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Original Article

Targeting STAT3 signaling alleviates severity of fungal keratitis by suppressing ICAM-1 and IL-1β

Yuanyuan Liu, Kuixiang Liu, Guiqiu Zhao

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

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Abstract: *Aim:* Our aim was to investigate expression and regulation of STAT3, ICAM-1 and IL-1β, further understanding the pathogenesis of fungal keratitis (FK) in order to develop more effective therapeutic approaches. *Methods:* Corneas of C57BL/6 mice were intrastromally inoculated with 1×10^5 *Candida albicans* blastospores. Slit-lamp microscopy and histopathological analysis were used to observe development of corneal lesions and inflammatory responses at 1 day and 5 days post-infection. Reverse transcription-polymerase chain reaction, Western blot, and enzyme-linked immunosorbent assay (ELISA) analyses were used to detect expression levels of STAT3, ICAM-1, and IL-1β in infected corneas. For interventional experiments, small interfering (si) RNA transfection was used to target STAT3 or neutralizing antibodies were used to target ICAM-1 and IL-1β. Experimental mice were subconjunctivally injected with 100 nM STAT3 siRNA or 5 μL (0.5 ng/μL) of ICAM-1 or IL-1β polyclonal antibody, 1 hour before and 24 hours after surgery. Reestablishment of the FK murine model was performed following injection. Effects of STAT3 siRNA transfection, ICAM-1 antibodies, or IL-1β polyclonal antibodies on corneal disease were observed with slit-lamp microscopy, histopathological analysis, and ELISA. *Results:* STAT3 expression was increased during corneal fungal infection followed by activation of ICAM-1 and IL-1β, which were reversed by targeting STAT3. Specific polyclonal neutralizing ICAM-1 or IL-1β antibodies or targeting STAT3 decreased clinical scores and inflammatory responses, effectively relieving FK induced injury. *Conclusions:* Targeting STAT3 or administration of specific polyclonal neutralizing antibodies, to inhibit ICAM-1 or IL-1β, effectively relieves FK-induced corneal injury.

Keywords: Fungal keratitis, STAT3, ICAM-1, IL-1β

Introduction

Fungal keratitis (FK) is a sight-threatening ocular disease with a growing incidence rate, especially in developing countries [1], accounting for approximately 1% of corneal ulcers and up to 35% of all corneal ulcers in temperate and tropical regions of developed industrialized countries, respectively [2]. In China, up to 60% of corneal ulcers are attributable to fungal infection [3], with *Fusariumsolani* and *Aspergillus fumigates* as the most common pathogens [4]. Although new clinical therapies have been designed, FK remains challenging to ophthalmologists due to delayed diagnosis and lack of standard treatment guidelines. In addition, the exact mechanisms of this process, such as specific inflammatory mediators, have not yet been fully elucidated. Therefore, a better understanding of the pathogenesis of FK is necessary to improve diagnosis and treatment [5, 6].

Previous studies have demonstrated that fungal infection activates hostimmune response, which plays an essential role in the pathogenesis of FK [7-9]. Neutrophils are the first cells to infiltrate the infected cornea, indicating that neutrophils are the main effector cells required for killing of fungal hyphae. Studies have reported that following corneal infection, intercellular cell adhesion molecule-1 (ICAM-1), macrophage inflammatory protein-2 (MIP-2), and some cytokines are then produced. These recruit neutrophils from peripheral limbal blood vessels to peripheral corneal stroma, which then migrate to the infected area of the cornea [10-12].

Gamma delta T-cells are also essential in innate immunity and play an important role in recruitment and activation of neutrophils [11]. These cells also take part in the immune response against FK by secreting cytokines and chemokines such as CC chemotactic factor (CCL)-3, 4,

and 5 [11] and interleukin (IL)-1β, 6, and 8, and MCP-1, suggesting that inflammatory cytokines are involved in FK [12, 13]. However, mechanisms underlying how the fungus activates the immune system and subsequent release of inflammatory cytokines in FK are not very clear.

The signal transducers and activators of transcription (STAT) family of latent cytoplasmic proteins is involved in transmitting extracellular signals to the nucleus [14, 15]. Bacterial endotoxins may induce tyrosine phosphorylation of STAT3 [16]. Activated STAT3 induces NF-κB translocation and, thus, triggers cytokine production [17]. Stattic, a STAT3 inhibitor, has been found to suppress STAT3 tyrosine phosphorylation *in vivo* and inhibit systemic inflammation, thereby increasing survival of mice with experimental sepsis [18]. These results implicate the STAT3 signaling pathway in inflammatory diseases.

Taylor et al. found that regulation of IL-17-induced production of neutrophils by JAK/STAT3 inhibitors impairs production of reactive oxygen species and fungal-killing activities but also blocks the activities of elastase and gelatinase, possibly causing tissue damage [19]. Another study reported that high expression levels of IL-1β and ICAM-1 are major factors in the corneal pathogenesis of FK. Specific polyclonal neutralizing antibodies against IL-1β and ICAM-1 have been reported to effectively relieve FK-induced corneal injury [20]. Studies have also reported that the immune system and inflammatory cytokines may be regulated by STAT3 [21, 22].

In our present study, we tested the role of STAT3 activation and the resulting expression of ICAM-1 and IL-1β in experimental FK. Results of STAT3, ICAM-1, and IL-1β expression studies showed that ICAM-1 and IL-1β were inhibited by small interfering (si) RNA targeting STAT3 or polyclonal antibodies to neutralize inflammatory effects. Histopathological changes in the corneal lesion were observed and expression of major inflammatory cytokines in the infected cornea was detected.

Materials and methods

Mice

This study protocol was approved by the Ethics Committee of Qingdao University (Qingdao, China). Female C57BL/6 (B6) mice (8 weeks) were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and housed in accordance with guidelines of the National Institutes of Health. The study protocol also conformed to standards described by the Association for Research in Vision and Ophthalmology and Statement for the Use of Animals in Ophthalmic and Vision Research.

Design of a mouse FK model

Corneas of the right eyes of mice were superficially scarified [23]. A 5-μL inoculum of either *Candida albicans* (1×10^6 CFU) or sterilized phosphate-buffered saline (PBS) was topically applied to the eyes of the infected and control groups, respectively. For interventional experiments using neutralizing antibodies or STAT3 siRNA transfection, mice were subconjunctivally injected with 100 nM STAT3 siRNA or ICAM-1 polyclonal antibody (ICAM-1 treated group), 5 μL of the IL-1β polyclonal antibody (IL-1β treated group), or sterile saline (infected group), both 1 hour before and 24 hours after surgery. The mice were then photographed and clinically scored before being sacrificed at 5 days post-infection (dpi), as follows [24]: 0, normal cornea; 1, mild corneal haze; 2, moderate corneal opacity; 3, severe corneal opacity; 4, opaque cornea and ulcer; and 5, corneal rupture. Additionally, images of mouse corneas were acquired using a photo slit-lamp microscope equipped with a digital camera. Normal, uninfected, and infected eyes of mice were enucleated and processed for histological, reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and enzyme-linked immunosorbent assay (ELISA) analyses.

Histological analysis

For histological analysis, the eyes were fixed in formaldehyde and embedded in paraffin. They were then cut into 5 μm-thick sections and stained with periodic acid-Schiff solution (Sigma-Aldrich, St. Louis, MO, USA), counterstained with hematoxylin, and examined with light microscopy. Degree of inflammation in microscopic corneal sections was graded by an examiner, blinded to any knowledge of the experimental procedure, using a scale from 0 to 4 (0, no sign of inflammation; 1, minimal inflammatory cell infiltration and minimal structural changes; 2, mild inflammatory cell infil-

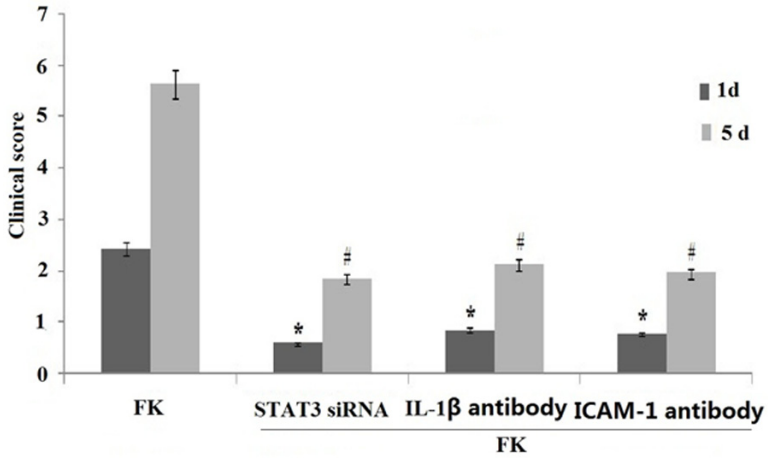

Figure 1. Clinical scores of *C. albicans*-induced FK in mice. Clinical scores were significantly decreased at 1 and 5 dpi in the STAT3 siRNA-transfected groups and ICAM-1 or IL-1β antibody-treated groups. $^{*}p < 0.05$ and $^{\#}p < 0.05$ vs. the FK-infected (untreated) group (ANOVA followed by post hoc test).

tration and mild structural changes; 3, moderate inflammatory cell infiltration and moderate structural changes; and 4, severe inflammatory cell infiltration and severe structural changes).

RNA extraction and real-time RT-PCR

Total RNA was isolated from individual corneas using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) and reverse transcribed into complementary DNA, according to the manufacturer's instructions. Primers for mouse IL-1β and ICAM-1 were purchased from Bersin Bio (Guangzhou, China). IL-1β and ICAM-1 mRNA expression levels were measured with quantitative real-time PCRs on a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA, USA). Relative quantities of IL-1β and ICAM-1 transcripts were calculated using $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference.

ELISA

Corneal samples were collected from different treatment groups (n = 5/group/time) at 5 dpi and homogenized in 0.5 mL of PBS containing 0.1% Tween-20. The supernatant was removed and centrifuged for 5 minutes at 13,000 × *g* at 4°C. Then, cells were lysed using a specific buffer (pH 8.0) and the supernatants were collected. ICAM-1and IL-1β protein levels were detected in the collected samples using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions, with use of a standard curve. Sensitivity of ELISA was 2.5 pg/mL for ICAM-1 and 1.5-1.92 pg/mL for IL-1β, respectively.

Western blot analysis

Corneas collected from BALB/c eyes at 1 and 5 dpi were lysed and sonicated in radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc., Beverly, MA, USA) containing phosphatase inhibitor cocktail (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Lysates were centrifuged at 10,000 × *g* for 10 minutes at 4°C. Pellets were discarded and total protein concentrations of the supernatants were detected using DC Protein Assay Reagents Package kit (Bio-Rad Laboratories). The samples were boiled for 5 minutes at 97°C. 25 μg of protein was then loaded per lane for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, which was blocked with blocking buffer (Thermo Fisher Scientific, Waltham, MA, USA) and then incubated overnight at 4°C with primary antibodies against STAT3 and β-actin (Cell Signaling Technology, Inc.). Afterward, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) for 1 hour at room temperature. Labeled proteins were identified with a chemiluminescence detection kit (Cell Signaling Technology, Inc.) and imaged on x-ray film. Optical densities were quantified using Image J software (https://imagej.net/) and data were reported as a percent of the control.

Statistical analysis

All statistical analyses were performed using unpaired, two-tailed Student's *t*-test, or analysis of variance (ANOVA) followed by post hoc test using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). A probability (p) value < 0.05 was considered statistically significant.

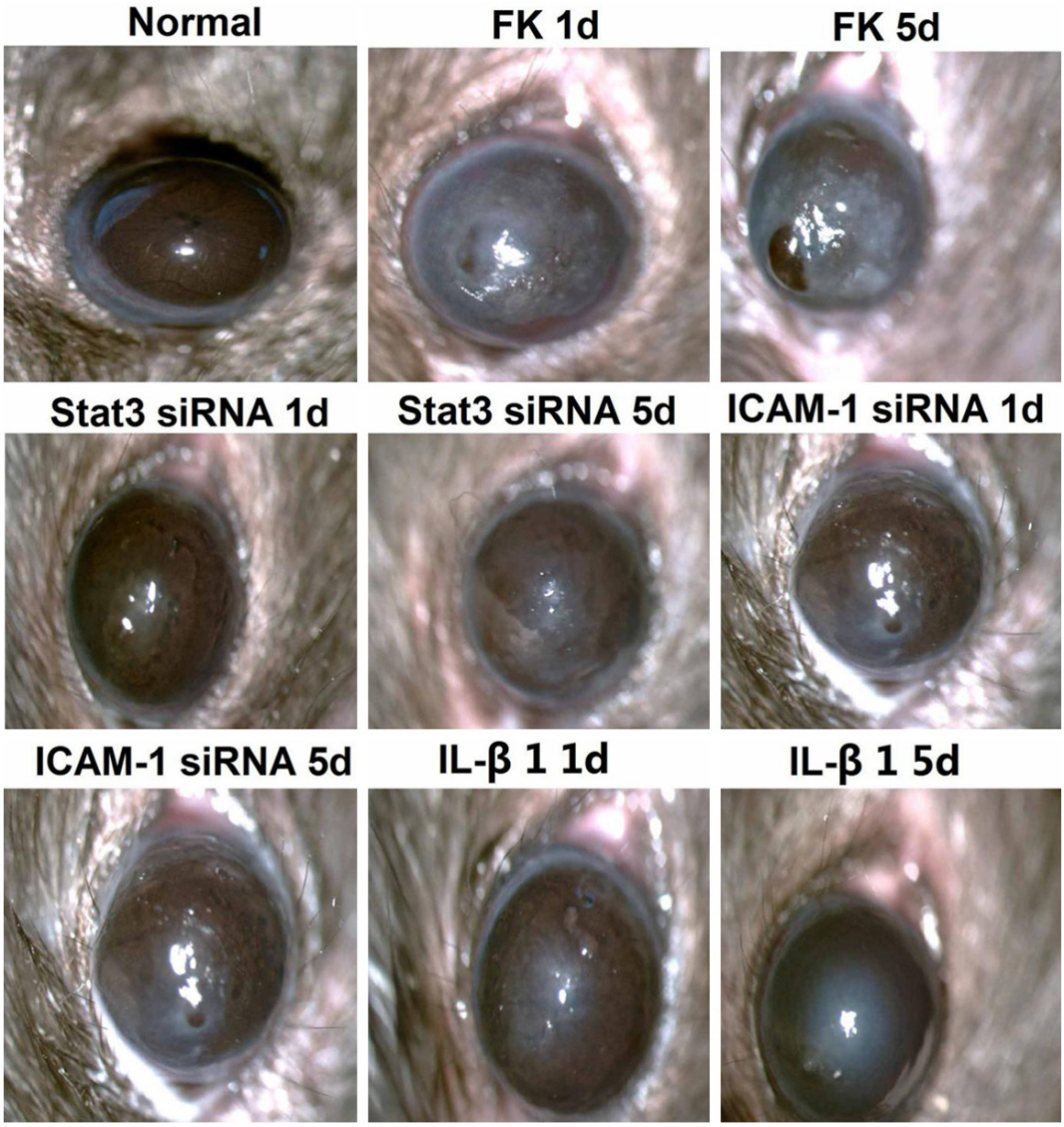

Figure 2. Clinical progression of *C. albicans*-induced FK in the STAT3 siRNA-transfected groups and ICAM-1- or IL-1β antibody-treated groups. Corneas were observed under a slit-lamp microscope.

Results

Experimental FK

All eyes inoculated with *C. albicans* developed clinical signs of keratitis. **Figure 1** shows the mean clinical scores of mice (n = 6), as determined by slit-lamp microscopy. Corneal inflammation began at 1 dpi. The mean clinical score was 2.4 ± 0.2 and the corneas appeared cloudy with mild surface irregularity (**Figure 2**). At 5 dpi, corneas were cloudy with significant edema and dense infiltrates with non-uniform opacity (**Figure 2**), with a mean clinical score of 5.7 ± 0.8. However, in the STAT3 siRNA-transfected groups and ICAM-1 or IL-1β antibody-treated groups, clinical scores of each eye were significantly lower than of eyes with infected lesions alone (**Figure 1**). In addition, in STAT3 siRNA-transfected groups and ICAM-1 or IL-1β antibody-treated groups, corneas were significantly improved (**Figure 2**).

Histological analysis

Histological analyses of the sections indicated obvious infiltration of inflammatory cells into corneas of the inflammation group (**Figure 3**,

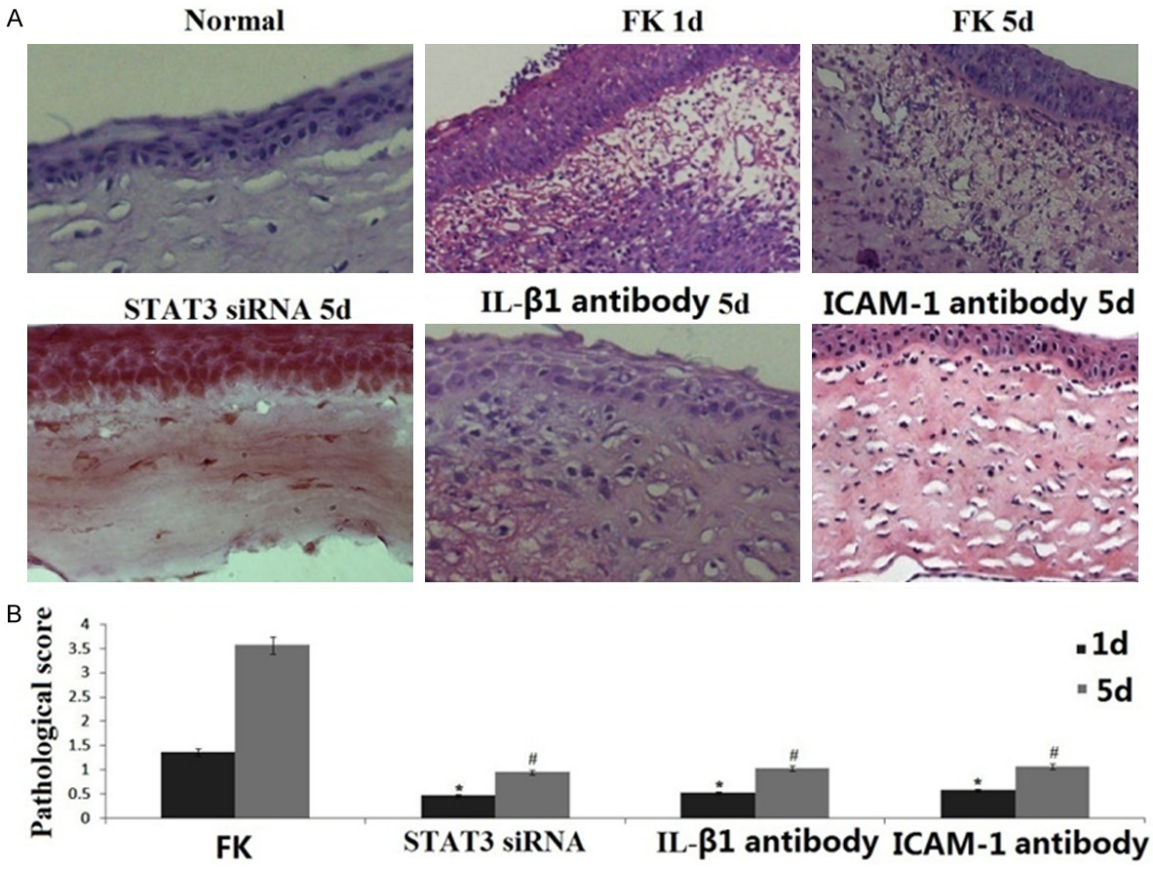

Figure 3. Histopathological analysis of *C. albicans*-induced FK in mice. Inflammatory changes observed at 1 and 5 dpi (magnification 100 ×). Here, the inflammatory responses were significantly reduced at all time points in the STAT3 siRNA-transfected and ICAM-1 or IL-1β polyclonal antibody-treated groups. The bar chart shows semi-quantitative pathological scores.$^{*}p < 0.05$ and $^{\#}p < 0.05$ (ANOVA followed by post hoc test).

some data not shown). Following STAT3 siRNA transfection or treatment with IL-1β or ICAM-1 antibodies, inflammatory responses (**Figure 3A**) and pathological scores (**Figure 3B**) were significantly lower ($p < 0.05$) than those observed in the infected group.

STAT3 expression

Western blot analysis showed that STAT3 expression was significantly increased in *C. albicans*-infected groups on 1 and 5 dpi (**Figure 4A**). After STAT3 siRNA transfection, STAT3 expression was decreased in *C. albicans*-infected groups.

ICAM-1 and IL-1β expression

IL-1β and ICAM-1 were significantly upregulated in corneas of the infected group, as compared to normal control group (**Figure 4B**). Expression levels of IL-1β and ICAM-1 were highest in the infected group at 5 dpi, which was in agreement with ELISA results (**Figure 4C**). However, when STAT3 was inhibited by siRNA transfection, IL-1β and ICAM-1 expression levels were significantly lower than in the FK-infected only groups (**Figure 4B**, **4C**; $p < 0.05$). In addition, protein expression levels of IL-1β or ICAM-1 in the IL-1β or ICAM-1 polyclonal antibody-treated groups were significantly reduced (data not shown). However, administration of polyclonal antibodies against IL-1β or ICAM-1 did not affect STAT3 expression in FK-infected corneas, as indicated by Western blot assay results (**Figure 4D**).

Discussion

The results of our present study suggest that transcription factor STAT3 and inflammatory cytokines IL-1β and ICAM-1 play important pathological roles in FK at gene and protein levels. Moreover, expression levels of STAT3,

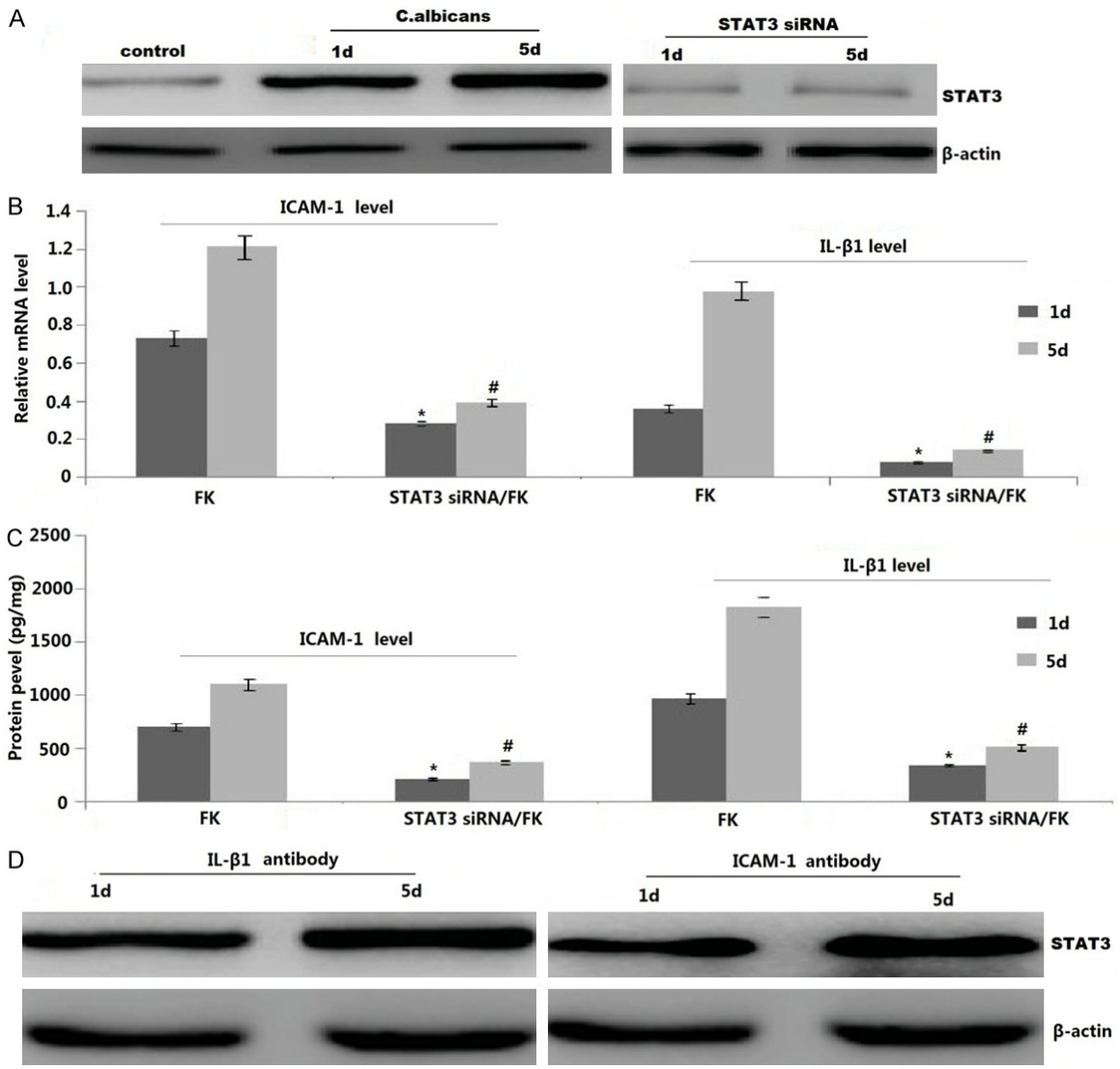

Figure 4. Detection of STAT3, ICAM-1, and IL-1β in *C. albicans*-induced FK of mouse corneas. A. STAT3 expression was detected by Western blot analysis. B. Relative mRNA levels of *ICAM-1* and *IL-1β* were detected by qRT-PCR analysis. C. Protein levels of *ICAM-1* and *IL-1β* were detected with ELISA. D. STAT3 expression in FK-infected corneas was detected by Western blot analysis after administration of the IL-1β or ICAM-1 polyclonal antibody. $^{*}p < 0.05$ and $^{\#}p < 0.05$ vs. the FK-infected (untreated) group.

ICAM-1, and IL-1β were positively correlated with severity and progression of FK, demonstrating that these factors are responsible for the pathogenesis of FK. The aforementioned association between low levels of STAT3 and decreased clinical scores, inflammatory responses, and expression of IL-1β and ICAM-1 has been demonstrated in observational studies. In addition, following treatment with an antibody against ICAM-1 or IL-1β, corneal clinical scores and inflammatory responses were also decreased but STAT3 expression was not affected.

IL-1β, a pro-inflammatory cytokine produced by epithelial cells, macrophages, and inflammatory cells as well as resident corneal cells, plays important roles in multiple infectious and acute and chronic inflammatory diseases. It is a target for anti-inflammatory therapies [25, 26]. The innate immune response is the first line of defense against pathogens and plays critical roles in activation and regulation of adaptive immune response. Neutrophils are active in innate immune response. Once a cornea is infected, neutrophils are the first immune cells to infiltrate the cornea to protect against

fungi. ICAM-1 and some cytokines can attract neutrophils to the inflammatory site of the cornea [27, 28].

Our study is the first to demonstrate that ICAM-1 and IL-1β were significantly upregulated in corneas in response to fungal infection. Moreover, expression levels of ICAM-1 and IL-1β were closely associated with severity and the pathological process of corneal damage. In addition, when mice received subconjunctival injections of antibodies against ICAM-1 or IL-1β, corneal inflammatory responses and severity of corneal disease were significantly reduced, indicating that ICAM-1 and IL-1β may play central roles in the pathogenesis of FK. Therefore, targeting of ICAM-1 and IL-1β may be a potential strategy for treatment of FK.

STAT3 plays a crucial role in normal development, acute phase response, chronic inflammation, autoimmunity, metabolism, and cancer progression [22]. STAT3 is activated by a family of cytokines including IL-6, IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), ciliary neurotrophic factor, cardiotrophin-1, and cardiotrophin-like cytokines which share the same gp130 signal transducer [29]. Furthermore, STAT3 is a key molecule in regulation of genes responsible for the production of pro-inflammatory cytokines and chemokines [30].

In the present study, a *C. albicans* keratitis model was used to test the hypothesis that STAT3 plays a role in profungal responses. The results of this study demonstrate that *C. albicans* infection stimulates STAT3 production in the corneal epithelial. Targeting STAT3 by siRNA, applied prior to *C. albicans* inoculation, greatly reduced severity of FK and corneal inflammation. Also, STAT3 induced ICAM-1 and IL-1β expression in the damaged cornea and targeted STAT3-inhibition of ICAM-1 and IL-1β expression. Although some inflammatory cytokines and chemokines are known to activate STAT3 [30], treatment with polyclonal antibodies against ICAM-1 or IL-1β did not affect STAT3 expression, suggesting that ICAM-1 and IL-1β are regulated by STAT3.

In conclusion, use of siRNA targeting STAT3 or specific polyclonal antibodies, to inhibit ICAM-1 or IL-1β, could effectively relieve FK-induced corneal injuries. This result is encouraging. Therefore, further exploration of this information will be important in the development of new therapeutics aimed at inflammatory cytokines in FK.

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Disclosure of conflict of interest

None.

Address correspondence to: Yuanyuan Liu, Department of Ophthalmology, The Affiliated Hospital of Qingdao University, No 16, Jiangsu Road, Qingdao 266003, Shandong, China. Tel: +86-532-8291-6783; E-mail: wujiangqdsd@sina.com

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