*) and 35 for FK group. The right eyes were chosen as the experiment ones. Levofloxacin eyedrops were given three times one day to the eyes of rats, and ofloxacin ointment was used every night before experiment 10% chloral hydrate 3 mL/kg for intraperitoneal injection, 0.4% oxybuprocaine hydrochloride eyedrops for surface anesthesia. Cleaning of conjunctival sac was done by using Yasuji iodine (0.5%) for routine disinfection. Central epithelium of cornea was scraped about 3 mm-4 mm in diameter, and the surface of cornea was scratched with a gauze sterile needle into stromal layer under microscope. Then the surface of cornea was smeared with colonies of *A. fumigatus* (about 3-4 mm in diameter), and covered with contact lenses to prevent the loss of fungus in the eyes. Finally, 5-0 black silk suture closed eyelid. Nothing was done for the left cornea, except for laying a plane contact lens before closing the eyelids. The contact lenses were removed after 24h. Fungal hyphae could be found by PAS stain in each observation point. The diagnoses of FK models were confirmed by fungal culture, staining of corneal scrapings, or confocal microscopy.

Four rats in sham group and seven rats in FK group were executed randomly at 8, 16, 24, 48 and 72h respectively after the experimental model being established. Two rats in control group were executed at the same time with other groups. The eyeball was removed under sterile conditions. The corneas were divided into three parts: one part was fixed with 40 g/L formaldehyde solution for immunofluorescence observation. The corneal epithelial scrapings of the other two parts were harvested and saved at -80℃ for quantitative RT-PCR and Western blot analysis.

Evaluation of Inflammation The severity of keratomycosis in the animals was scored visually with the aid of a dissecting microscope and slit lamp[12]. A grade of 0 to 4 was assigned to each of the following three criteria: area of opacity, density of opacity, and surface regularity (Table 1). A normal, unscarified cornea was given a score of 0 in each category and thus had a summation score of 0. The scores from all three categories were tallied daily for each eye to yield a possible total score ranging from 0 to 12.

Quantitative Reverse Transcription –Polymerase Chain Reaction The corneal epithelial scrapings were harvested and saved at -80℃ . Total RNA of the epithelia were extracted using RNAiso plus reagent (TaKaRa, Dalian, Liaoning Province, China) and rapidly quantified using spectrophotometry. Complementary DNA was generated by

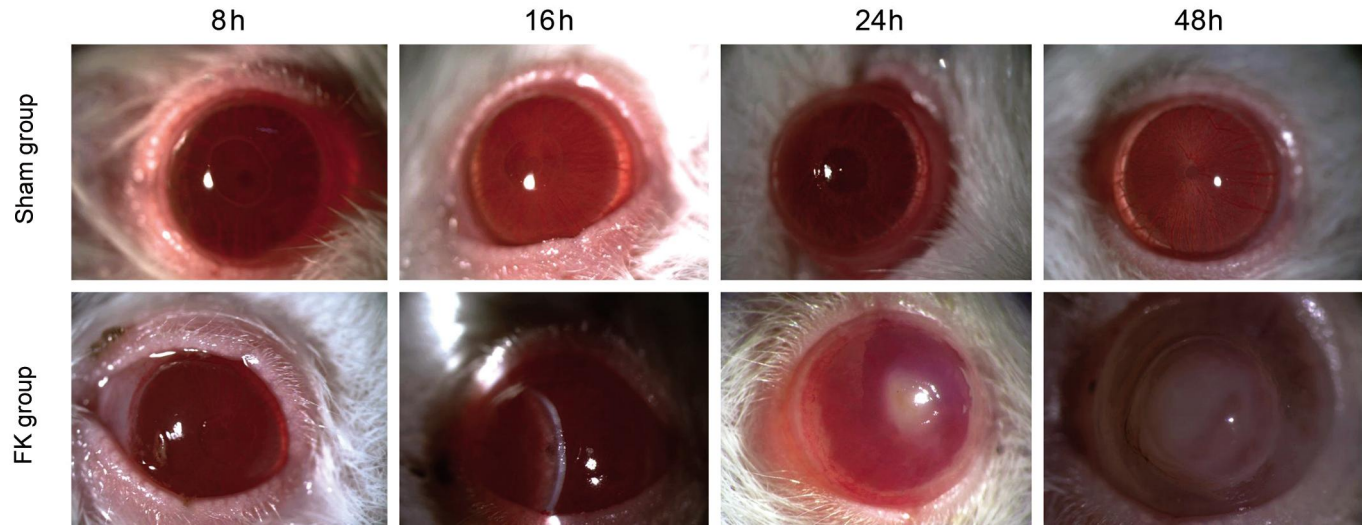

Figure 1 Corneal inflammation at different times in sham group and FK group In Sham group, mild inflammation reduced with time and nearly disappeared at 24h. In FK group, mild inflammation aggravated with time until 48h.

reverse transcription 2 μg of total RNA and then used in following quantitative PCR with SYBR Green using specific primers: 95℃ for 30s, followed by 40 cycles of 95℃ for 5s, 60℃ for 30s, followed by a final stage of 95℃ for 15s, 60℃ for 30s, and 95℃ for 15s. The oligonucleotide primers used were as follows: β-actin 5-GACTCATCGTACTCCTGCTTGCTG-3, 5-GGAGATTACTGCCCTGGCTCCTA-3, TREM-1 5-AAGTATGCCAGAAGCAGGAAGG-3 and 5-GGTAGGGTCATCTTTCAGGGTGT-3. The gene expression levels were quantified by RT-PCR using the housekeeping gene β-actin as an internal control. Quantification was performed using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated at least three separate times.

Immunocytochemical Localization Paraffin-embedded cornea sections (4 μm) were mounted on superfrost plus glass slides, heated to 60℃ for 30min, deparaffinized in xylene, and then hydrated through a series of ethanol/water solutions. Sections were then placed in a 10 nmol/L citric acid solution (pH 6.0) and high pressure for 1min. After cooled to room temperature, sections were transferred to PBS for 10min. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10min. The sections were rinsed in PBS for 10min and then blocked in goat serum for 20min before overnight incubation at 4℃ with rabbit anti TREM-1 (1:100), goat anti-rabbit IgG-FITC secondary antibody (1:100) was applied for 1.5h followed by PI (1:100) for 10min for nuclear counterstaining at 37℃. The results were photographed with an epifluorescence microscope using a digital camera.

Western Blot Analysis Proteins of corneal epithelial scrapings were extracted *via* RIPA lysis buffer plus 1 mmol/L PMSF at 4℃ for 40min. The lysate was centrifuged every 10min, followed by centrifugation at 14 000 rpm for 15min at 4℃.

Total protein was quantified *via* bicinchoninic acid assay, denatured with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Sample Loading Buffer at 95℃ for 5min. Proteins (40 μg/well) were separated by 12% SDS-PAGE in Tris/glycine/SDS buffer and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking by Western Blocking Buffer for 2h, the membranes were incubated with rabbit anti-TREM-1, mouse anti-β-actin, at 4℃ overnight and then incubated with secondary antibody for 1h. All blots were detected with BeyoECL Plus (Beyotime, Shanghai, China). Band intensity was measured by Quantity One Software (Bio-Rad, CA, USA).

Statistical Analysis The results were shown as mean ± standard deviation (SD). Data analysis was performed by one-way analysis of variance or multivariate analysis of variance, further pairwise comparisons were made using the least significant differences test, and correlation analysis performed by Pearson product moment correlation using SPSS17.0 software (SPSS, Chicago, IL, USA). Statistical significance was set at the $P<0.05$ level.

RESULTS

Corneal Inflammation Level of Rat Models in Different Time Points To further explore the corneal inflammation in FK, a well-characterized and accepted model of *A. fumigatus* keratitis was used to mimic human ocular infection. In FK model group, there were clinical features of FK after models were stablished. Significant corneal edema and rough surface could be seen at 16h after fungal infection and aggravated gradually until 48h. Then the corneal inflammation decreased with time. In Sham group, slight corneal inflammation weakened with time and was recovered at 24h. Hypha could not be seen by confocal microscopy in this group (Figure 1).

Table 2 The score of corneal inflammation between sham group and FK group $\bar{x} \pm s$

Groups	8h	16h	24h	48h	72h
Sham group	2.1±0.8	1.8±0.4	0.3±0.5	0.0±0.0	0.0±0.0
FK group[a]	2.5±0.5	3.8±0.7[b]	6.6±0.8[b]	8.8±0.7[b]	4.8±0.8[b]

[a]Compared with sham group, there was an obvious difference on the whole (F=137.78, P=0.000); [b]There was significant difference between two groups at 16, 24, 48 and 72h (All P<0.01).

As shown in Table 2, corneal inflammation scores increased with time after fungal infection, and there was an obvious difference on the whole (F=49.74, P=0.000). The scores in FK group were obviously higher than those in sham group on the whole (F=137.78, P=0.000). Furthermore, the score after 16h in FK group was higher than that in sham group (P<0.01).

TREM-1 Expression in Rat Corneal Epithelium after *Aspergillus fumigatus* Infection To determine the effect of *A. fumigatus* infection on TREM-1 expression in rat FK model, the corneal epithelial scrapings from control group, FK group and sham group were collected and the mRNA was analyzed using quantitative RT-PCR. As shown in Figure 2, levels of TREM-1 in the infected rat corneal epithelium had elevated at 8h, significantly upregulated in a time-dependent manner and peaked at 48h (P<0.001, compared with control group). Although the expression of TREM-1 also upregulated at 8h and 16h in sham group, elevation degrees were lower than those in FK group (P<0.001). Western blot analysis also showed an obviously elevated TREM-1 level in rat corneal epithelium at 24 and 48h after fungal infection (Figure 3).

To further determine the expression and distribution of TREM-1 in rat cornea, the corneas infected after 24h from FK group were detected by immunofluorescence technique, compared with normal rat corneas. As shown in Figure 4, the increased TREM-1 positive cells in FK group compared with those in control group, indicated that the TREM-1 protein expression was enhanced after fungal infection. This was in agreement with the above analysis. Moreover, TREM-1 positive cells mainly existed in corneal epithelium and infected corneal stroma of rats.

Correlation Between Fungal Keratitis Inflammation and Expression of TREM-1 The correlation analysis (Figure 5) showed that the corneal inflammation scores of rat models after *A. fumigatus* infection were significantly related to the expression of TREM-1 mRNA (r=0.942, P=0.000), which indicated that there was a significant relation between severity of FK inflammation and TREM-1 expression in FK.

DISCUSSION

When exposed to pathogens infection, host's cells begin to initiate inflammatory responses, and generate specific and consistent adaptive immune response to resist the invasion of pathogens[13]. The immune defense system provides important protection against pathogens. In the present study we explored the expression of TREM-1 an adaptor protein in a well-established model of FK. Although there was an

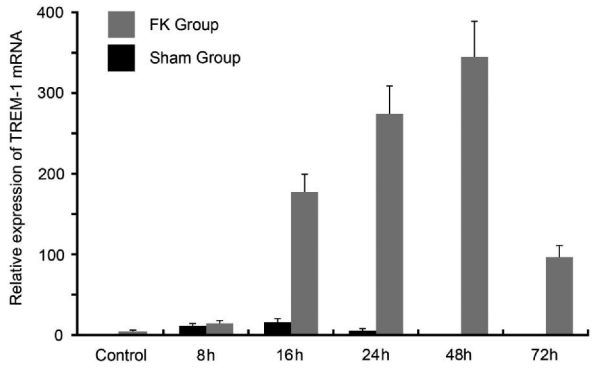

Figure 2 Triggering receptor expressed on myeloid cells-1 (TREM-1) expression in rat corneal epithelium In FK group, relative expression of TREM-1 mRNA was determined with real-time polymerase chain reaction at 8, 16, 24, 48 and 72h after infected by *A. fumigatus*. *A. fumigatus* infection increased TREM-1 mRNA expression in rat corneal epithelium and peaked at 48h. There were significant differences among these time points (P< 0.001). In sham group, relative expression of TREM-1 mRNA was determined with real-time polymerase chain reaction. TREM-1 mRNA expression increased at 8, 16, 24h after the models were completed. There were significant differences among these time points (P<0.001). No difference was detected in TREM-1 mRNA expression at 8h between sham group and FK group. However, significant differences were detected at 16 and 24h between these two groups (P<0.001).

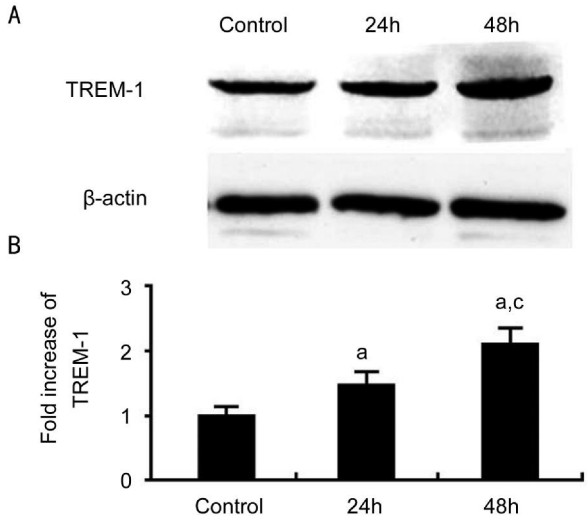

Figure 3 Triggering receptor expressed on myeloid cells-1 (TREM-1) expression in rat corneal epithelium in FK group Western blotting confirmed that *A. fumigatus* infection increased TREM-1 protein expression at 24 and 48h. The data represent the mean±standard deviation of three independent experiments. [a]P<0.05 compared with control; [c]P<0.05 compared with 24h.

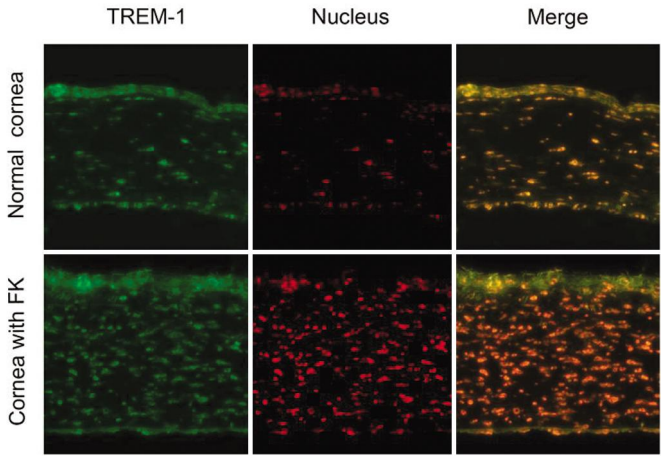

Figure 4 Immunofluorescence staining of TREM-1 in rat corneas before and after *A. fumigatus* infection The positive staining of TREM-1 (green) was mainly located in the membrane of corneal epithelial cells in normal corneas. More positive staining of TREM-1 was detected in infected corneas (corneal epithelium and stroma). Propidium iodide (PI) staining (red) indicates nuclei. The representative images were from three independent experiments. Magnifications ×200.

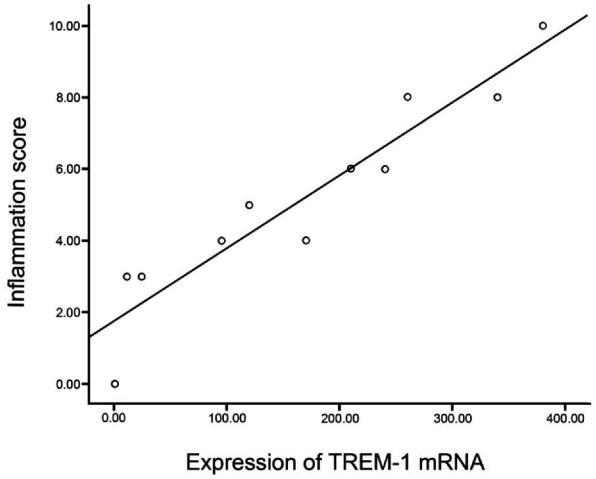

Figure 5 Pearson product moment correlation showed significant relation between scores of rat models and the expression of TREM-1 mRNA after *A. fumigatus* infection.

increase of TREM-1 expression in sham group compared with control group, more significant enhancement of TREM-1 expression was detected in rat corneal epithelium infected with *A. fumigatus* compared with those in other groups without fungal infection. Highly upregulated expression of TREM-1 mRNA in our experiments in animal models of FK suggested that TREM-1 may played a role in the innate immune response needed for effective clearance of fungal from corneal epithelium.

In this study, we detected that TREM-1 expression on rat corneal epithelium in control group, as well as the rat corneal epithelium and stroma in FK group. Moreover, we also observed obvious enhancement of TREM-1 expression on rat corneas infected with *A. fumigatus* compared with control group. These results indicate that TREM-1, as a cell-membrane receptor, mainly expressed on epithelial cells and infiltrated inflammatory cells (*e.g.* neutrophils and macrophages) [14], and fungal infection could strengthen its expression in the cornea.

Bouchon *et al* [9] demonstrated that TREM-1 expression on neutrophils and monocytes/macrophages increased in the presence of bacteria. Subsequent studies gradually unveiled that an endogenous TREM-1 ligand has been identified on human platelets but the identity of this ligand is not presently clear [15]. Activated TREM-1 binds to unknown ligands, stimulates production of inflammatory cytokines and chemokines (*e.g.* TNF-a, IL-1) and suppresses production of the anti-inflammatory cytokine IL-10 to amplify the inflammation[16,17]. This increase was consistent with that seen in other studies showing that the TREM-1 level was elevated after bacterial infection, and during the course of experimental fungal asthma in mice[9,18-20].

The role of TREM-1 as an inflammatory amplifier has been confirmed in many studies of bacterial inflammation [9]. TREM-1 blockade has been shown to reduce inflammation and increase survival in animal models of bacterial infections [8,11]. In this study, TREM-1 expression on infected rat corneal epithelium elevated in a time-dependent manner and peaked at 48h. This increase is consistent with the clinical development of FK models. Furthermore, it is also significantly correlated to the inflammation scores of FK. These findings suggest that TREM-1 may also function as an amplifier of the inflammatory response in FK, and TREM-1 pathway modulation may alter the inflammatory response associated with FK.

In conclusion, we observed that TREM-1 may contribute to amplify the inflammation in the cornea infected with *A. fumigatus* and play critical roles in the battle against *A. fumigatus* in the innate immune responses. A synthetic peptide that we called LP17 mimicking short highly interspecies conserved domains of TREM-1 attenuated the cytokine production of human monocytes and protected animals from hyperresponsiveness and death during acute or chronic inflammatory conditions[11]. Our hypothesis is that the it maybe represent a potential therapeutic target for the inflammation of FK.

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