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Original Article

The role of Mincle in innate immune to fungal keratitis

Guiqiu Zhao*, Qiang Xu*, Jing Lin, Wenjun Chen, Tingting Cui, Liting Hu, Nan Jiang

Department of Ophthalmology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China

* Both authors contributed equally to this work.

Abstract

Introduction: This study aimed to detect the early expression of macrophage-inducible C-type lectin (Mincle) and investigate its role in the innate immune response of fungal keratitis (FK).

Methodology: Wistar rats were used to make fungal keratitis models. The inflammatory responses and corneal lesions were observed by slit-lamp microscope.RT-PCR, immunohistochemistry, and immunofluorescence were used to detect the expression of Mincle in the rat corneal epithelium. The expression of eight cytokines (CXCL1, CXCL2, IL-1β, IL-6, IL-10, TNF-α, CCL2, CCL3) were detected by real-time RT-PCR and immunohistochemistry. Lastly, corneal epithelium of 54 patients with *Aspergillus fumigatus* keratitis and 13 ocular trauma patients were collected to detect expression of Mincle by real-time RT-PCR, and 12 FK and 10 ocular trauma paraffin samples were collected to confirm expression of Mincle by immunohistochemistry.

Results: The expression of Mincle was significantly upregulated at 4, 8, 16, and 24 hours after fungal infection. There were significant differences in the expression of the eight inflammatory cytokines between the blank control group and the fungus-infected group. Mincle expression was correlated with the expression of TNF-a, IL-1β, IL-10, and CCL3 in the cornea. The mRNA expressions of Mincle in the corneas of both normal and FK patients were significantly different.

Conclusions: The expression of Mincle increases significantly during the early period of *Aspergillus fumigatus* infection, while expression of eight corresponding cytokines changes. Mincle, as a pattern recognition receptor, may play a role in the early innate immune response of the corneal resistance against fungus.

Key words: Mincle; fungal keratitis; innate immune; corneal epithelium; *Aspergillus fumigatus.*

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Introduction

Fungal keratitis (FK) is a major cause of blindness in developing countries of the world. As a result of corneal injury, long-term usage of antibiotics or corticosteroids, and the decreased resistance of the body because it is immunocompromised or malnourished, the incidence rate of fungal keratitis has been higher in recent years [1]. Fungal keratitis continues to be a therapeutic challenge to oculists due to the lack of sensitive drugs and effective treatment. The nature of the host response to fungus in the cornea is not well understood. Therefore, further studies on the pathogenesis and the evolution mechanism need to be done.

Mincle exhibits the highest sequence similarity to group II C-type lectin members, and is mainly expressed in activated macrophages [2]. As a member of the C-type lectin family, recent reports have shown that Mincle plays a role in antifungal immunity [3,4]. The purpose of this study was to investigate the early expression of Mincle in *Aspergillus fumigatus* keratitis of rats and humans, and to explore the role of Mincle in the innate immunity of fungal keratitis.

Methodology

The preparation of Aspergillus fumigatus suspension

Aspergillus fumigatus standard strain (CPCC 3.0772) was grown on Sabouraud dextrose agar (Oxoid, Haoranbio, UK) at 30°C for 5 days. *Aspergillus fumigatus* antigens was prepared according to the previously described protocol [5]. Conidia of *Aspergillus fumigatus* were inoculated 200 mL Erlenmeyer flasks containing Sabouraud liquid medium (Oxoid, Haoranbio, UK) (8 g glucose, 2 g mycopeptone) by inoculating loopin. Flasks were shaken at 37°C and 200 rpm for 48 hours. *Aspergillus fumigatus* was harvested, and the mycelia were disrupted into 20–40 μm pieces and yielded 5×10^6 pieces/mL. The density of the fungal spores was read in a hemocytometer separating counter and reached the

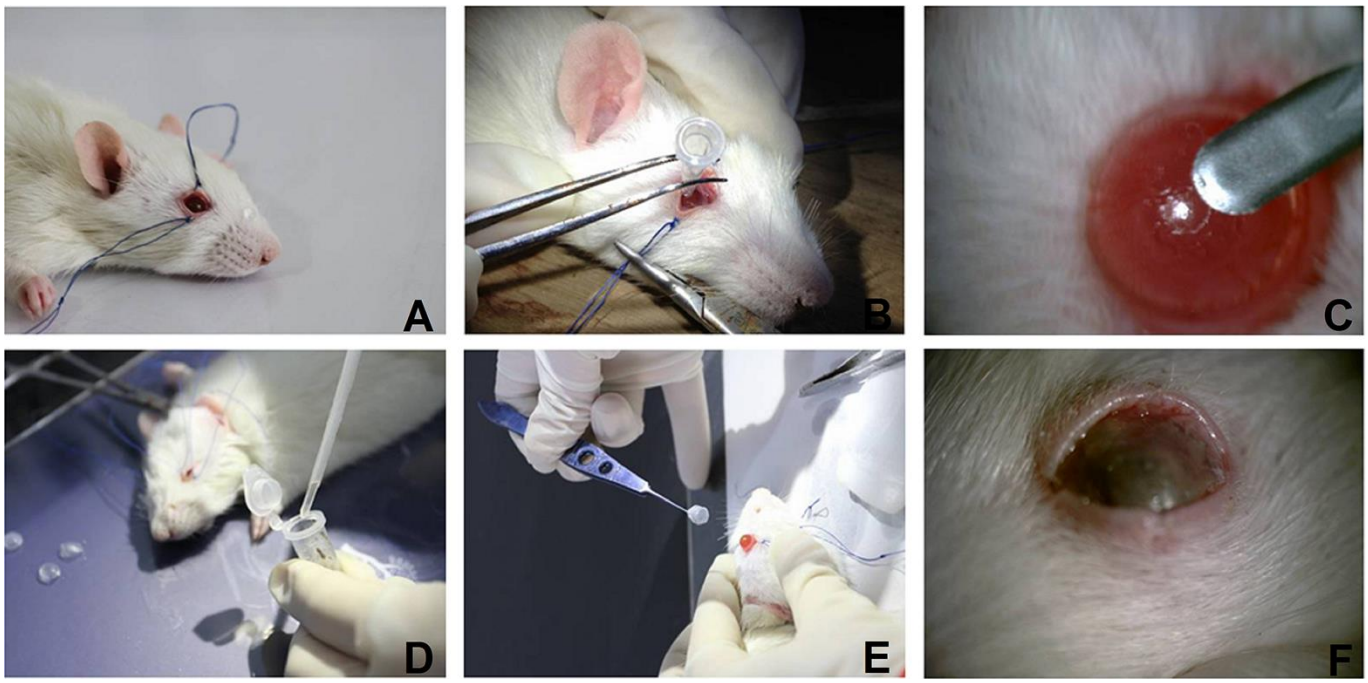
final concentrations of 10^8 colony-forming units per 5 μL inoculum.

Establishment of a rat fungal keratitis model

A total of 72 Wistar rats (both male and female) weighing 200–300 g each were used in this study. Rats with corneal disease were identified by slit-lamp examination and excluded from the study. The rats were randomly divided into three groups: group A served as a blank control group (12 eyes), group B was the sham group (60 eyes), and group C was the fungus-infected group (60 eyes). The right eyes were selected for the experiment in groups B and C. The animal research protocol was approved by the Center for Comparative Medicine at Baylor College of Medicine. All rats were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO)'s Statement for the Use of Animals in Ophthalmic and Vision Research.

Levofloxacin eye drops (Santan, Ishikawa, Japan) were given three times per day before the experiment for two days to allow fungi to establish infection in an otherwise healthy environment. The rats were anesthetized with 10% chloral hydrate 3 mL/kg for intraperitoneal injection and 0.4% oxybuprocaine hydrochloride eye drops for topical anesthesia. For conjunctival sac cleaning, 0.5% Yasuji iodine was used. The upper and lower eyelids of the rat were fixed by suture (Figure 1A). Central epithelium of the cornea was scraped, about 2 mm in diameter, by microkeratome (Figure 1B, C), then the surface of cornea was smeared with colonies of *Aspergillus fumigatus* (5 uL about 3–4 mm in diameter) (Figure 1D) in group C. Contact lenses made by Parafilm M membrane were covered after that (Figure 1E, F) in groups B and C [6]. Parafilm M membrane is a product that has characteristics of penetration performance, vapor permeability, and anti-corrosive properties. Finally, a 5-0 black silk suture was used to close the eyelid [7]. Group A served as control group without treatment. In group B, the cornea epithelium was scraped but not inoculated with *Aspergillus fumigatus*. The sutures were opened, and the change of rats' corneas at 0, 4, 8, 16, and 24 hours after surgery was observed. Cornea epithelium, except the central 2 mm, were collected at every observed time (n = 6 eyes at each observed time). The eyes were fixed with 40g/L formaldehyde solution for hematoxylin-eosin, immunohistochemical, and immunofluorescence observation at 0 and 24 hours after infection. The standard of clinical scoring of Wu [8] was used to score. A total score of 5 or less was categorized as mild eye disease, a total score of 6 to 9 was considered moderate, and a total score of more than 9 was considered severe.

Figure 1. A: Eyelids were fixed by suture. **B**, **C**: Central epithelium of cornea was scraped about 2 mm in diameter; **D**: Smeared with colonies of *Aspergillus fumigatus*. **E**, **F**: Contact lenses made by Parafilm M membrane were covered.



Human sample collection

Corneal epithelium tissue about 0.5 mm outside the infiltration edge was collected from 54 patients who were clearly diagnosed with fungal keratitis according to their case history, clinical characteristics, identification of fungal elements, and cultivation from corneal scraping elements in the Affiliated Hospital of Qingdao University, and who had not been administered antifungal drug treatment, while avoiding the focal necrotic tissue and purulent secretion. Corneal epithelium of 13 ocular trauma patients was collected as a blank control group. All these were reviewed and approved by the ethics committee of the Aravind Medical Research Foundation. The aims and methodology were thoroughly explained to the patients, and the samples were collected after informed consent was obtained.

Immunohistochemistry

Cornea specimens were made into serial sections of 4 μm thickness and sliced into 100 pieces continuously. A total of 20 slices were selected at an interval of five slices. Immunohistochemical staining was done using the SP and DAB staining method. Primary antibodies Mincle (1:100) was applied overnight at 4°C. Negative control was performed by 0.01 mol/L phosphate-buffered saline (PBS) instead of primary antibody. Isotype controls were performed by normal rabbit IgG (1:400). The samples were mounted with neutral gum and observed and photographed under the microscope. Cells with brownish-yellow granules in the cytoplasm and cell membrane were counted as positive.

Immunofluorescence

Cornea specimens were made into serial sections of 7 μm thickness. They were flushed by PBS after being dewaxed. Primary antibodies against rat Mincle (1:100) anti-Mincle mAb was purchased from MBL, Nagoya, Japan was applied overnight at 4°C. After washed by PBS, the fluorescein-labeled goat anti-rabbit biotin-labeled secondary antibody were dropped into the specimens and then incubated at 37°C for 15 minutes. Then they were observed and photographed under a fluorescence microscope.

Total RNA extraction, reverse transcription (RT) and quantitative real-time PCR

To measure the expression of mRNA, RT followed by real-time PCR was performed using the Taqman (Takara, Dalian, China) method. Total RNA was extracted from samples using RNA plus reagent, according to the manufacturer's protocol. The value of total RNA prepared was between 1.8 and 2.0, measured by UV spectrophotometer to indicate RNA purity. The cDNA was synthesized from 2 μg of total RNA by PrimeScript RT Reagent Kit (Takara, Dalian, China) with gDNA Eraser (Takara, Dalian, China). Primers were synthesized by Shanghai Biological Engineering Technology Engineering Service Co. Real-time PCR was performed in a 20 μL volume with 2 μL of respective cDNA and 1.5 μL of TaqMan gene expression assay. The thermocycler parameters were 42°C for 2 minutes, 37°C for 15 minutes, 85°C for 5 seconds, followed by 40 cycles of 95°C for 20 seconds and 65°C for 45 seconds. The results were analyzed by the comparative threshold cycle (CT) method and normalized by GAPDH.

Statistical analysis

All data were presented as mean ± standard deviation (SD) from independent experiments. The data were analyzed using SPSS version 17.0 statistical software (IBM, Armonk, USA). One-way analysis of variance (ANOVA) test was used to make comparisons among three or more groups, and least significant difference (LSD) was used to identify between two groups. $P < 0.05$ was considered to be statistically significant. The correlation analysis was performed using Pearson's correlation between gene expression and protein of Mincle and eight cytokines [9].

Figure 2. Clinical progression of rats' cornea induced by *Aspergillus fumigatus*. The images of A, B, C, and D represented the disease process of 4, 8, 16, and 24 hours after *Aspergillus fumigatus* infection, respectively.

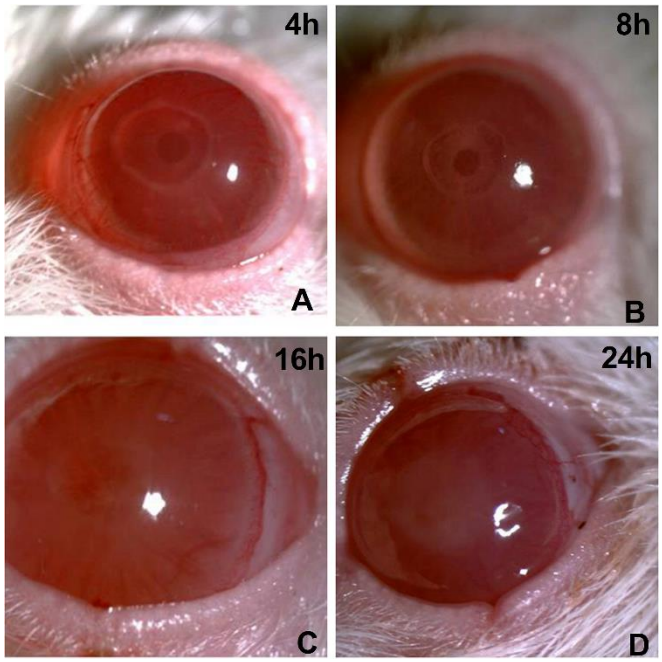


Figure 3. Relative mRNA levels of Mincle in rat at 4, 8, 16, and 24 hours in group B (surgical control group SC) and group C (*Aspergillus fumigatus* induced). Data were expressed as the mean ± SD. Mincle were found to be significantly upregulated in group C compared to the group B in every observed time. The asterisks indicate significance levels assessed via ANOVA followed by post-hoc tests (*p < 0.05; **p < 0.01).

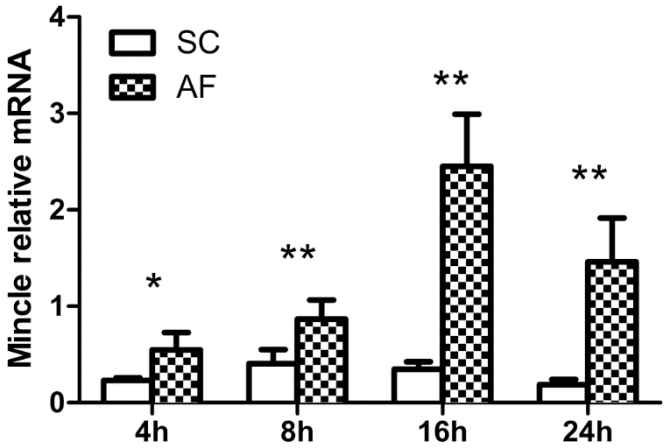


Figure 4. Relative mRNA levels of TNF-α, IL-1β, IL-6, IL-10, CCL2, CCL3, CXCL1, and CXCL2 in rats group A (normal control group) and at 4, 8, 16, and 24 hours in group C (*Aspergillus fumigatus*). Data were expressed as the mean ± SD. The asterisks represent p < 0.01 levels assessed via ANOVA followed post-hoc tests (* *p < 0.01).

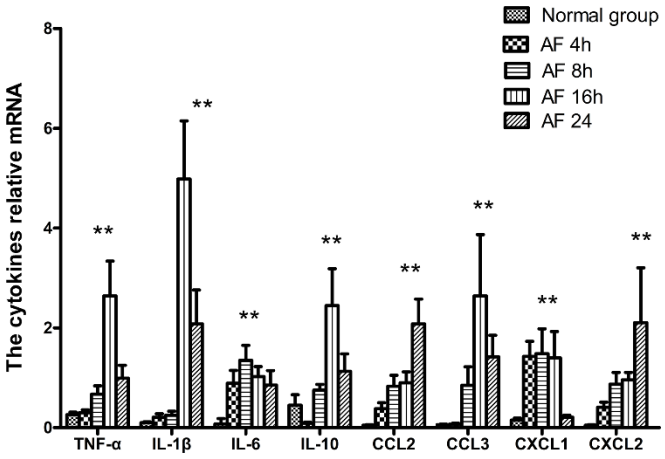
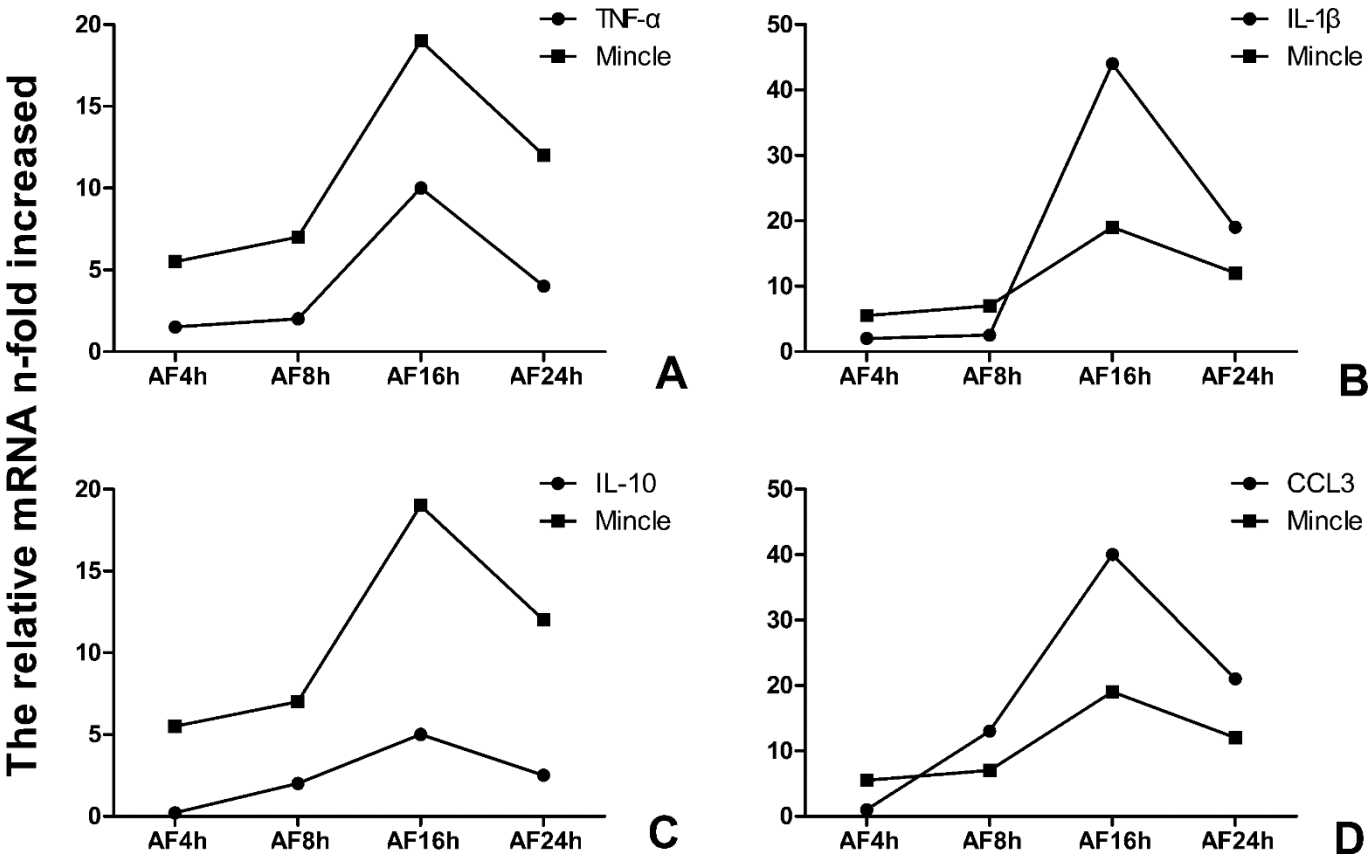


Figure 5. The relative mRNA expression increase of TNF-a, IL-1β, IL-10 and CCL3 compared with that of Mincle is shown in each chart, respectively. The correlation analysis was performed using Pearson's correlation between Mincle and cytokines. Mincle mRNA expression correlated with the expression of TNF-α (r = 0.695, p = 0.000), IL-1β (r = 0.642, p = 0.001), IL-10 (r = 0.615, p = 0.001), and CCLl3 (r = 0.607, p = 0.002).



Results

Morphological observation of animal models

As is shown in Figure 2A, the cornea was slightly edematous at 4 hours post-infection. As time went on, the lesions of corneas that were infected by *Aspergillus fumigatus* were more and more serious in clinical features (Figure 2B, C, D). The cornea was characterized by severe inflammation and marked edema, and the ulceration was induced at 24 hours (Figure 2D).

Real-time RT-PCR analysis

The reference GAPDH could be detected stably in every group. The mRNA expression of Mincle in the untreated blank group A was at a relatively low level, but it was largely induced after exposure to *Aspergillus fumigatus* at every observed time point (Figure 3). Its expression reached a peak at 16 hours after stimulation. Differences of Mincle transcript between group B and group C were 2.97-fold at 4 hours (p = 0.025), 3.28-fold

Figure 6. The mRNA expression of Mincle in cornea of both normal and fungal keratitis (FK) patients could be detected. Mean ± SD in FK group was 7.1613 ± 2.8347, while in normal group it was 1.1392 ± 0.5922. There was a statistically significant difference between them (t = -14.36, p < 0.05).

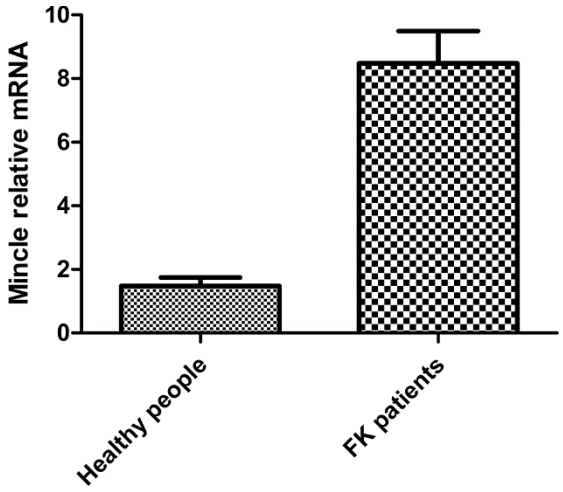
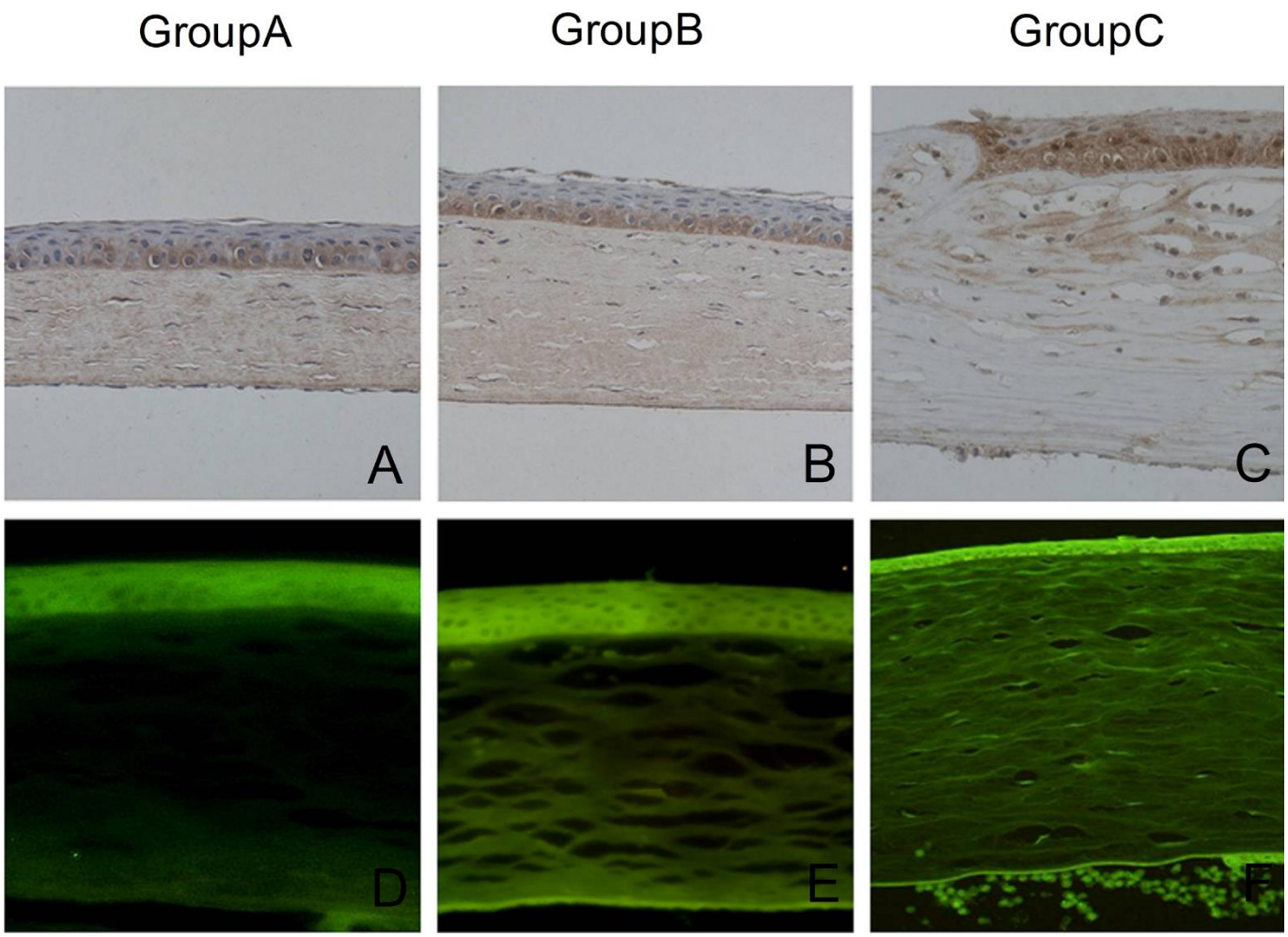


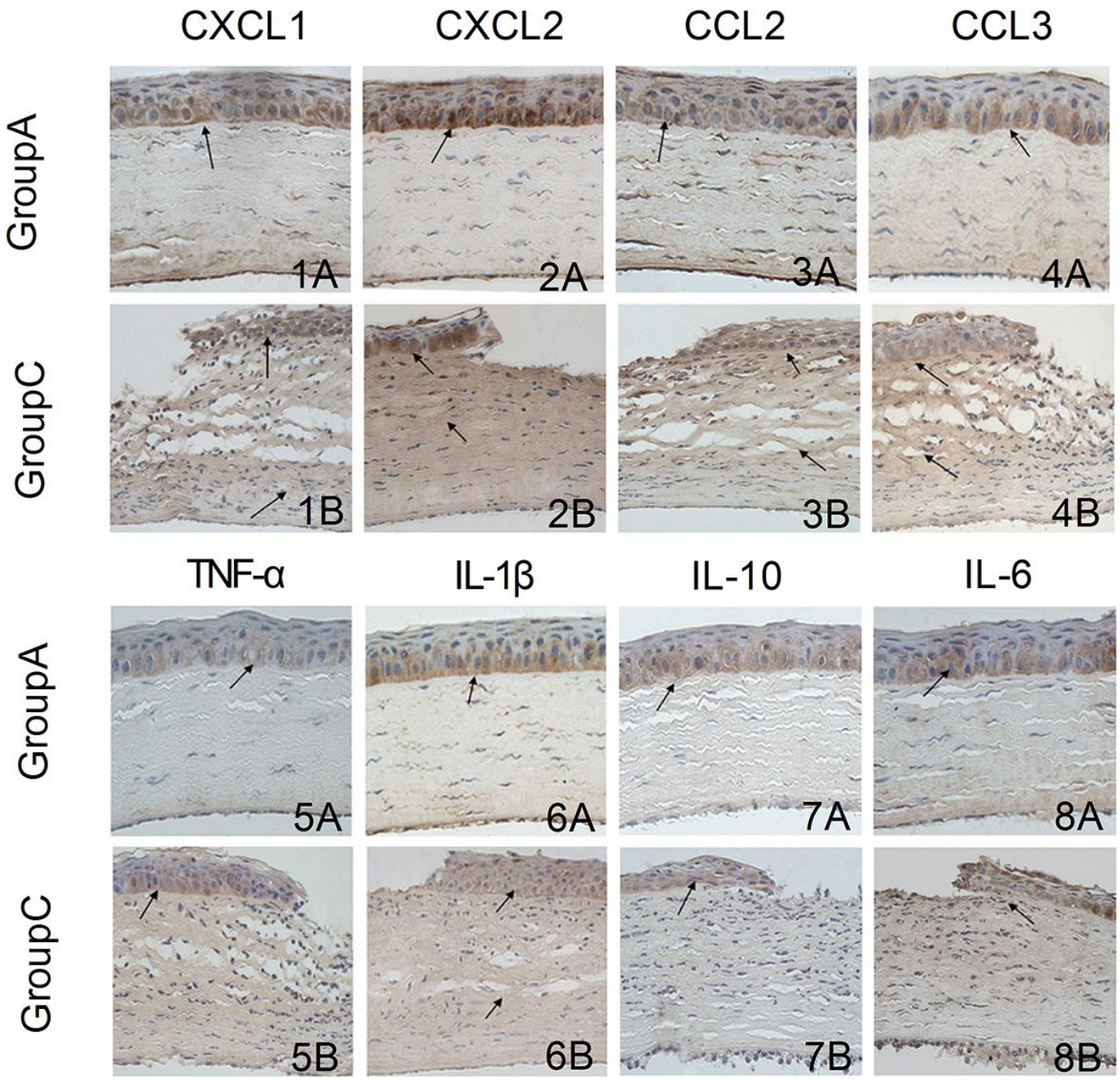
Figure 7. Mincle expression in rats evaluated by immunohistochemical and immunofluorescence staining. **A**, **D**: normal group (group A); **B**, **E**: scarified control group (group B); **C**, **F**: *Aspergillus fumigatus*-infected 24-hour group (group C) (magnification 400 ×).



at 8 hours (p = 0.004), 6.97-fold at 16 hours (p < 0.001), and 7.36-fold at 24 hours (p < 0.001) (Figure 2A). The relative mRNA expressions of CXCL1, CXCL2, CCL2, CCL3, IL-1β, TNF-α, IL-6, and IL-10 are shown in Figure 4. There was statistically significant difference of all the above factors between groups A and C (p < 0.05). The relative mRNA increase of TNF-a, IL-1β, IL-10, and CCL3 compared with that of Mincle is shown in Figure 5. Expression of Mincle mRNA correlated with the expression of TNF-α (r = 0.695, p = 0.000), IL-1β (r = 0.642, p = 0.001), IL-10 (r = 0.615, p = 0.001), CCL3 (r = 0.607, p = 0.002), IL-6 (r = 0.238, p = 0.262), CCL2 (r = 0.202, p = 0.345), CXCL1 (r = 0.017, p = 0.938), and CXCL2 (r = 0.220, p = 0.300).

In humans, the mRNA expression of Mincle in the corneas of both normal and FK patients could be detected, and the mean ± SD in the FK group was 7.1613 ± 2.8347, while in the normal group it was

Figure 8. Cytokines expression in rat corneal epithelium evaluated by immunohistochemical (1 to 8 CXCL1, CXCL2, CCL2, CCL3, IL-1β, TNF-α, IL-10, and IL-6) **A**: normal group (group A); **B**: *Aspergillus fumigatus* group (group C) (magnification 400 ×).



1.1392 ± 0.5922, and the difference was statistically significant ($t = -14.36$, $p < 0.05$) (Figure 6).

Immunohistochemistry and immunofluorescence technique results

In Figure 7, brown staining indicates the expression of Mincle. In Figure 7A, the brown staining can only be found in the basal cells of normal rat corneal epithelium. The staining in group B in 24 hours was similar to that in group A (Figure 7B). In group C, at 24 hours after infection, the positive staining was obviously increased. The inflammatory response was observed in the infected *Aspergillus fumigatus* group, such as minimal inflammatory cell infiltration, damaged corneal tissue, epithelial defects, and corneal ulceration (Figure 7C, F). From Figure 7D and 7E, the immunofluorescent staining in the epithelium was visible in groups A and B, but in group C, the staining of corneal epithelium was stronger than in groups A and B. As shown in Figure 7F, a large number of inflammatory cells were infiltrated in the anterior chamber and were visible by immunofluorescence staining. In groups A and C of rats, the cytokines of CXCL1, CXCL2, CCL2, CCL3, IL-1β, TNF-α, IL-6, and IL-10 were all expressed (Figure 8).

The brown staining that indicates the expression of Mincle was only found in the basal corneal epithelium of ocular trauma patients and fungal keratitis.

Discussion

Aspergillus is widely presented in the environment and it is one of the main pathogens of fungal keratitis in China [9]. Fungal keratitis comprises a dynamic interaction between host and microorganisms [10]. The innate immune system as the first line is important to the occurrence, development, and prognosis of host inflammation, and it can recognize distinct pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Activation of PRRs triggers cell signaling, leading to the production of chemokines, pro-inflammatory cytokines, and the induction of antimicrobial and inflammatory responses. Corneal epithelial cells play an important role in innate immune response after being infected by pathogenic microorganisms [11]. Many studies have shown that the toll-like receptor (TLR) and nucleotide oligomerization domain 2 (NOD2) expressed by corneal epithelial cells may recognize PAMPs in fungal keratitis, contributing to the induction of the innate immune response to the pathogen [12,13]. Other than the members of the TLR family, the C-type lectin family is most prevalent in immune responses in fungal keratitis [14].

C-type lectin can recognize pathogens, and it can be classified as inhibitory or activating molecules in the immune response. Mincle, as a key macrophage surface expressed C-type lectin, is an inducible early response gene in macrophages responding to pathogens [2]. A typical carbohydrate recognition domain (CRD) of Mincle contains an EPN (Glu-Pro-Asn) motif, which can putatively bind mannose [15]. Mincle may recognize specific geometry of α-mannosyl residues of some fungi, and may play a certain role in immune response to fungi [16]. In this study, we found the expression of Mincle mRNA and proteins in both rat and human normal corneal epithelium. After fungal infection, its expression increased at 4 hours and peaked at 16 hours. The results of immunohistochemical and immunofluorescence staining showed that neutral granular cells infiltrated into the cornea and the expression of Mincle increased in corneal epithelium at 24 hours after infection. These results suggest that there are base amounts of Mincle in corneal epithelium, and Mincle increases when fungal infection happens, which suggests that Mincle may take part in the process of innate immune and be associated with fungal invasion in cornea epithelium. Lee *et al*. [17] advocated that Mincle was necessary for neutrophils recruitment during the initial stage of TDM (trehalose 6,6'-dimycolate)-induced lung inflammation by enhancing neutrophil integrin expression, cytoskeleton remodeling, and cell adhesion. In this study, Mincle was strongly expressed on the inflammatory cells gathered in the anterior chamber (Figure 7). It may be a significant influence in recruiting neutrophils to fungus.

After being activated by the ligands, Mincle can effectively activate NF-κB signaling through Syk/Card actions, and can increase the expression levels of some cytokines [18]. Recent studies have shown that coculture of increasing amount of *Malassezia* activated BMM (bone marrow-derived macrophages) to produce MIP-2, TNF- a, KC, and IL-10 in a dose-dependent manner. The inflammatory cytokine/chemokine mRNA expression was significantly suppressed in Mincle$^{-/-}$ mice [16]. Wells *et al*. [19] reported that Mincle could recognize *C. albicans* and promoted the production of TNF-α by macrophages. There was significantly increased susceptibility to systemic *C. albicans* when the mice lacked Mincle. In this study, we found that Mincle was upregulated during fungal infection and that its expression correlated with TNF-a, IL-1β, IL-10, and CCL3. TNF-a, produced by neutrophils and macrophages, is a Th1-type cytokine and an essential factor in antifungal inflammation. It acts as a molecular switch in antifungal immunity, regulates leukocyte

recruitment, and promotes fungicidal activity [4,20]. IL-1β is produced by inflammatory cells and resides in corneal cells [21]. Runder *et al*. [22] reported that the expression levels of IL-1β mRNA and protein were significantly elevated in mice with *Pseudomonas aeruginosa* keratitis. The severity of corneal disease was reduced when mice were treated with anti-IL-1β polyclonal antibody. The expression levels of MIP-2, KC, and IL-1β may be closely related to the performance of the disease severity and inflammatory reaction of fungal keratitis [17]. IL-10 is a Th2-type inflammatory factor, which can suppress the reaction of the Th1-type factor. Previous reports showed that IL-10 could promote the activation of Th2 cells, inhibit the phagocytosis of macrophage, and weaken the body's ability to resist fungus by inhibiting the proliferation of Th1 cells [7]. In our experiment, IL-10 mRNA expression level was higher in group A at 4 hours after infection, but the expression level was increased from 4 hours to 16 hours. This indicates that Th1-type cytokine plays a major role in the early period of cornea after fungal infection. As time went on, IL-10 appeared increasingly, inhibiting the influence of Th1-type cytokine. Xue *et al*. [18] concluded that CCL2 and CCL3 could play a critical role in the *Pseudomonas aeruginosa*-induced model of corneal infection in mice. They are critical regulators of polymorphonuclear neutrophil (PMN) recruitment, and may lead to therapeutic strategies via targeting of the CC chemokines. CCl3 plays important roles in the acute inflammatory response to *C. albicans* corneal infection [24]. Our study proved the involvement of the Mincle pathway in the expression of TNF-α, IL-1β, IL-10, and CCL3 during the development of fungal keratitis.

Corneal epithelial cells acting as the first barrier can protect the cornea from infection by pathogenic microorganisms. The ability of corneal epithelial cells to recognize pathogenic microorganisms starts the innate immune response. It is important to the occurrence, development, and prognosis of corneal inflammation. In our study, Mincle existed and expressed in human and rat corneal epithelial cells, and contributed to the innate immune responses triggered by fungus. Moreover, it correlated with inflammatory cytokines, such as TNF-α, IL-1β, IL-10, and CCL3, to affect the initiation and progression of fungal keratitis. To investigate the mechanism of fungal keratitis, it is important to further discover and study the PRRs that can bind pathogens and regulate the innate immune. Mincle and other PRRs may become targets for immune intervention in the treatment of keratitis.

Conclusions

We detected Mincle in both protain and mRNA level in normal corneal tissue in humans and rats. Its expression increases significantly during the early period of *Aspergillus fumigatus* infection, and its expression correlated with TNF-a, IL-1β, IL-10, and CCL3. Mincle may play a role in the early innate immune response of the corneal resistance against fungus.

Acknowledgements

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Corresponding author

Zhao Guiqiu, PhD
Department of Ophthalmology, The Affiliated Hospital of Qingdao University
16th Jiangsu Road, 266003
Qingdao, Shandong Province, China
Phone: +86 0532-82911211
Fax: +86 0532-82911097
Email: zhaoguiqiu_good@126.com

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