Therapeutic Effects of STAT3 Inhibition on Experimental Murine Dry Eye

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PURPOSE. To investigate the therapeutic effects of targeting signal transducer and activator of transcription-3 (STAT3) activation on the ocular surface damage of dry eye in mice.

METHODS. Adult Balb/C and C57BL/6 mice with benzalkonium chloride (BAC) treatment, lacrimal gland excision, and meibomian gland dysfunction were used as dry eye models. The levels of phosphorylated STAT3 (p-STAT3) were detected with immunofluorescence staining and Western blotting. STAT3 inhibition was performed by topical application of STAT3 inhibitor S3I-201. Corneal epithelial barrier function, tear production, and conjunctival goblet cell density were quantified with fluorescein sodium staining, phenol red cotton test, and histochemical staining. The expressions of matrix metalloproteinase (MMP)-3/9, TUNEL, and inflammation cytokines were assessed with immunofluorescence staining, qPCR, and ELISA assays. The therapeutic effect of S3I-201 was further compared with the Janus kinase inhibitor tofacitinib and ruxolitinib.

RESULTS. Elevated levels of nuclear p-STAT3 were detected in the corneal and conjunctival epithelium of three dry eye models. Topical application of S3I-201 improved corneal epithelial barrier function, increased tear production and conjunctival goblet cell density in BAC-induced dry eye mice. Moreover, S3I-201 decreased the expression of MMP-3/9, suppressed the apoptosis of corneal and conjunctival epithelial cells, and reduced the levels of IL-1β, IL-6, IL-17A, and IFN-γ. Compared with tofacitinib and ruxolitinib, the STAT3 inhibitor S3I-201 showed superior improvement of tear production and inflammatory cytokine expression in lacrimal gland.

CONCLUSIONS. Elevated STAT3 activation is involved in the pathogenesis of dry eye, while targeting STAT3 effectively alleviates BAC-induced ocular surface damage.

Keywords: JAK-STAT3, dry eye, pathogenesis, ocular surface

Dry eye disease (DED) affects hundreds of millions of people worldwide with varying severity, commonly characterized by symptoms of ocular discomfort, visual disturbance, tear film instability, and ocular surface damage.1,2 According to the International Dry Eye Workshop II of Tear Film and Ocular Surface Society, the definition of dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles.3 Currently, several commercially available eye drops have been developed for treatment of DED, such as cyclosporin A, rebamipide, and diquafosol.4–7 Although the mechanism of DED remains not entirely understood, inflammation has been recognized as the key feature of DED development.

Signal transducer and activator of transcription 3 (STAT3) is a latent cytoplasmic protein that mediates extracellular signals into the nucleus upon stimulation with the ligands, such as IL-6, epidermal growth factor, and ciliary neurotrophic factor (CNTF). Cytokines of IL-6 family members binding to specific receptors leads to the activation of tyrosine kinases including Janus kinase (JAK) and subsequent phosphorylation of STAT3 (p-STAT3). The activation of STAT3 plays critical roles in multiple phases of wound healing, such as cell proliferation, migration, survival, and inflammation regulation.8–10 In the cornea, we had demonstrated that reduced STAT3 activation was involved in the delayed epithelial wound healing of diabetic mice, which was improved by exogenous CNTF application through STAT3 reactivation.11 However, persistent STAT3 activation was frequently observed in breast cancer, atherosclerosis, chronic inflammation,12–14 and psoriasis.15,16 In cornea, constitutive STAT3 activation resulted in squamous metaplasia, inflammatory infiltration, and neovascularization in mice.17 Moreover, therapeutic effect of tofacitinib, a selective inhibitor of JAK1/3, had been reported in a mouse corneal thermocautery model and in human DED.18–20

Given the crosstalk between STAT3 activation and inflammation, it is hypothesized that the aberrant activation of STAT3 may be involved in the pathogenesis of dry eye. Therefore, the phosphorylated STAT3 levels of corneal and conjunctival epithelium in three dry eye models were detected first. The therapeutic effects of STAT3 inhibitor were further evaluated and compared with JAK1/3 inhibitor tofacitinib and JAK2/3
ruxolitinib on the alleviation of ocular surface damage in benzalkonium chloride-induced dry eye mice.

MATERIALS AND METHODS

Animal Models
Adult C57BL/6 and Balb/C mice (6- to 8-weeks old; Institute of Laboratory Animal Sciences, CAMS & PUMC, Beijing, China) were maintained in the animal center of Shandong Eye Institute. All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the experimental protocol was approved by the Ethics Committee of Shandong Eye Institute. The dry eye mouse model with benzalkonium chloride (BAC) treatment, lacrimal gland excision (LGE), and meibomian gland dysfunction (Tabby mice) were used to examine the phosphorylation level of STAT3 in ocular surface epithelium. For BAC-induced dry eye, 0.2% BAC was applied topically in Balb/C mice for 10 days as previous description, with 0.2% PBS as vehicle control. For LGE-induced dry eye, the extraorbital lacrimal gland was completely removed in C57BL/6 mice and was monitored after 4 days as previous description, with the sham mice as control. The ectodysplasin A mutant Tabby mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) with wide-type C57BL/6 mice as control, the mice represents the dry eye caused by meibomian gland dysfunction as our previous study. Only the right eye of each animal was treated and used for all experiments.

Animal Treatment
The mice with BAC treatment for 10 days were randomly divided into six groups: blank control group, vehicle control group, STAT3 inhibitor S3I-201 (Selleck Chemicals, Houston, TX, USA) group, STAT3 inhibitor peptide (Merck-Millipore, Darmstadt, Germany) group, JAK inhibitor tofacitinib (Selleck Chemicals) group, and JAK inhibitor ruxolitinib (Selleck Chemicals) group. The mice without BAC treatment were used as healthy controls. The vehicle control group was administered with 5 μL PBS as solvent control for STAT3 inhibitor peptide or 0.2% dimethyl sulfoxide (DMSO) as solvent control for STAT3 inhibitor S3I-201, tofacitinib, and ruxolitinib. The mice were administered with 5 μL of peptide (0.1%), S3I-201 (0.004%), tofacitinib (0.003%), and ruxolitinib (0.003%) three times per day, accompanied with the continuous BAC application for another 10 days. The mice were quantified with corneal fluorescein staining grade and tear production. Mouse eyes after euthanization were harvested for real-time qPCR, immunofluorescence staining, ELISA, and Western blotting analysis.

Corneal Fluorescein Staining
Fluorescein staining was used to assess the barrier function damage of corneal epithelium. The staining was performed by
instilling 0.25% fluorescein sodium (Jingming, Tianjin, China) and visualized under slit lamp (BQ900; Haag-Streit, Bern, Switzerland). For grading of the fluorescein staining, the cornea was separated into four quadrants, staining score was evaluated individually as previous description, positive fluorescein plaque, 4; very dense dot-like fluorescence, 3; dense dot-like pattern, 2; slightly fluorescence resembling sparse dots, 1; no fluorescence, 0. The scores were summed and analyzed for each eye (minimum = 0, and maximum = 16) with 10 eyes per group. All fluorescein staining was blindly scored by single observer (LW).

**Tear Production**

Tear production of 10 eyes per group was examined using the phenol red thread (Jingming) test as previously described. In brief, the thread was placed on the palpebral conjunctiva of the lower eyelid at one-third of the distance from the lateral canthus for 15 seconds. The length of wet portion was measured in millimeters.

**Immunofluorescence Staining**

Tissue cryosections of five eyes per group were fixed with ice methanol for 10 minutes at −20°C, blocked with 5% BSA.
Sigma-Aldrich Corp., St. Louis, MO, USA), and incubated with the primary antibodies, including rabbit anti-p-STAT3 (ab76315, 1:100; Abcam, Cambridge, MA, USA), rabbit anti-matrix metalloproteinase (MMP)-3 (ab73955, 1:100; Abcam), and rabbit anti-MMP-9 (ab38898, 1:200; Abcam) at 4°C overnight. The samples were subsequently incubated with Alexa Fluor 488-labeled donkey anti-rabbit IgG antibody (A21206, 1:300; Invitrogen, Carlsbad, CA, USA) for 1 hour at 37°C and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corp.). The staining was observed and captured with an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan).

Western Blotting

Corneal or conjunctival tissues of five eyes were collected and pooled as one sample, and three samples were used in each group. Nuclear and cytoplasmic protein were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo, Rockford, IL, USA). The protein samples (20 μg each sample) were separated on SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck-Millipore). After blocking in 5% BSA for 1 hour, the blots were incubated with rabbit anti-p-STAT3 (ab76315, 1:2000; Abcam), rabbit anti-STAT3 (ab68153, 1:2000; Abcam) overnight at 4°C, and HRP-conjugated goat anti-rabbit IgG at room temperature (ZB-2301, 1:3000; Zsbio, Beijing, China). The bands were visualized by enzyme-linked chemiluminescence using the ECL kit (Chemicon, Temecula, CA, USA), and the image intensity was calculated with ImageJ Software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).
Periodic Acid-Schiff (PAS) Staining

Mouse eyeballs were fixed in 10% formalin and embedded in paraffin. The sections were stained with PAS staining kit (MXB, Fuzhou, China). Representative images of the conjunctiva were captured with a light microscope (Nikon). The number of goblet cells in the conjunctiva were counted from five eyes per group.

Real-Time qPCR

Corneal or conjunctival tissues of five eyes were collected and pooled as one sample, and three samples were used in each group. For the detection of lacrimal gland inflammatory cytokines, extraorbital lacrimal glands were excised, five samples per group were tested, and one sample consisted of pooled glands from the same animal. Total RNA was extracted using the Nucleospin RNA Kits (BD Biosciences, Palo Alto, CA, USA). Isolated RNA was reverse transcribed into cDNA using the Primescript First-Strand cDNA Synthesis kit (TaKaRa, Dalian, China). Quantitative PCR was performed using SYBR Green reagents (Applied Biosystems, Foster City, CA, USA). The primer sequences are summarized in the Table. The results were analyzed with the Sequence Detection System software (Applied Biosystems) using \( \beta\)-actin as the reference gene.

In Situ TUNEL Staining

To detect the apoptosis of corneal and conjunctival epithelium, in situ TUNEL assay (Roche Diagnostics GmbH, Mannheim, Germany) was performed on frozen sections of eyes according to the manufacturer’s instructions. Sections were counterstained with DAPI and photographs were captured with the fluorescence microscopy (Nikon). The TUNEL-positive cells in the corneal epithelium and conjunctiva were counted from three samples per group.

ELISA Analysis

Corneal or conjunctival tissues of five eyes were collected and pooled as one sample, and three samples were used in each group. Total protein was extracted with cold RIPA buffer (Beyotime, Shanghai, China), the concentrations were calculated with the proteins measured by using the BCA kit (Beyotime). Sixty and 10 \( \mu \)g of protein was used for the quantification of IL-6 (R&D, San Diego, CA, USA) and IFN-\( \gamma \)

Table. Mouse Primer Sequences Used for qPCR

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<th>Genes</th>
<th>Forward Primer</th>
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Figure 4. Effects of STAT3 inhibition on BAC-induced MMP-3 and -9 expression. (A) Representative immunofluorescence staining of MMP-3 and -9 in corneal epithelium after 10 days of S3I-201 treatment, with DMSO as vehicle control. (B, C) Comparison of MMP-3 and MMP-9 mRNA transcript levels in corneal epithelium. Data were shown as mean ± SD. * \( P < 0.05 \).
(R&D) according to the manufacturer's instructions of ELISA Kit. The optical absorbance was measured at 450 nm with the microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

Results in this present study were performed at least three independent experiments and presented as mean ± SD. Student’s t-test was conducted for statistical comparison between two individual groups using SPSS 17.0 software (SPSS, Chicago, IL, USA). Statistical significances were accepted as *\(P<0.05\).

RESULTS

Mouse Dry Eye Models

The mice treated with BAC, LGE, and Tabby mice were used as dry eye models. Fluorescein staining showed apparent positive staining in the mouse cornea of three models (Figs. 1A–C). According to the assessment, the fluorescein score increased by 8.63-, 6.25-, and 6-fold, while tear production reduced to 44%, 64%, and 41% in BAC-induced mice (Fig. 1A), Tabby mice (Fig. 1B), and LGE mice (Fig. 1C) when compared with control mice, respectively. The results confirmed the presence of damaged epithelial barrier function and reduced tear production in the mouse dry eye models.

Elevated p-STAT3 in the Ocular Surface Epithelium of Dry Eye Mice

The phosphorylation levels of STAT3 in ocular surface epithelium were examined by immunofluorescence staining and Western blotting. Immunofluorescence staining showed the apparent nuclear p-STAT3 translocation in the corneal and conjunctival epithelium of dry eye mice, while weakly expressed in the cytoplasm in the control mice (Figs. 2A–C).

To quantify the levels of nuclear translocated p-STAT3, the nuclear and cytoplasmic proteins of mouse tissues were extracted and assessed by Western blotting (Figs. 2D–F). Quantitative results showed that the nuclear levels of corneal and conjunctival p-STAT3/STAT3 were elevated with 1.45- and 1.64-fold in BAC-induced mice, 1.2- and 3.3-fold in Tabby mice, and 1.78- and 1.3-fold in LGE mice when compared with control mice (Figs. 2G–I). The results suggest that the levels of activated STAT3 were elevated in the ocular surface epithelium of three dry eye mice.

STAT3 Inhibition Attenuates the Severity of Ocular Surface Damage

To examine whether the inhibition of STAT3 activation can attenuate the severity of dry eye, STAT3 inhibitor S3I-201 and peptide were applied topically for 10 days in the BAC-induced dry eye mice (Fig. 3A). Fluorescein staining showed positive staining in the BAC-induced blank group and DMSO or PBS vehicle control groups, while STAT3 inhibitor S3I-201 or peptide treatment improved the epithelial barrier function when compared with the vehicle control (Fig. 3B). Fluorescein scores of S3I-201- and peptide-treated mice reduced to 33% and 41% of the vehicle control levels (Fig. 3C), respectively, and tear production showed 1.45- and 1.44-fold increasing (Fig. 3D). Meanwhile, PAS staining (Fig. 3E) showed that conjunctival goblet cell density was significantly increased after the treatment with S3I-201 and peptide (Fig. 3F). Taken together, topical application of STAT3 inhibitor attenuated epithelial barrier function damage, increased tear production, and goblet cell density in BAC-induced dry eye mice.

STAT3 Inhibition Decreases MMP-3 and -9 Expression in Corneal Epithelium

Increased production of MMPs, particularly MMP-3 and -9, has been reported to be associated with the disruption of corneal
epithelial barrier in dry eye. Therefore, the expression of MMP-3 and -9 was examined in corneal epithelium using immunofluorescence staining and qRT-PCR. Immunofluorescence staining showed a strong fluorescence density in the corneal epithelium of BAC-induced mice and vehicle DMSO treatment, while it was alleviated after S3I-201 treatment (Fig. 4A). Quantitative analysis further confirmed that the increased MMP-3 and -9 expression in BAC-induced mice was significantly decreased and approached the normal levels after topical S3I-201 application (Figs. 4B, 4C), suggesting the improvement of corneal epithelial barrier function by STAT3 inhibition.

STAT3 Inhibition Suppresses Apoptosis of Ocular Surface Epithelium

Corneal and conjunctival epithelial cell apoptosis has been reported in the BAC-induced dry eye model. Therefore, the changes in epithelial cell apoptosis were further examined when treated with STAT3 inhibitor S3I-201 by in situ TUNEL staining. As shown in Figure 5A, TUNEL-positive cells were found in the corneal and conjunctival epithelium in both BAC-induced blank and vehicle control group, while very few apoptotic cells were detected after 10 days of topical S3I-201 application. Quantitative analysis confirmed that S3I-201 significantly decreased the number of apoptotic cells in both corneal (Fig. 5B) and conjunctival epithelium (Fig. 5C).

STAT3 Inhibition Attenuates Dry Eye–Related Inflammation

Dry eye is characterized by increased proinflammatory cytokine levels of ocular surface, including IL-1β, IL-6, IL-17A, and IFN-γ. To evaluate the anti-inflammatory effects of STAT3 inhibition in dry eye mice, corneal and conjunctival samples were collected. Quantitative PCR showed that BAC treatment resulted in elevated mRNA transcripts of IL-1β, IL-6, IL-17A, and IFN-γ in both corneal and conjunctival tissues (Fig. 6A), while topical S3I-201 application significantly reduced their expressions when compared with the vehicle control group (Fig. 6A). The reduced protein levels of IL-6 and IFN-γ in cornea and conjunctiva of S3I-201–treated mice were further confirmed with ELISA assay (Fig. 6B).

Comparison of STAT3 and JAK Inhibition on Dry Eye Mice

The JAK1/3 inhibitor tofacitinib has been used to treat dry eye in animal model and clinical trials with remarkable improvement. To compare the therapeutic effects of JAK inhibition and STAT3 inhibition, S3I-201 and two JAK inhibitors, tofacitinib and ruxolitinib, were applied for 10 days in BAC-induced dry eye mice. Fluorescein staining showed that three inhibitors improved the epithelial barrier function when
FIGURE 7. Comparisons of STAT3 inhibition and JAK inhibition on BAC-induced ocular surface damage. (A) Representative fluorescein staining of BAC-induced dry eye mice after the treatment of STAT3 inhibitor S3I-201 or JAK inhibitor tofacitinib and ruxolitinib. (B) Fluorescein staining score analysis. (C) Tear production analysis. (D) Comparisons of mRNA transcript levels of proinflammatory factor in cornea. (E) Comparisons of mRNA transcript levels of proinflammatory factor in conjunctiva. (F) Comparisons of mRNA transcript levels of proinflammatory factor in lacrimal gland. Data were shown as mean ± SD. *P < 0.05, ns, means no significance.
compared with vehicle control group (Fig. 7A). Fluorescein score reduced to 23%, 45%, and 32% of vehicle control levels, without significant difference among three treatment groups (Fig. 7B). However, tear production increased by 2.54-, 1.74-, and 1.81-fold, with a more significant improvement for S3I-201 treatment than that of tofacitinib or ruxolitinib treatment (Fig. 7C).

Furthermore, the expressions of inflammatory cytokines of cornea, conjunctiva, and lacrimal gland were analyzed by quantitative PCR. In cornea, all three inhibitors reduced the expression of IL-1β, IL-17A, and IFN-γ. However, only the treatment of S3I-201, but not two JAK inhibitors, significantly decreased the IL-6 expression (Fig. 7D). In conjunctiva, all three inhibitors significantly attenuated the upregulated expression of IL-1β, IL-6, IL-17A, and IFN-γ when compared with vehicle control, without significant difference among three treatment groups (Fig. 7E). In lacrimal gland, only S2I-201 treatment significantly reduced all the expressions of IL-1β, IL-6, and IFN-γ. Tofacitinib treatment showed no reduction when compared with vehicle control, while ruxolitinib treatment only reduced the expression of IFN-γ, not IL-1β and IL-6 (Fig. 7F). Taken together, compared with two JAK inhibitors, STAT3 inhibitor showed similar attenuation of epithelial barrier function damage and conjunctiva inflammation, but superior in the improvement of tear production and lacrimal gland inflammation in BAC-induced dry eye mice.

**DISCUSSION**

Multiple signaling pathways, such as the stress-activated mitogen activated protein kinases, c-Jun N-terminal kinase, nuclear factor-kB, and oxidative stress, have been reported to be involved in the pathogenesis of dry eye. 51–54 In the present study, the presence of aberrant STAT3 activation in the ocular epithelium of three dry eye mouse models was confirmed. Moreover, topical application of STAT3 inhibitor alleviated dry eye–related symptoms, including epithelial barrier function, tear production, conjunctival goblet cell density, epithelial cell apoptosis, and ocular inflammation. In addition, compared with JAK inhibitors tofacitinib and ruxolitinib, the STAT3 inhibitor exhibited a superior improvement in tear production and lacrimal gland inflammation in BAC-induced dry eye mice.

Abnormal STAT3 activation had been reported in the epidermal keratinocytes of human psoriatic lesions. Transgenic mice with keratinocytes expressing a constitutively active STAT3 developed skin lesions that closely resemble psoriasis. 15 Similarly, sporadically expression of p-STAT3 had been described in the keratinized cornea of patients with severe ocular surface disease. Constitutive activated STAT3 in mice lead to chronic corneal inflammation and epithelial squamous metaplasia. 17 More importantly, enhanced STAT3 activation was also found in the salivary glands of Sjögren’s syndrome patients, 35 which suggests that targeting STAT3 inhibition may be effective for dry eye disease. Consistently, here, direct evidence is provided to confirm the abnormal STAT3 activation in the ocular surface epithelium in three dry eye mouse models, including BAC treatment, LGE, and Tabby mice. These results suggest that the abnormal STAT3 activation may be a common pathogenic mechanism of dry eye, although further experiments should be conducted in clinical samples. IL-6 is a classical ligand of JAK-STAT3 pathway through the binding to IL-6 receptor on the gp130 complex. 18 Previous studies had indicated the increased corneal IL-6 levels in a BAC-induced dry eye rabbit model and scopolamine-induced mouse dry eye model. 20,25 Similarly, an increased IL-6 expression was detected in both cornea and conjunctiva of BAC-induced dry eye mice. Therefore, the abnormal STAT3 activation may be caused by the increased IL-6 expression in the ocular surface of dry eye.

Activated JAK-STAT signaling plays an important role in the regulation of inflammation and represents a potential target for inflammatory diseases. 15,14 Recently, the inhibition of JAK family (including JAK1/2/5) and inhibition of STAT3 have been used as for the treatment of several autoimmune and inflammatory diseases, such as rheumatoid arthritis, 38 psoriasis, 39 and transplant rejection. 39 Topical JAK1/3 inhibitor, tofacitinib, has been reported to treat DED in animal models and clinical trials with an improvement in ocular surface inflammation, tear production, and epithelial barrier function. 19,20,30 Comparatively, it was found in this study that tofacitinib and ruxolitinib showed similar effects in the attenuation of fluorescein staining scores, but less effects in the improvement of tear production when compared with the STAT3 inhibitor in BAC-induced dry eye mice. Interestingly, to explore why STAT3 inhibitor had better influence on the improvement of superior tear production in DED syndrome, the functions of the three above-mentioned inhibitors were further examined in the expressional control of inflammatory cytokines in cornea, conjunctiva, and lacrimal gland, respectively. In the cornea and conjunctiva, the treatments with three inhibitors induced a comparable reduction of inflammatory cytokine expression. However, in lacrimal gland, only STAT3 inhibitor showed a significant suppression of all tested inflammatory factors, including IL-1β, IL-6, and IFN-γ, while tofacitinib exhibited no reduction and ruxolitinib only reduced the expression of IFN-γ, but not of IL-1β or IL-6. Therefore, the different anti-inflammatory effects in lacrimal gland may contribute to the different tear production between JAK and STAT3 inhibitions. In addition, tear production may be a gatekeeper for dry eye, because the clinical aspect of the fluorescein score is similar.

In conclusion, the results confirm the presence of aberrant STAT3 activation in the ocular epithelium of three dry eye models, while targeting STAT3 activation attenuates the ocular surface damage, which may represent a potential therapeutic approach to controlling DED.

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