· Basic Research ·

Interactions of thymic stromal lymphopoietin with interleukin-4 in adaptive immunity during *Aspergillus fumigatus* keratitis

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Abstract
● **AIM:** To investigate the potential interactions of thymic stromal lymphopoietin (TSLP) with interleukin-4 (IL-4) in adaptive immunity during fungal keratitis (FK).
● **METHODS:** An FK mouse model was induced with *Aspergillus fumigatus* (AF) hyphal infection. Mice were divided into several groups: untreated, phosphate buffer saline (PBS), infected with AF, and pretreated with a scrambled siRNA, a TSLP-specific siRNA (TSLP siRNA), murine recombinant TSLP (rTSLP), immunoglobulin G (IgG), murine recombinant IFN (rIFN-γ), murine recombinant IL-4 (rIL-4), rIL-13, murine recombinant IL-17A (rIL-17A), and murine recombinant IL-17F (rIL-17F) groups. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) or Western blot were performed to determine mRNA and protein levels in the inflamed cornea. Cytokine locations were observed by immunofluoresence staining after AF hyphal infection.
● **RESULTS:** Compared to those in the untreated group, TSLP and T helper type 1 (Th1) cytokine levels in the AF group were upregulated at 24h post infection (hpi), and those of T helper type 2 (Th2) and T helper type 17 (Th17) cytokines were increased at 5d post infection (dpi). Th2 cytokine levels were decreased in the TSLP siRNA-pretreated group and increased in the rTSLP-pretreated group compared with the AF group. The TSLP level was increased in the rIL-4-pretreated group, but there were no significant changes among the other groups. Immunofluorescence staining showed cytokine locations after AF hyphal infection.
● **CONCLUSION:** TSLP induces a Th2 immune response and promots Th2 T cell differentiation *in vivo*. IL-4 promotes TSLP secretion. Therefore, TSLP with IL-4 regulates adaptive immunity in FK.
● **KEYWORDS:** *Aspergillus fumigatus*; keratitis; thymic stromal lymphopoietin; Th2 immune response; interleukin-4
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INTRODUCTION
Fungal keratitis (FK) is a serious vision-impairing ocular surface infection that accounts for 65% of all corneal ulcers on a global scale[1]. *Aspergillus* is a major pathogen genus in FK[2]. Due to its rapid progression, delayed diagnosis, and limited drug options, FK is the major cause of corneal blindness in China[3-5]. Therefore, studies of the mechanism underlying FK are significant in the development of a mechanism-based therapy to treat FK.

The cornea is the first line of the innate immune defense system that exerts antifungal infection functions. The innate immune system identifies pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors[6-10]. *Aspergillus fumigatus* (AF) can induce the production of inflammatory factors through nuclear factor κB (NF-κB) and the release of antimicrobial cytokines, such as thymic stromal lymphopoietin (TSLP)[11-13].

TSLP, a four-helix bundle that function as an interleukin (IL)-17-like cytokine, is mostly expressed by epithelial cells in the airways and ocular tissues[14]. TSLP receptors (TSLPRs) are present in T cells, B cells, and dendritic cells (DCs)[15-18]. Studies have shown that TSLP can promote the secretion of

Table 1 Fungal keratitis clinical score

Score	Corneal opacity range	Degree of surface irregularities of the cornea	Degree of corneal opacity
1	1%-25%	Cornea mildly irregular	Mild corneal opacity, pupil, and iris vessels clearly visible
2	26%-50%	Stromal edema, raised or recessed surface	Moderate corneal opacity, pupil profile visible
3	51%-75%	Matrix edema or sag obvious or posterior elastic layer bulging	Corneal uneven turbidity, pupil, and the organizational structure not visible
4	76%-100%	Corneal perforation	Corneal uniformly cloudy, pupil, and the organizational structure not visible

T helper type 2 (Th2) chemokines by activating DCs and the Th2 transcription factor GATA binding protein 3 (GATA-3), which recruits and differentiates $CD4^+$ T cells into Th2 cells, which secrete cytokines, such as IL-4 and IL-13, in allergic inflammatory diseases[16,19-20].

Th2 cells are mainly involved in the humoral immune response against parasitic diseases and can secrete the cytokines IL-4, IL-5, and IL-13. In normal circumstances, the numbers and functions of T cells in the body maintain a dynamic balance. Additionally, in allergic inflammation, the expression of IL-4 promotes TSLP production[21].

Previously, we found that TSLP interacted with toll-like receptors (TLRs) to regulate antifungal innate immunity and activate DCs in AF-induced FK[22-23]. However, the interactions between TSLP and $CD4^+$ T cells have not been examined, especially the interactions with IL-4. Therefore, we aimed to explore the roles of TSLP and $CD4^+$ T cells in AF-induced adaptive immunity.

In this study, we found that the levels of $CD4^+$ T cells [T helper type 1 (Th1), Th2, and T helper type 17 (Th17) cells] increased, TSLP promoted the transcription of the Th2 transcription factor GATA-3 and Th2 cytokines (IL-4 and IL-13), and IL-4 in turn stimulated the expression of TSLP in AF keratitis.

MATERIALS AND METHODS

Ethical Approval The experimental conditions and treatments were in line with the Association for Research Vision and Ophthalmology (ARVO) guidelines on animal use.

Preparation of AF Hyphae The AF strain CCTCC 93024 purchased from the China Centre for Type Culture Collection was cultured on Sabouraud dextrose agar on a shaking table at a speed of 200 rpm for 24h at room temperature (RT). The next day, hyphal conidia were collected and seeded into Sabouraud fluid medium at a concentration of 10^8 microorganisms per milliliter.

Animal Experiments Wild-type C57BL/6 mice (female, 6-8 weeks old) were provided by the Shandong University Experimental Animal Center.

Construction of Animal Models To establish a model of AF infection, mice were narcotized with 0.2 mL pentobarbital (10 mg/mL) by intraperitoneal injection, and the central corneas were scratched with three parallel 1-mm scratches made with a sharp needle. Then, the mice were inoculated with 5 μL of AF hyphae suspension at a concentration of 10^6 colony forming unit (CFU)/mL, and before the eyelid was sewn closed, a molded parafilm contact lens was placed. In addition, 5 μL of AF hyphae suspension were added into every eye through the palpebral fissure after the eyelid was sewn closed. After 24h, the lenses were removed. The blank control group of mice received no treatment. The negative control group of mice received phosphate-buffered saline (PBS).

Clinical Evaluation After the construction of animal models, the mice eyes were observed at 12h post infection (hpi), 24 hpi, 72 hpi, 5d post infection (dpi), and 7 dpi though a slit lamp (Carl Zeiss, Germany) to diagnosis infection, and the keratitis damage severity was described (Table 1)[24].

Preparation of TSLP-Specific siRNA TSLP-specific small interfering RNA (TSLP siRNA) genes were constructed by RiboBio Corporation (Guangdong Province, China). The sequences of the TSLP siRNAs were 1) 5'-CACAAGAAGUCCAAAACAUTT-3' and 5'-AUGUUUUGGACUUCUUGUGTT-3'; 2) 5'-GGGAGAAACUGCUGAGAUCTT-3' and 5'-GAUCUCAGCAGUUUCUCCCTT-3'; and 3) 5'-CCUCACAAAUUCUAAGAUUTT-3' and 5'-AAUCUUAGAAUUUGUGAGGT T-3'[25]. The final concentration of TSLP siRNA was 10 μmol/L in sterile water. Twenty-four hours before corneal infection, one eye was subconjunctivally injected with 5 μL of TSLP siRNA or scrambled siRNA. In addition, 5 μL of TSLP siRNA or scrambled siRNA was again injected at 72 hpi[26].

Recombinant Protein Pretreatment Recombinant TSLP (rTSLP), murine recombinant IFN-γ (rIFN-γ), murine recombinant IL-4 (rIL-4), murine recombinant IL-13 (rIL-13), murine recombinant IL-17A (rIL-17A), and murine recombinant IL-17F (rIL-17F) were designed by PeproTech Corporation (Rocky Hill, NJ, USA). Mice were subconjunctivally injected with 5 μL rTSLP, rIFN-γ, rIL-4, rIL-13, rIL-17A, or rIL-17F at 24h before infection. Additionally, 5 μL of the same recombinant protein was again injected at 72 hpi.

Histopathological Examination Corneal tissue samples were stained with hematoxylin and eosin (H&E). The pathological morphology was observed under a microscope, and images were acquired.

Table 2 List of forward and reverse primers used for qRT-PCR analysis

Gene	Forward	Reverse
IL-4	CCATATCCACGGATGCGACAA	TGGTGTTCTTCGTTGCTGTGA
IL-13	AAACTGCAGCAAGACCGTGA	CCACCGGGATACTGACAGAC
GATA-3	CTGGAGGAGGAACGCTAATG	AGATGTGGCTCAGGGATGAC
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA
TSLP	ACGGATGGGGCTAACTTACAA	AGTCCTCGATTTGCTCGAACT
IFN-γ	CCATCGGCTGACCTAGAGAAGA	GCAGTGTGTAGCGTTCATTGTC
T-bet	GTCTGGGAAGCTGAGAGTCG	AATGGGAACATTCGCCGTCC
IL-17A	TACCTCAACCGTTCCACGTC	TTTCCCTCCGCATTGACACA
IL-17F	CGTGAAACAGCCATGGTCAAGT	GCTGCTACCTCCCTCAGAAT
STAT-3	AGTTCTCGTCCACCACCAAG	CCAGCCATGTTTTCTTTGCAG
Foxp3	ATATGCGACCCCCTTTCACC	TGTGGCGGATGGCATTCTTC

Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction To obtain total RNA, the RNeasy Mini Kit (Qiagen, Germantown, USA) was used, and complementary deoxyribonucleic acid (cDNA) was produced with the cDNA Synthesis Kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. The quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) system contained 2 μL of cDNA, 10 μL of SYBR Green real-time polymerase chain reaction Master Mix (Toyobo Co.), 1 μL of each oligonucleotide primer, and 6 μL of sterile water. qRT-PCR was performed on a CFX96 instrument (Bio-Rad Company). The qRT-PCR protocol followed the manufacturer's protocol. The relative mRNA quantities of samples were calculated using the $2^{-\Delta\Delta CT}$ method, and all expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression data. All tests were repeated three times, with three duplicate samples each time. The polymerase chain reaction (PCR) primers used in these experiments are shown in Table 2.

Enzyme-Linked Immunosorbent Assay According to the manufacturer's instructions, to analyze TSLP, INF-γ, T-bet, IL-4, IL-13, GATA-3, IL-17A, IL-17F, signal transducer and activator of transcription (STAT)-3, and Forkhead box P3 (FoxP3) concentrations in corneal tissue, dissolution supernatants were evaluated with ELISA detection kits (Annuoruikang Co., Ltd., Beijing, China). By using standard curves, all concentrations were calculated, and all samples were tested in triplicate.

Immunofluorescence Staining Optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura, Torrance, CA, USA) was used to embed eyes and frozen at -80°C. Tissue specimens were cut into 5-μm-thick sections and placed on microscope slides. The microscope slides were fixed in 4% buffered paraformaldehyde for 15min at RT, and the specimens were blocked with 5% bovine serum albumin (BSA) for approximately 1h and incubated with primary antibodies (1:50) against IFN-γ (Servicebio, polyclonal, GB11107-1), IL-4 (Abcam, monoclonal, ab11524), IL-13 (Abcam, polyclonal, ab106732), and IL-17 (Abcam, polyclonal, ab79056) overnight at 4°C. The next day, the slides were incubated with secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or tetrametrylrhodarnine isothiocyante (TRITC; 1:200, Zhongshan Technologies, Beijing, China) for 1h at RT and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5min. An anti-fluorescence attenuator was added to the slides, and cover slips were applied. The slides were observed and captured by fluorescence microscopy at a magnification of 200× (Olympus fluorescent convert microscope; Olympus Optical, Tokyo, Japan).

Western Blotting Analysis Total protein was extracted from corneal samples using radio immunoprecipitation assay (RIPA) lysis buffer and phenylmethylsulfonyl fluoride (PMSF; 1:100), and a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was used for quantification. The extracted proteins were subjected to protein denaturation using loading buffer. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel. The proteins from the samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA, USA), and the membranes were blocked with 5% milk for 2h at RT. Then, the membranes were washed, cut, and incubated with primary antibodies, such as anti-β-actin (Abways, USA) and anti-TSLP (NB110-55234, Novus, USA) on a shaking table at 4°C overnight. The next day, the membranes were washed with Tris-buffered saline Tween (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4000, Beyotime) at RT for 2h. The membranes were detected with an enhanced chemiluminescence reagent (Millipore) and evaluated with an imager (Bio-Rad, Hercules, CA, USA).

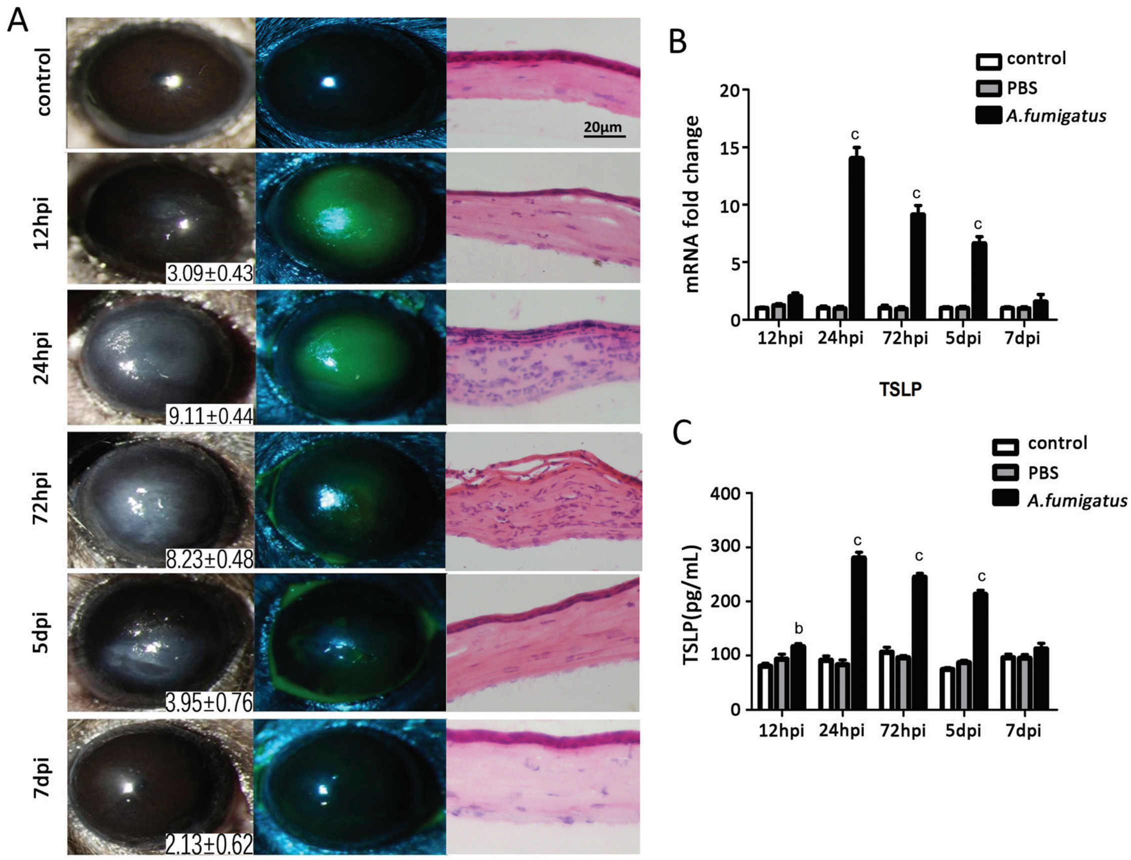

Figure 1 Expression of TSLP in AF-infected corneas and the evaluation of corneal FK C57BL/6 mice corneas were gently scratched with 26-gauge needles (1-mm long; 3 lines), followed by treatment with 1×10^8 CFU of AF or PBS, which was used as a control, or by no treatment. The severity of keratitis was evaluated by slit-lamp examination, clinical scoring, and histopathological examination staining (A) at 12, 24, 72 hpi, 5 and 7 dpi. Corneas were excised and processed for real-time PCR (B) and ELISA (C) to determine the levels of TSLP in AF-infected corneas. B: The fold increase in the level of TSLP mRNA in corneas after AF treatment was assessed. GAPDH was included as an internal control; error bars show standard deviations. C: The levels of TSLP in different AF treatment groups is shown ($^bP<0.01$, $^cP<0.001$).

Statistical Analysis All measurements and calculations are expressed as the mean±standard deviation. Statistical differences between two groups were analyzed by Student's *t* test; those among three or more groups were identified using one-way analysis of variance (ANOVA). Analysis of the variance in clinical scores was performed with a nonparametric Mann-Whitney *U* test. $P<0.05$ was considered statistically significant. All data were analyzed with GraphPad Prism 7 software.

RESULTS

Expression of TSLP Increased in *Aspergillus Fumigatus* Fungal Keratitis in Wild-Type C57BL/6 Mice We used a fungal conidia suspension to infect mice and observed the mice with a slit lamp at 12, 24, 72 hpi, 5 and 7 dpi to observe the disease progression in the corneas after AF infection, and clinical scores were obtained. Corneal ulcers worsened as infection progressed during the early stage and healed with a scar in the later stages. Fluorescein staining showed damage to the corneal epithelium. The different times at which clinical scores were calculated are shown in Figure 1A. Epithelial damage was most severe at 12 hpi, then the progression of infection was reduced, and the corneas were finally healed at 7 dpi. H&E staining showed the severity of cellular infiltration into the corneas with AF infection at 12-72 hpi, and this infiltration was resolved by 7 dpi.

To investigate the expression of TSLP in mice corneas with AF infection, we collected corneas at different times (12, 24, 72 hpi, 5 and 7 dpi). qRT-PCR and ELISA showed that the expression of TSLP was highest at 24h after fungal conidia suspension infection (Figure 1B and 1C).

Expression of Th1, Th2, and Th17 Cytokines Increased After AF Infection To clarify the expression patterns of Th1, Th2, Th17, and regulatory T cell (Treg) cytokines and transcription factors in corneas challenged with AF, we stimulated mice corneas with AF hyphae and collected corneal tissue samples at various periods (12, 24, 72 hpi, 5 and 7 dpi) for analysis by qRT-PCR and ELISA. IFN-γ expression was elevated approximately 6-fold (Figure 2A), T-bet expression approximately 2-fold, GATA-3 expression approximately 2-fold, and STAT-3 expression approximately 2-fold in

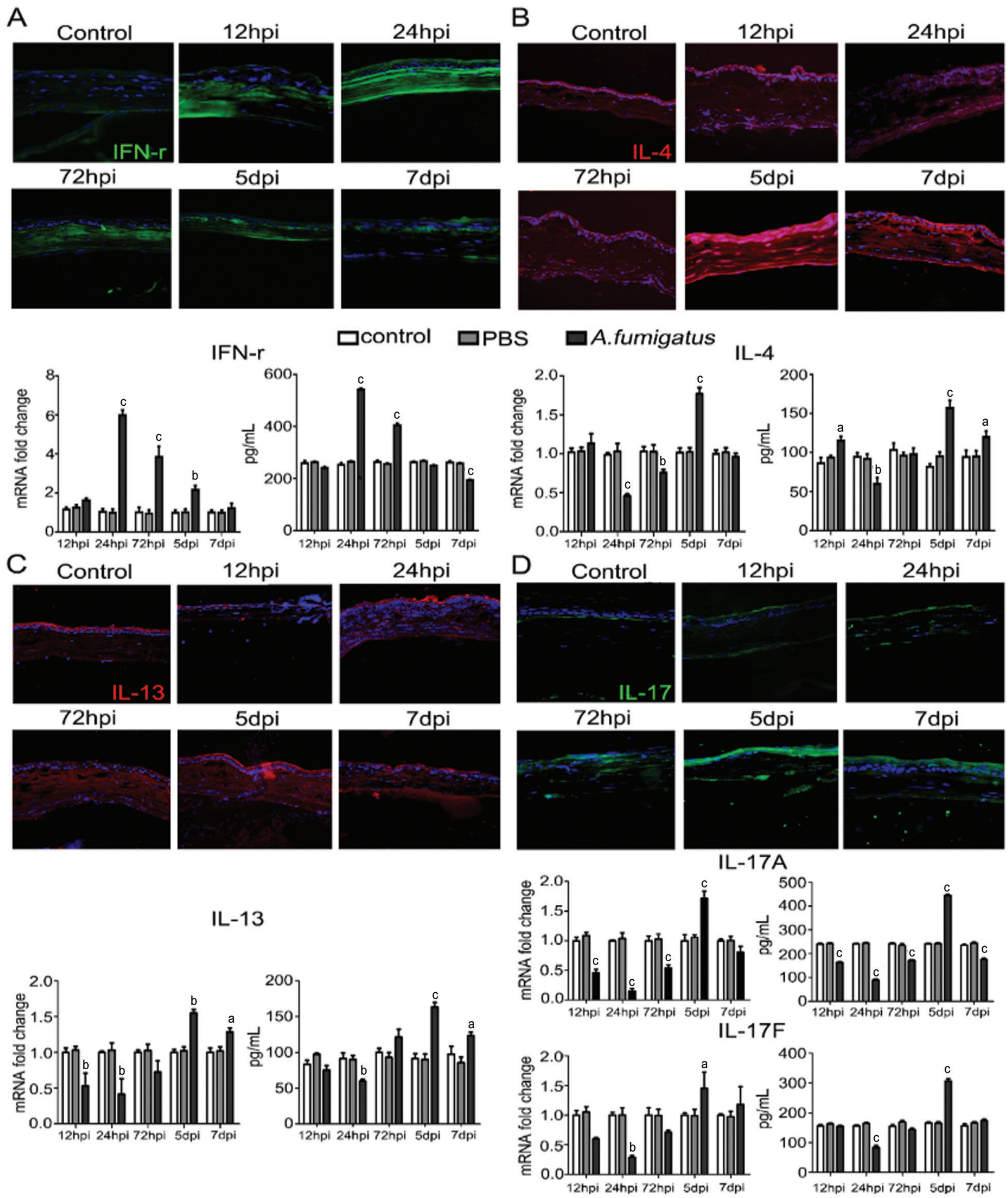

Figure 2 The expression of Th1, Th2, and Th17 cytokines was increased in AF-infected corneas Mice were infected with AF hyphae and euthanized at 12, 24, 72 hpi, 5 or 7 dpi showing the expression and locations of IFN-γ (A), IL-4 (B), IL-13 (C), and IL-17 (D). GAPDH was included as an internal control; mean±SD of three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$ compared with the control or negative control (with PBS).

infected samples compared with blank controls at 24 hpi, and IL-4 expression was increased approximately 1.7-fold (Figure 2B), IL-13 expression approximately 1.5-fold (Figure 2C), IL-17A expression approximately 1.7-fold (Figure 2D), and IL-17F expression approximately 1.5-fold (Figure 2D) at 5 dpi. Immunofluorescence staining revealed that IFN-γ, IL-4, IL-13, and IL-17 were abundant in the corneal stroma at 24 hpi or 5 dpi. There was no significant effect on the expression of the Treg transcription factor FoxP3 in FK.

TSLP Affected the Inflammatory Response in Fungal Keratitis To determine the function of TSLP in adaptive immunity against FK, TSLP siRNA or rTSLP was used to knockdown or upregulate TSLP expression, respectively. TSLP siRNA, a scrambled siRNA, rTSLP, or immunoglobulin G (IgG) was subconjunctivally injected into the eyes at 24h before infection with AF hyphae. qRT-PCR showed that TSLP expression was dramatically decreased or increased in the TSLP siRNA or rTSLP-pretreated corneas compared with untreated AF-infected corneas (Figure 3A). On this basis, the following results were obtained.

To confirm the role of TSLP in FK, TSLP siRNA or rTSLP was subconjunctivally injected into the eyes at 24h before infection with AF hyphae and again at 72 hpi. Corneal inflammation and cellular infiltration were more reduced at 24 hpi and 5 dpi in the TSLP siRNA-pretreated infected corneas than in untreated AF-infected corneas and increased with rTSLP pretreatment. Clinical scoring of FK was performed at 24 hpi (Figure 3B) and 5 dpi (Figure 3C). Compared to those of the AF group, the average clinical scores of the TSLP siRNA group were lower (9.11±0.44 *vs* 3.43±0.31 at 24 hpi and 3.95±0.76 *vs* 0.23±0.25 at 5 dpi), and those of the rTSLP group were higher (9.11±0.44 *vs* 11.67±0.58 at 24 hpi and 3.95±0.76 *vs* 6±1 at 5 dpi).

TSLP Promoted the Expression of Th2 Cytokines and Transcription Factors in Fungal Keratitis To determine whether TSLP induces Th2 cytokines and transcription factors in FK, we detected the expression of Th2 cytokines and transcription factors at 24 hpi or 5 dpi. qRT-PCR and ELISA showed that the expression of IL-4, IL-13 (Figure 4A and 4B), and GATA-3 was decreased approximately 2-fold in TSLP siRNA-pretreated corneas and increased approximately 1.5-fold in rTSLP-pretreated corneas. Immunofluorescence staining (Figure 4C and 4D) also showed that less IL-4 and IL-13 were secreted in the TSLP siRNA-pretreated group than in the control group and more were secreted in the rTSLP-pretreated group. To study the roles of TSLP in Th1 and Th17 adaptive immunity, corneas were evaluated at 24 hpi or 5 dpi. TSLP did not significantly affect the expression of IFN-γ, T-bet, IL-17A, IL-17F, or GATA-3 at either the gene or protein level (Figure 4E and 4F).

Up-regulated the Expression of IFN-γ, IL-4, IL-13, IL-17A, and IL-17F Expression by rIFN-γ, rIL-4, rIL-13, rIL-17A, and rIL-17F Proteins TSLP aggravates inflammatory responses in AF FK. Then, to probe whether IFN-γ, IL-4, IL-13, IL-17A, and IL-17F promote the expression of TSLP during FK, we used rIFN-γ, rIL-4, rIL-13, rIL-17A, and rIL-17F proteins to pretreat corneas. The eyes were subconjunctivally injected with rIFN-γ, rIL-4, rIL-13, rIL-17A, or rIL-17F 24h before infection. Slit-lamp examination, HE staining, and qRT-PCR showed that IFN-γ expression was increased approximately 3-fold at 24 hpi and 7-fold at 72 hpi (Figure 5A), IL-4 expression was increased approximately 2- and 15-fold, respectively (Figure 5B), IL-13 expression was increased

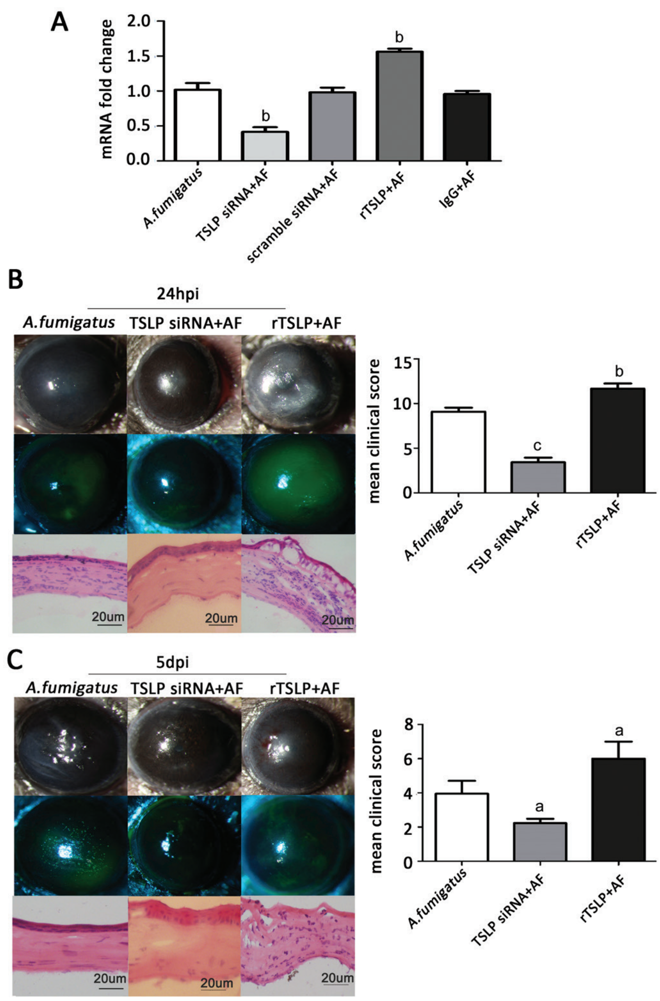

Figure 3 TSLP affected the inflammatory response in FK Mice eyes were subconjunctivally injected with TSLP siRNA or rTSLP and infected with a fungal conidia suspension after efficiently knocking down or upregulating TSLP expression, respectively. A: Mice eyes were subconjunctivally injected with TSLP siRNA, a scrambled siRNA, rTSLP, or IgG and infected with a fungal conidia suspension. qRT-PCR was performed to assess TSLP mRNA expression at 24 hpi. Slit-lamp examination was used to assess clinical manifestations, fluorescein staining was used to estimate the degree of epithelial injury, and H&E staining was used to evaluate the severity of cellular infiltration. According to the clinical manifestations, the mean clinical scores at 24 hpi (B) and 5 dpi (C) were determined. Experiments were performed in triplicate. GAPDH was included as an internal control; bars: mean±SD; $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$ compared with AF-infected corneas.

approximately 3- and 1.5-fold, respectively (Figure 5C), IL-17A expression was increased approximately 14-fold (Figure 5D), and IL-17F expression was increased approximately 1.5-fold (Figure 5E) after rIFN-γ, rIL-4, rIL-13, rIL-17A, or rIL-17F pretreatment compared with no treatment in AF-infected corneas.

IL-4 Enhanced the Expression of TSLP in AF Keratitis To analyze the role of IL-4 in inducing the secretion of TSLP by AF-induced cornea infection, the eyes were subconjunctivally

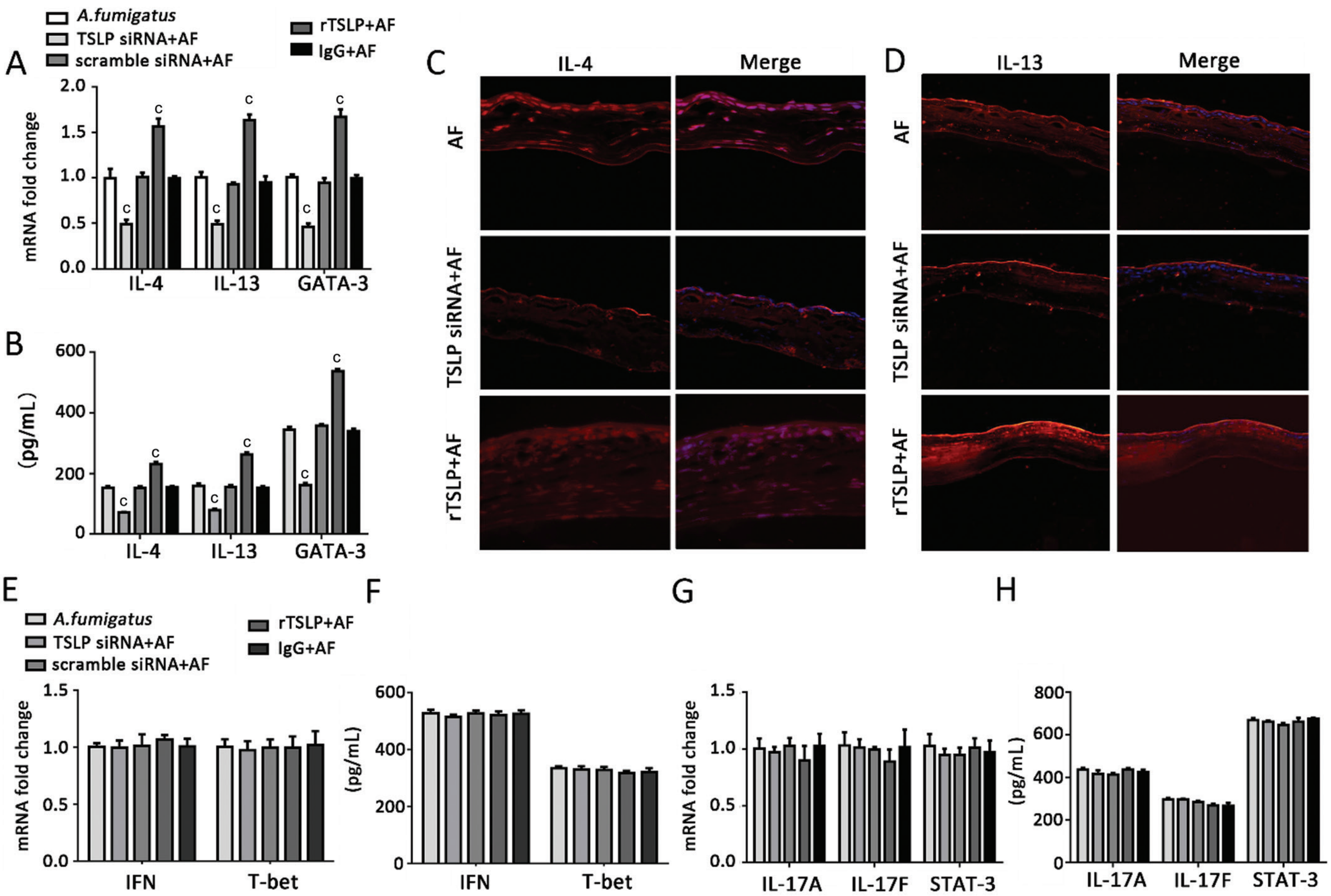

Figure 4 Thymic stromal lymphopoietin promoted the Th2 immunoreaction Mice eyes were subconjunctivally injected with TSLP siRNA, a scrambled siRNA, rTSLP, or IgG and infected with a fungal conidia suspension. qRT-PCR (A) and ELISA (B) were used to assess IL-4, IL-13, and GATA-3 mRNA and protein expression at 24 hpi or 5 dpi. IFN-γ, T-bet (E, F), IL-17A, IL-17F, and STAT-3 (G, H) mRNA and protein expression was assessed at 24 hpi or 5 dpi. Immunofluorescence staining shows the expression and locations of IL-4 (C) and IL-13 (D) at 5 dpi. Experiments were performed in triplicate. GAPDH was included as an internal control; bars: mean±SD; $^cP<0.001$ compared with AF-infected corneas.

injected with the rIFN-γ, rIL-4, rIL-13, rIL-17A, or rIL-17F protein 24h before infection with an AF suspension. qRT-PCR (Figure 6A), ELISA (Figure 6B), and Western blotting (Figure 6C) were used to evaluate the expression of TSLP in corneal tissue specimens at 24 and 72 hpi. The expression of TSLP was 7-fold and 10-fold higher in the rIL-4-pretreated group than in the untreated AF-infected group. rIFN-γ, rIL-13, rIL-17A, and rIL-17F had no significant effects on TSLP mRNA or protein expression in the cornea as determined by qRT-PCR and ELISA, respectively.

Building a Model of the Adaptive Immune Response in AF Keratitis Based upon our studies, we constructed a model of the relationship between TSLP and adaptive immune responses (Figure 7). TSLP is generated through the TLR2/MyD88/NF-κB pathway in AF-infected mice and leads to adaptive immune responses by DC-induced OX40. The body exhibits induced adaptive immune responses and $CD4^+$ T cells activated by antigen-presenting cells when the cornea is infected with AF. Additionally, exogenous IL-4 leads to increased expression of TSLP. Overall, TSLP-activated DCs induce Th2 inflammation in FK, and IL-4 promotes the expression of TSLP and constitutes a positive feedback pathway.

DISCUSSION

FK is a severe destructive corneal disease, but there is very little literature on its mechanism. Previous reports have shown that TSLP can affect the inflammatory status by regulating the immune status, but its role in FK has not been studied. Aspergillus keratitis is the most common type of FK so we used AF hyphae to infect the corneal epithelium of mice as a suitable model for exploring the mechanisms underlying innate and adaptive immunity during each stage of corneal infection. An initial objective of the study was to identify the role of TSLP in regulating Th2 adaptive immune responses, and IL-4 can positively regulate TSLP *via* feedback.

TSLP is produced by thymocytes and is a novel cytokine that is similar to IL-17. It is mainly produced by epithelial cells and is found in allergic diseases[27-28]. In allergic asthma, TSLP is highly expressed and has the ability to promote Th2 immune responses[16,29]. Additionally, studies have found that TSLP can promote Th2 responses in lung-specific and skin-

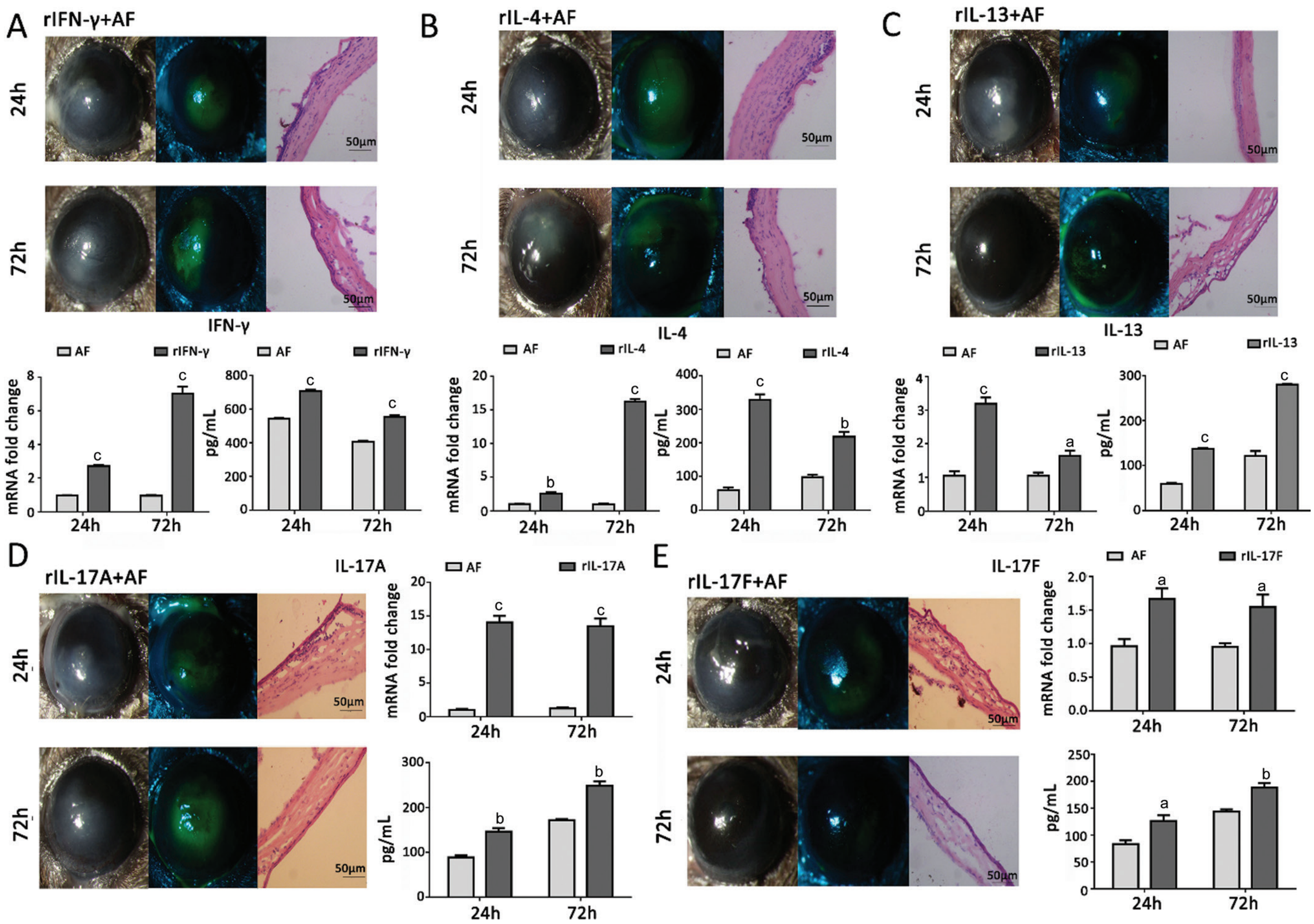

Figure 5 Efficient knockdown or upregulation of IFN-γ, IL-4, IL-13, IL-17A, and IL-17F expression by recombinant proteins Mice eyes were subconjunctivally injected with recombinant proteins and infected with a fungal conidia suspension. Slit-lamp examination, histopathological examination staining, qRT-PCR and ELISA were performed to assess the expression of IFN-γ, IL-4, IL-13, IL-17A and IL-17F at 24 and 72 hpi. Experiments were performed in triplicate. GAPDH was included as an internal control; bars: mean±SD; $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$ compared with AF-infected corneas.

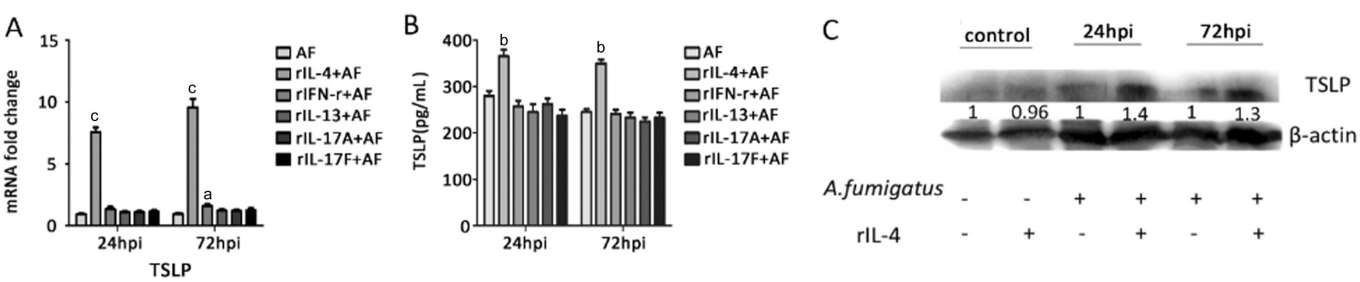

Figure 6 IL-4 enhanced the expression of thymic stromal lymphopoietin in AF hyphal infection Mice eyes were subconjunctivally injected with a recombinant IFN-γ, IL-4, IL-13, IL-17A, or IL-17F protein and infected with a fungal conidia suspension. qRT-PCR (A), ELISA (B) and Western blotting (C) were performed to assess TSLP expression at 24 and 72 hpi. Experiments were performed in triplicate. GAPDH or β-actin was included as an internal control; mean±SD; $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$ compared with AF-infected corneas.

specific allergic diseases and is an important initiating factor. TSLP is widely found in various cells and can induce DCs and promote $CD4^{+}$ T cell differentiation into Th2 cells[30-31]. Our initial research showed that the expression of TSLP was increased in AF-infected corneal epithelial cells[32-33]. In this study, we further investigated whether, how, and what TSLP joins in the adaptive immune response to corneal fungal infection *in vivo*. We showed that the expression of TSLP was increased at 24 hpi in corneal tissues infected with AF hyphae and induced Th2 immune responses. We also found that the inflammatory response in the cornea was reduced with TSLP siRNA. Our data confirmed that TSLP was released in corneas infected with AF hyphae, that TSLP siRNA treatment regulated the antifungal infection response in the cornea and

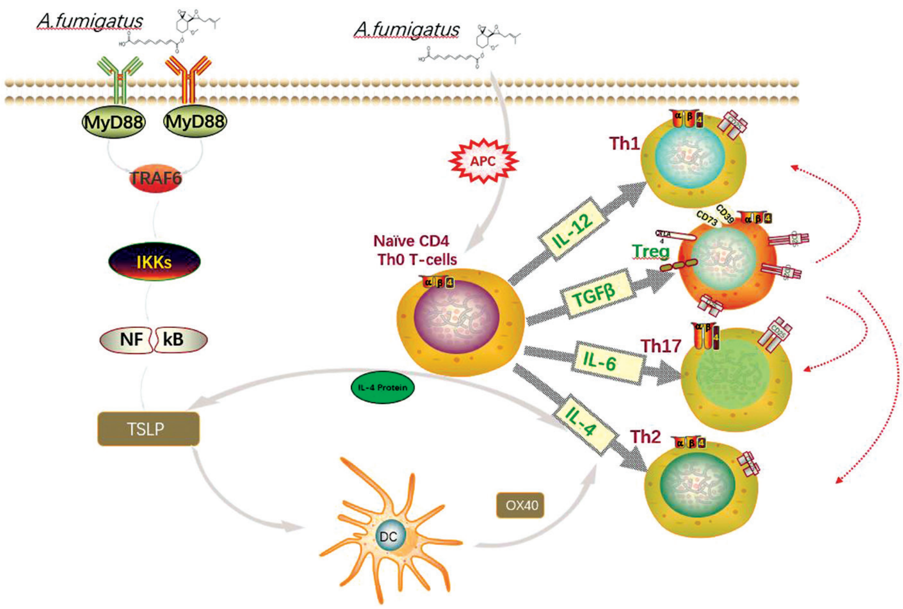

Figure 7 Role of TSLP in adaptive immune responses to FK AF leads to increased expression of TSLP *via* the TLR/MyD88/TSLP pathway. High levels of TSLP are recognized to induce Th2 immune responses by activating DCs. AF also leads to CD4$^+$ T cell activation by antigen-presenting cells. CD4$^+$ T cells are divided into Th1, Th2, Th17, and Tregs. If exogenous IL-4 protein exists, the expression of TSLP increases in FK.

that TSLP increased Th2-type cytokine levels in mice. These results imply that TSLP has the capability to induce Th2 cell infiltration into corneas with fungal infection.

T cells, specifically CD4$^+$ T lymphocytes, have been implicated in mediating stromal pathology[34]. A previous article showed that corneal opacity resulting from herpes simplex virus (HSV) infection represents a T cell-mediated inflammatory response[35]. A previous study showed that Th1 immunity can involve IFN-γ release to attenuate allergic asthma, whereas Th2 immune responses aggravate this disease by promoting the secretion of Th2 cytokines. The level of IL-17A and IL-17F in the airways of patients with severe asthma are increased[36], and the number of Th17 cells in the airways of patients with chronic obstructive pulmonary disease (COPD) is increased. Therefore, intervention with IL-17A and IL-7F is a good target for treatment of severe neutrophilic asthma and COPD[37]. Tregs express the transcription factor FoxP3 to maintain immune tolerance and restrict anti-inflammatory responses by secreting anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and IL-10[38]. Previous studies have shown that AF-specific Th2 cell differentiation and expansion can restrict the accumulation of Tregs[39-40]. Our data confirmed that in AF FK, Th1, Th2, and Th17 inflammation was induced at different times, that large amounts of IFN-γ, IL-4, IL-13, IL-17A and IL-17F were produced and that there was no significant change in the expression of the Treg transcription factor FoxP3.

One of the most important CD4$^+$ T cell subsets is the Th2 subset, which contributes to allergic inflammatory disorders, such as allergic asthma, allergic rhinitis, atopic dermatitis (AD), and anaphylaxis[41]. IL-4, the key cytokine for Th2 differentiation, together with IL-13 stimulates the secretion of IgE from B cells, promotes eosinophilic inflammation in the airway epithelium, and further upregulates the expression of TSLPR on CD4$^+$ T cells. Therefore, IL-4 together with TSLP is an inflammatory amplification loop[37]. IL-4, through IL-4Rα, activates the transcription factor STAT6, which, in turn, induces the expression of GATA3, which encodes a key Th2 cell-specific transcription factor. STAT6 and GATA3 act on the regulation and activated expression of Th2 cytokines[42-46]. Previous studies have also demonstrated that IL-4 upregulates TSLP mRNA expression in bronchial mast cells (MCs) in asthmatic subjects[46]. As shown in Figure 7, IL-4 treatment of AF-infected corneas shows a clear upregulation of TSLP expression at the mRNA and protein levels. Our results confirm that IL-4 has the ability to enhance the expression of TSLP in corneas with AF infection.

In conclusion, our findings imply that TSLP can facilitate Th2 cell recruitment and infiltration, induce Th2 adaptive immune responses, increase CD4$^+$ T cell numbers in FK, and in turn, increase IL-4 expression to enhance TSLP expression in FK. TSLP siRNA treatment regulates antifungal adaptive immunity, and rIL-4 treatment upregulates TSLP expression, suggesting a novel method for antifungal infection treatment of the cornea and prevention of injury caused by excessive inflammation.

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