Topical TSG-6 Administration Protects the Ocular Surface in Two Mouse Models of Inflammation-Related Dry Eye

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Purpose. To investigate the therapeutic potential of TNF-α stimulated gene/protein (TSG)-6 in two mouse models of inflammation-mediated dry eye syndrome (DES).

Methods. We created inflammation-mediated DES in mice by injecting concanavalin A (ConA; 10 mg/mL) into intraorbital and extraorbital lacrimal glands. Recombinant TSG-6 (1 μg in phosphate-buffered solution [PBS]) or the same volume of PBS was administered topically to eyes of the mice four times a day (QID) for 1 week. In parallel experiments, we topically applied TSG-6 (1 μg) or PBS QID to eyes of 12-week-old NOD.B10.H2^b mice, a model for primary Sjögren's syndrome. Seven days later, tear production was measured, and the corneal surface was observed for epithelial defects. The number of goblet cells was evaluated in the forniceal conjunctiva. The levels of proinflammatory cytokines were analyzed in the cornea, conjunctiva, and lacrimal glands. Also, in vitro experiments were performed using cultures of corneal epithelial cells (CECs) to test the effects of TSG-6 on cell proliferation and migration.

Results. Topical TSG-6 administration improved tear production and reduced corneal epithelial defects both in ConA-injected mice and NOD.B10.H2^b mice. The conjunctival goblet cell density was higher in TSG-6–treated eyes than in PBS-treated eyes. The expression of proinflammatory cytokines in the cornea, conjunctiva, and intraorbital gland was repressed by TSG-6, while the levels of proinflammatory cytokines in the extraorbital gland were not changed. In vitro experiments revealed that TSG-6 promoted the migration of CECs, but did not affect the proliferation.

Conclusions. Topical TSG-6 protected the ocular surface by suppressing inflammation and promoting corneal epithelial wound healing.

Keywords: cornea, dry eye syndrome, inflammation, TNF-α stimulated gene/protein-6, TSG-6

Dry eye syndrome (DES) is one of the most common ocular diseases and causes symptoms of dryness, irritation, and visual disturbance in 7% to 33% of the population worldwide.[1] One of the most severe forms of DES is associated with Sjögren's syndrome (SjS), an autoimmune disorder characterized by inflammatory destruction of lacrimal glands in the absence or presence of other rheumatic diseases.[2] Although the etiology of DES is not fully understood, a plethora of studies indicate that inflammation of lacrimal glands and the ocular surface plays a key role in the pathogenesis of DES and SjS.[3–5] Currently, several anti-inflammatory agents including corticosteroid and cyclosporine are used to treat ocular symptoms in DES and SjS.[6] However, corticosteroid eye drops are not suitable for long-term use because of adverse effects such as cataract and glaucoma. Cyclosporine ophthalmic emulsion produces noncompliance in 17% to 22% of patients because it contains hydrophobic vehicles that produce ocular stinging sensation and redness.[7,8]

Our group has previously reported that a periorbital injection of mesenchymal stem/stromal cells (MSCs) protected the ocular surface in mice with DES by suppressing inflammation in lacrimal glands and the ocular surface.[9] The anti-inflammatory mechanism of MSCs is not clear; however, recent data suggest that MSCs improve mouse models for myocardial infarction,[10] corneal injury,[11] or peritonitis[12] by secreting a multifunctional anti-inflammatory protein, TNFα-stimulated gene/protein 6 (TSG-6),[13–15] and TSG-6 can be used as a biomarker to predict the anti-inflammatory efficacy of MSCs.[16] Also, direct application of recombinant TSG-6 protein has been shown to have therapeutic effects in various animal models of diseases in the eye and other tissues.[17–21]

In this study, we evaluated the therapeutic effects of topical TSG-6 administration in DES using two mouse models of dry eye: (1) mice with inflammation-related DES induced by an intraorbital and extraorbital gland injection of concanavalin A (ConA), which is a T cell mitogen, and (2) NOD.B10.H2^b mice that spontaneously develop features of primary SjS without diabetes or other rheumatologic diseases.[22,23]

Materials and Methods

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital Biomedical Research Institute (IACUC No.12-0360 and

13-0162). Animals were treated in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female BALB/c and C57BL/6 (B6) mice were purchased from Orient Bio Inc. (Seongnam, Korea), and breeding pairs of NOD.B10.H2^b mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were bred and maintained at the Mouse Facility at Biomedical Research Institute of Seoul National University Hospital under a specific pathogen-free environment with free access to water and food.

Animal Model and Treatment

Two types of murine dry eye models were used for the present study.

First, DES was created in 7-week-old BALB/c mice by injecting ConA into lacrimal glands as our group previously reported.[9] Briefly, under anesthesia with an intraperitoneal injection of zolazepam-tiletamine (Zoletil, Virbac, Carros, France), 20 μL ConA (Sigma-Aldrich Corp., St. Louis, MO, USA), that was diluted in phosphate-buffered solution (PBS) at the concentration of 10 mg/mL, was injected into both intraorbital and extraorbital lacrimal glands of BALB/c mice using a Hamilton syringe with a 33-gauge needle (Hamilton, Reno, NV, USA; Fig. 1A). The same volume of PBS was injected into the glands as a negative control.

Second, 12-week-old NOD.B10.H2^b mice were used in this study as another model of DES. The NOD.B10.H2^b strain exhibits a SjS-like phenotype with inflammatory infiltration in the lacrimal glands without developing diabetes because the diabetogenic major histocompatibility complex (MHC) locus has been replaced with a diabetes resistance gene locus. Therefore, NOD.B10.H2^b mice serve as a model for studying primary SjS.[22,23] In experiments using NOD.B10.H2^b mice, 12-week-old B6 mice were used as controls.

For treatment, recombinant human (rh) TSG-6 (1 μg/10 μL PBS; R&D Systems, Minneapolis, MN, USA) was topically instilled four times a day (QID) to the ocular surface of the mice for 7 days. The same volume of PBS was instilled QID for 7 days in control mice.

Phenol Red Thread Test

To evaluate tear production, phenol red-impregnated cotton threads (FCI Ophthalmics, Pembrooke, MA, USA) were applied into the lateral canthus of mice for 60 seconds, and wetting of the thread was measured in millimeters.

Corneal Dye Staining

To evaluate the degree of corneal epithelial defects, one drop of 3% Lissamine Green B (Sigma-Aldrich Corp.) was administered to the inferior lateral conjunctival sac. The corneal surface was observed, and dye staining of the cornea was scored in a blinded manner as follows: score 0 for no punctuate staining; score 1 when less than one third of the cornea was stained; score 2 when two thirds or less was stained; and score 3 when more than two thirds was stained.[24]

Periodic Acid Schiff (PAS) Staining

The whole eyeball including the superior and inferior forniceal conjunctiva was excised and fixed in formalin. The tissues were sliced into 4-μm-thick sections through superior and inferior conjunctival fornices, and subjected to PAS staining. The number of PAS-stained cells was counted per 100 μm in four different sections of the eye from the same animal, and the average count was determined in each eye as the goblet cell density.

Real-Time RT-PCR

The intra- and extraorbital lacrimal glands and the ocular surface including the cornea and conjunctiva were extracted, lysed in RNA isolation reagent (RNA Bee, Tel-Test, Friendswood, TX, USA), and homogenized using a sonicator (Ultrasonic Processor, Cole Parmer Instruments, Vernon Hills, IL, USA). Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA) and quantified using a NanoDrop spectrophotometer. Equal amounts of RNA (3 μg) from each sample were used to synthesize double-stranded cDNA by reverse transcription (SuperScript III; Invitrogen/Life Technologies, Carlsbad, CA, USA). The cDNA was analyzed by real-time PCR (ABI 7500 Real Time PCR System, Applied Biosystems, Carlsbad, CA, USA) for the following cytokines: IL-2 (Taqman Gene Expression Assays ID, Mm00434256_m1), IFN-γ (Taqman Gene Expression Assays ID, Mm01168134_m1), TNF-α (Taqman Gene Expression Assays ID, Mm00443260_g1), IL-1β (Taqman Gene Expression Assays ID, Mm00434228_m1), IL-6 (Taqman Gene Expression Assays ID, Mm00446190_m1), and B-cell activating factor (BAFF; Taqman Gene Expression Assays ID, Mm00446347_m1). An 18s rRNA (Taqman Gene Expression Assays ID, Hs03003631_g1) was used for normalization of gene expression. For PCR probe sets, Taqman Gene Expression Assay kits were purchased from Applied Biosystems. The assays were performed in dual technical replicates for each sample.

Enzyme-Linked Immunosorbent Assay

For protein extraction, lacrimal glands were minced into small pieces, and sonicated in PRO-PREP Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea) on ice. The supernatant was collected after centrifugation at 12,000 rpm for 20 minutes, and assayed for the concentration of BAFF (R&D Systems) by ELISA according to the manufacturer's protocol.

Corneal Epithelial Cell Culture and Assays

Primary human corneal epithelial cells (CECs), which were obtained from CELLnTEC (Bern, Switzerland), were cultured in serum-free CnT-20 media (CELLnTEC) containing 1 ng/mL to 1000 ng/mL rhTSG-6 (R&D Systems) for 24 hours, and assayed for viability, proliferation, and migration. The cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (Vybrant MTT Cell Proliferation Assay Kit; Invitrogen/Life Technologies). The cell proliferation was quantitated by measuring 5-bromo-2-deoxyuridine (BrdU) uptake in cells using colorimetric immunoassay (Cell Proliferation ELISA, BrdU; Roche, Indianapolis, IN, USA). The cell migration was tested using a scratch-wound assay. Human CECs were cultured in 24-well plates, and a uniform scratch wound (in 150-μm width) was created in a confluent cell monolayer by a pipette tip. The cells were further incubated for 0, 6, and 24 hours in the presence of TSG-6 0, 10, 100, and 1000 ng/mL, and photographed. The cell migration was assessed by measuring the wound closure.

Statistical Analysis

GraphPad Software (GraphPad Prism, Inc., La Jolla, CA, USA) was used for statistical tests. Data were tested for a Gaussian distribution using the D'Agostino-Pearson omnibus test. To compare means of more than two groups, data were analyzed by one-way ANOVA or Kruskal-Wallis test. Tuckey's Honestly Significant Difference test or Dunn's test was used for a follow-up pairwise comparison of the groups after the null hypothesis

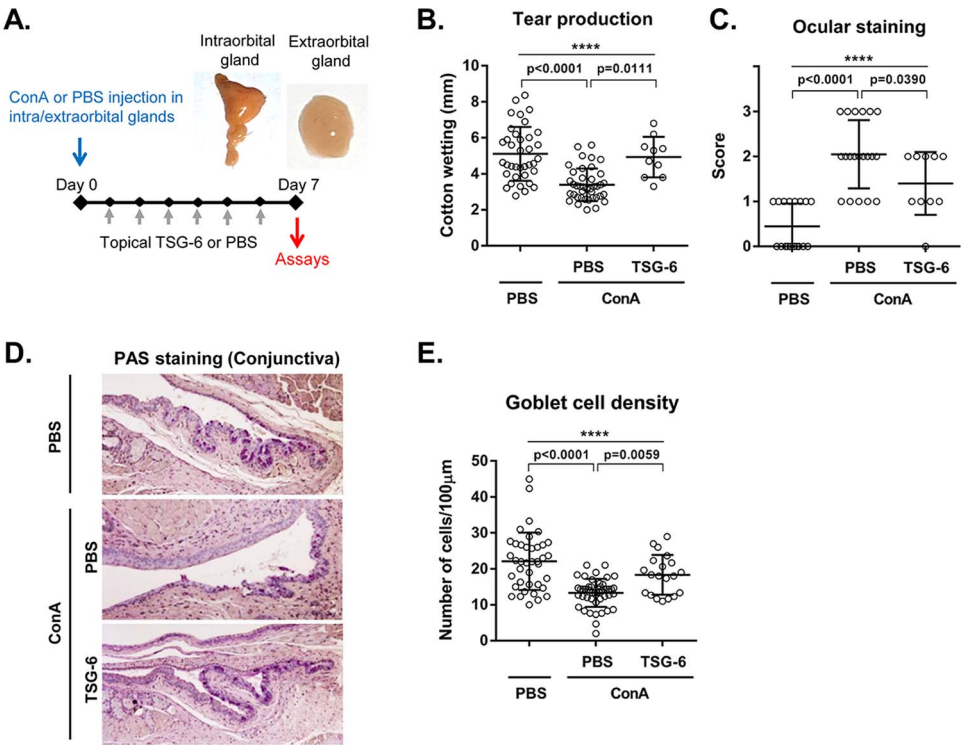

Figure 1. Effects of topical TSG-6 in ConA-induced dry eye model. (**A**) To induce dry eye, ConA or PBS was injected into both intraorbital and extraorbital lacrimal glands of BALB/c mice. For treatment, either recombinant TSG-6 (1 μg in 10 μL of PBS) or the same volume of PBS was topically administered to the ocular surface of the mice QID for 7 days. One week later, the ocular surface and lacrimal glands were assayed. (**B**) Phenol red thread test showed that aqueous tear production was markedly decreased by ConA injection. The tear production was significantly higher in TSG-6–treated eyes than in PBS-treated controls. (**C**) The ocular staining score was markedly increased by ConA injection, and significantly reduced by TSG-6 treatment. (**D**, **E**) Representative photographs of PAS staining of the forniceal conjunctiva. Original magnification ×100. The density of goblet cells in the conjunctiva was significantly higher in TSG-6–treated eyes than in PBS-treated controls. The data are presented as the mean ± SD. *Asterisk* indicates *P* value from one-way ANOVA analysis: $^{****}P < 0.0001$.

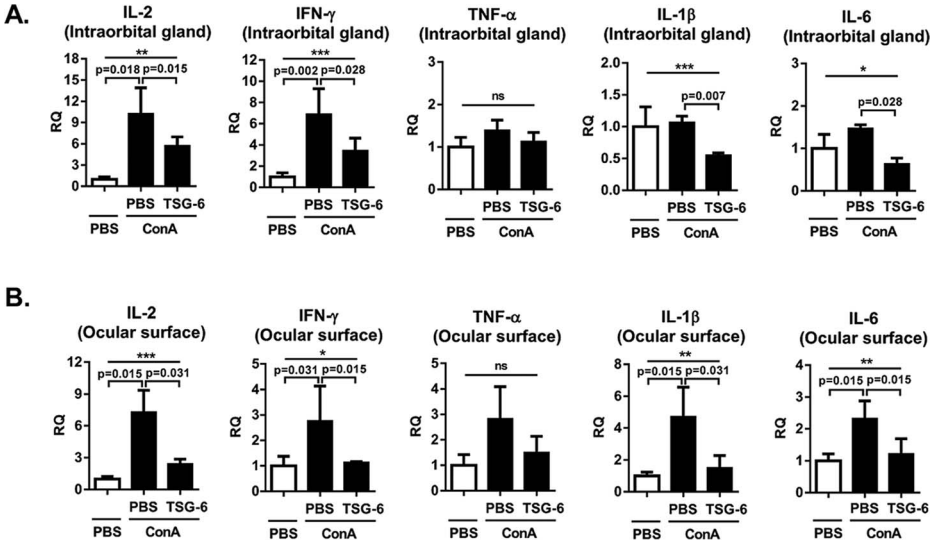

Figure 2. Effects of topical TSG-6 on levels of proinflammatory cytokines in the intraorbital gland and ocular surface in ConA-induced dry eye model. (**A**) Real-time RT PCR revealed that the levels of IL-2 and IFN-γ were markedly increased in the intraorbital gland by ConA injection, and were significantly lower in TSG-6-treated eyes than in PBS-treated controls. Topical TSG-6 application also reduced the levels of IL-1β and IL-6 transcripts. (**B**) In the ocular surface including the cornea and conjunctiva, the levels of IL-2, IFN-γ, IL-1β, and IL-6, which were increased by ConA injection, were significantly reduced by topical TSG-6 treatment. $n = 10$ in each group. RQ means a ratio of mRNA levels relative to those in controls without ConA injection. The data are presented as the mean + SD. *Asterisks* indicate *P* values from one-way ANOVA analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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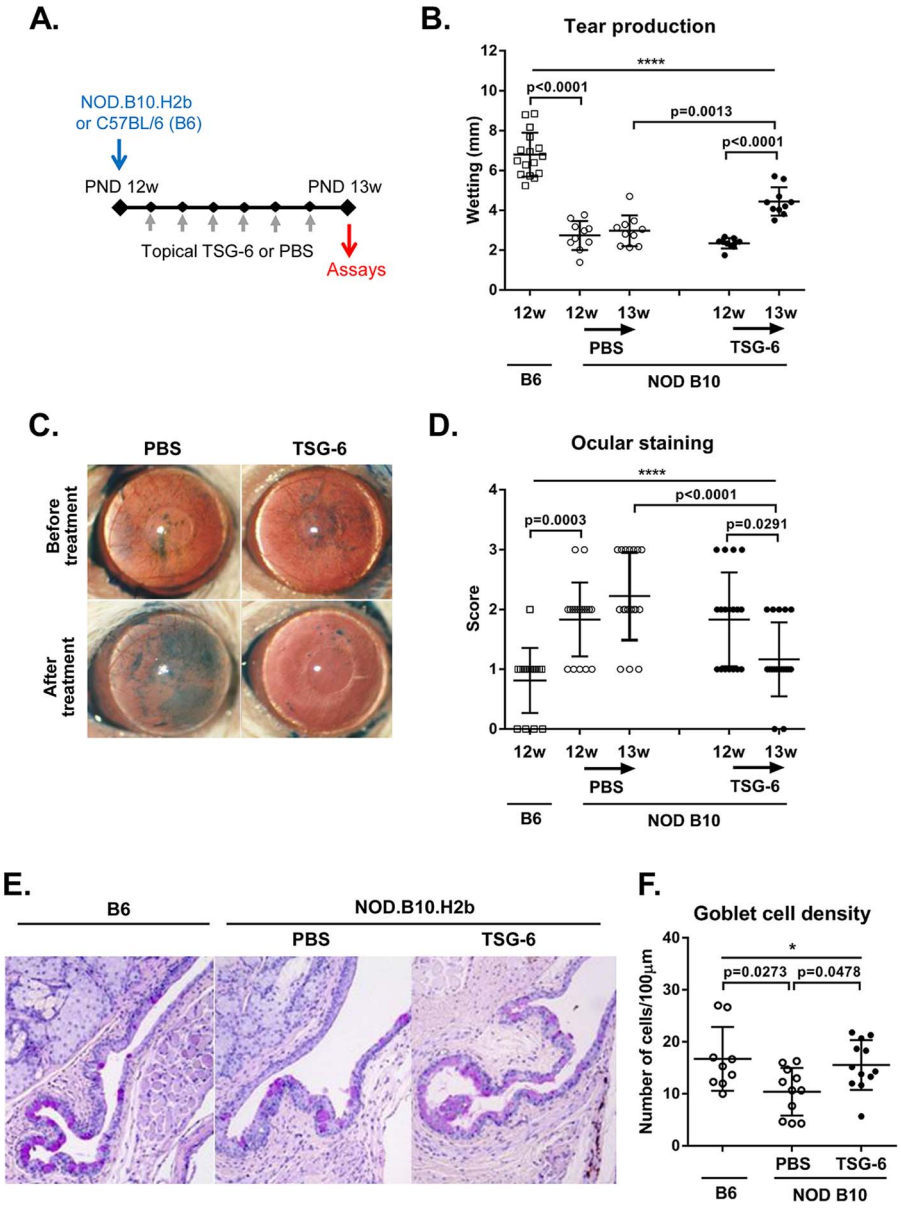

Figure 3. Effects of topical TSG-6 in NOD.B10.H2^b mice. (**A**) Recombinant TSG-6 (1 μg in 10 μL of PBS) or the same volume of PBS was topically administered QID to NOD.B10.H2^b mice at the postnatal day 12 weeks for 7 days. At postnatal day 13 weeks, the ocular surface and lacrimal glands were analyzed. (**B**) Phenol red thread test showed that tear production was markedly decreased in 12-week-old NOD.B10.H2^b mice, compared to C57BL/6 (B6) mice of the same age. Topical TSG-6 application for 1 week significantly increased the amount of tear production in NOD.B10.H2^b mice, while PBS application had no effect. (**C**, **D**) Representative corneal photographs after lissamine green staining demonstrated that TSG-6 treatment markedly suppressed the development of corneal epithelial defects in NOD.B10.H2^b mice, while PBS did not. The ocular staining score was significantly lower in TSG-6–treated eyes than in PBS-treated eyes. (**E**, **F**) PAS staining of the forniceal conjunctiva showed that the goblet cell density was significantly higher in TSG-6–treated eyes than in PBS-treated controls. Original magnification ×100. The data are presented as the mean ± SD. *Asterisks* indicate *P* values from one-way ANOVA analysis: $^*P < 0.05$, $^{****}P < 0.0001$.

was rejected ($P < 0.05$). The data are presented as the mean ± SD. Differences were considered significant at $P < 0.05$.

Results

The Effect of Topical TSG-6 in ConA-Induced Dry Eye Model

The injection of ConA into lacrimal glands markedly reduced tear production on day 7 compared to PBS injection as measured by phenol red thread test (Figs. 1A, 1B). Also, ConA injection induced epithelial defects in the cornea as reflected by increased ocular staining score (Fig. 1C), and decreased the number of PAS-stained goblet cells in the conjunctiva (Figs. 1D, 1E). Topical administration of TSG-6 to the ocular surface significantly improved tear production in mice with ConA injection, compared to topical PBS application (Fig. 1B). Also, ocular staining score was significantly lower in TSG-6–treated eyes than in PBS-treated controls (Fig. 1C). The goblet cell density was significantly higher in TSG-6–treated eyes (Fig. 1E). These results demonstrate that topical TSG-6 administration protected the ocular surface in mice with ConA-induced DES.

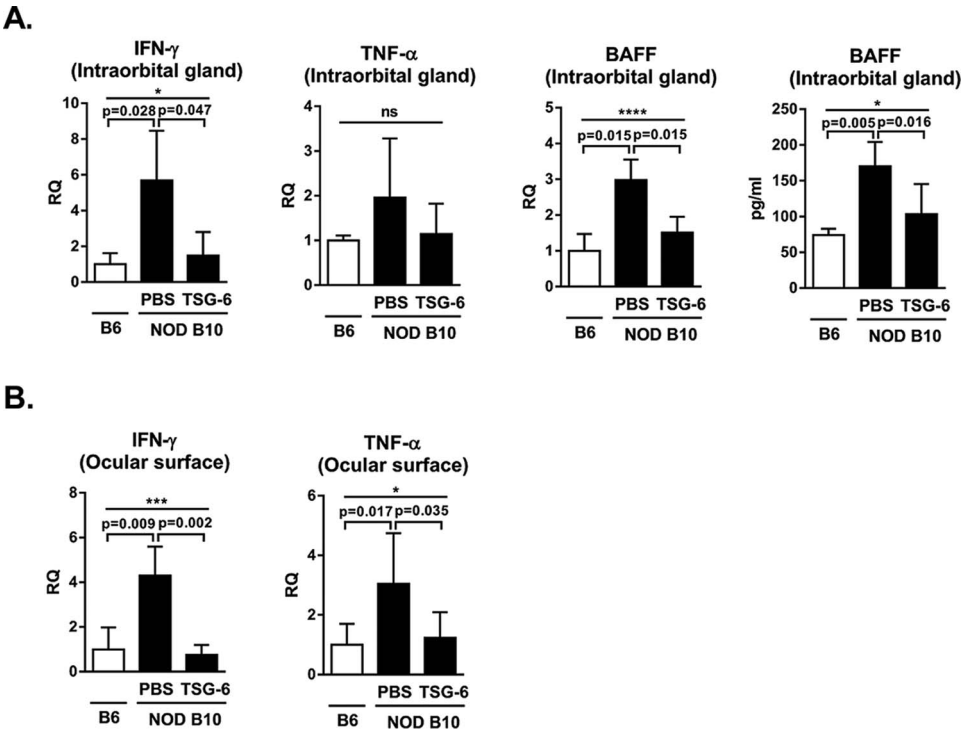

FIGURE 4. Effects of topical TSG-6 on levels of proinflammatory cytokines in the intraorbital gland and ocular surface in NOD.B10.H2^b mice. (**A**, **B**) Real-time RT PCR showed that the levels of IFN-γ or TNF-α were markedly increased in the intraorbital gland and ocular surface of NOD.B10.H2^b mice compared to C57BL/6 (B6) mice, and significantly reduced by topical TSG-6 treatment. Similarly, the transcript and protein levels of BAFF were increased in the intraorbital gland of NOD.B10.H2^b mice, and significantly lower in TSG-6–treated eyes, compared to PBS treatment. $n = 10$ in each group. RQ means a ratio of mRNA levels relative to those in B6 controls. The data are presented as the mean + SD. *Asterisks* indicate *P* values from one-way ANOVA analysis: $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Our group has previously shown that an intralacrimal injection of ConA, a T cell mitogen, induces DES by causing inflammation in lacrimal glands and the ocular surface.[9] Therefore, we next tested whether TSG-6 might improve DES by suppressing inflammation. Real-time RT PCR revealed that the transcript levels of T cell–related cytokines, IL-2, and IFN-γ were markedly increased in the intraorbital gland by ConA injection, and significantly repressed by topical TSG-6 (Fig. 2A). Also, the levels of other proinflammatory cytokines, IL-1β, and IL-6 in the intraorbital gland were reduced by TSG-6 treatment (Fig. 2A). Similarly, the levels of IL-2, IFN-γ, IL-1β, and IL-6 in the ocular surface including the cornea and conjunctiva were significantly lower in TSG-6–treated eyes than in PBS-treated controls (Fig. 2B). However, TSG-6 treatment did not affect the levels of the proinflammatory cytokines in the extraorbital gland (Supplementary Fig. S1).

Together, the data suggest that topical TSG-6 suppressed ConA-induced inflammation in the eye, and therefore prevented the development of DES.

The Effect of Topical TSG-6 in NOD.B10.H2^b Mice

To further confirm the effect of topical TSG-6 in DES, we employed another mouse model of dry eye: NOD.B10.H2^b mice. The NOD.B10.H2^b strain has a SjS-like phenotype without the occurrence of type 1 diabetes because the diabetogenic MHC locus has been replaced by the MHC locus of C57BL/10 strain that is not susceptible to diabetes.[22,23] First, we confirmed that 12-week-old NOD.B10.H2^b mice exhibited dry eye phenotype by observing that the mice had deficiency in tear production, corneal epithelial defects, and lower conjunctival goblet cell density compared to B6 mice (Fig. 3). Next, to evaluate the effect of topical TSG-6 in NOD.B10.H2^b mice, we applied either rhTSG-6 or PBS topically to the ocular surface of 12-week-old NOD.B10.H2^b mice QID for 1 week (Fig. 3A). Tear production and ocular staining score were not altered by topical PBS application (Figs. 3B, 3D). However, topical TSG-6 administration for 1 week significantly increased the tear production in NOD.B10.H2^b mice (Fig. 3B), suggesting that TSG-6 improved the function of lacrimal glands. Similarly, the ocular staining score was significantly reduced after TSG-6 treatment compared to before treatment (Figs. 3C, 3D), which indicated that TSG-6 promoted the healing of corneal epithelial defects. When compared between PBS- and TSG-6–treated groups, the amount of tear production was significantly higher in TSG-6–treated eyes (Fig. 3B), and the ocular staining score was significantly lower in TSG-6–treated eyes (Figs. 3C, 3D). Also, the number of goblet cells in the conjunctiva was significantly higher in TSG-6–treated eyes than in PBS-treated controls (Figs. 3E, 3F).

To examine whether TSG-6 might affect inflammation in eyes of NOD.B10.H2^b mice, we assayed the intra- and extraorbital glands and ocular surface containing the cornea and conjunctiva for the levels of proinflammatory cytokines. Among the cytokines analyzed, the levels of IFN-γ and BAFF were significantly increased in the intraorbital gland compared to B6 mice, and topical TSG-6 treatment markedly reduced the levels of IFN-γ and BAFF (Fig. 4A). Similarly, the levels of IFN-γ and TNF-α were increased in the ocular surface of NOD.B10.H2^b mice, and markedly reduced by TSG-6 application (Fig. 4B). However, the levels of the proinflammatory cytokines in the extraorbital gland were not different between NOD.B10.H2^b and B6 mice, and TSG-6 did not alter the

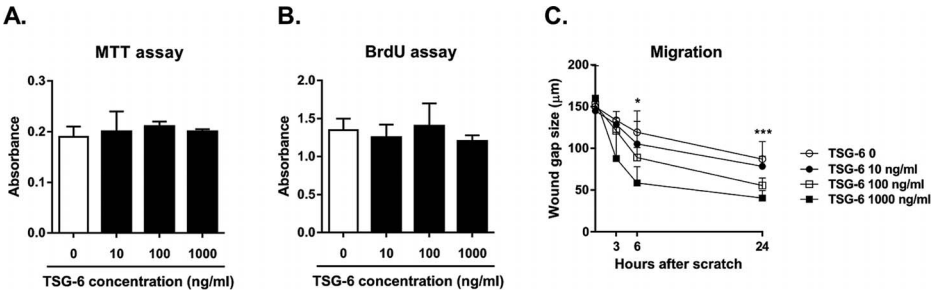

FIGURE 5. Effects of TSG-6 on the viability, proliferation, and migration of CECs in vitro. (**A**) MTT assay revealed that TSG-6 (1 to 1000 ng/mL) did not affect the number of metabolically active viable CECs. (**B**) Similarly, BrdU uptake assay showed that the proliferation of CECs was not changed by TSG-6 treatment. (**C**) However, a scratch-wound test demonstrated that TSG-6 promoted the migration of CECs to close the wound in a dose-dependent manner. The data are presented as the mean +/± SD. *Asterisks* indicate *P* values from one-way ANOVA analysis: $^{*}P < 0.05$, $^{***}P < 0.001$.

expression of the inflammatory cytokines in the extraorbital gland (Supplementary Fig. S2).

Effects of TSG-6 on CECs In Vitro

We observed that the area of corneal epithelial defects decreased after topical TSG-6 administration (Figs. 3C, 3D). To examine the possibility that TSG-6 might accelerate corneal epithelial wound healing by directly promoting the survival, proliferation, or migration of CECs, we tested the effects of TSG-6 on CECs in culture. MTT and BrdU uptake assays showed that TSG-6 did not have direct effects on the survival and proliferation of CECs (Figs. 5A, 5B). However, a scratch-wound test revealed that TSG-6 significantly accelerated the migration of CECs in a dose-dependent manner (Fig. 5C).

Together, the results suggest that topical TSG-6 repressed the inflammation and promoted corneal epithelial wound healing, therefore improving DES in NOD.B10.H2^{b} mice.

DISCUSSION

Understanding of the pathology of DES has dramatically improved over the past decade. Evidence from clinical and animal studies indicate that the pathogenesis of DES is closely linked to inflammation, and autoimmune inflammatory disease in the ocular surface and lacrimal glands manifests as DES.[3–6]

TSG-6 is a multifunctional endogenous protein expressed by a variety of cells including MSCs, and has anti-inflammatory activities.[10–16] In transgenic mice, inactivation of TSG-6 gene increased inflammatory response, and overexpression of the gene decreased inflammation and joint destruction following induced arthritis.[25–28] Also, administration of recombinant TSG-6 reduced inflammation, and improved tissue function in models for arthritis, myocardial infarction, peritonitis, brain injury, and lung injury.[10–12,17–19,28] In the eye, our group previously reported that systemic or local administration of rhTSG-6 was effective in suppressing corneal inflammation in mice with chemical injury, corneal allotransplants, or freezing-induced corneal endothelial injury.[20,21,29] In line with these reports, the present study suggests that topical TSG-6 improves DES by suppressing inflammation in the ocular surface and intraorbital lacrimal gland.

One of interesting observations made here was that TSG-6 was effective in suppressing inflammation of the intraorbital lacrimal gland but did not affect the extraorbital lacrimal gland. This might be related to the route of TSG-6 administration. We applied TSG-6 topically to the ocular surface. Therefore, topical TSG-6 might exert its anti-inflammatory action on the intraorbital gland by transconjunctival absorption, but not on the extraorbital gland, which is located underneath the skin at the base of ear. However, without the action on the extraorbital lacrimal gland, TSG-6 achieved a significant improvement in DES in two mouse models as reflected by an increase in tear production and goblet cell density and a reduction of corneal epithelial damage. Further studies would be necessary to determine the optimal dose, frequency, and route of TSG-6 administration for treatment of DES.

We here used two mouse models of DES. One is a model induced by ConA injection into lacrimal glands. Our group recently demonstrated that an intraorbital injection of ConA (20 μL, 10 mg/mL) created DES in mice by causing inflammation, reducing tear secretion, and disrupting corneal epithelium.[9] In the present study, we used the same model, and started TSG-6 application right after ConA injection. Therefore, the beneficial effects of TSG-6 in this model reflect the preventive action of TSG-6 in the development of inflammation and DES in mouse eyes. Another model we used here is NOD.B10.H2^{b} mice. NOD.B10.H2^{b} mice spontaneously develop primary SjS without diabetes and have many characteristics similar to those of human SjS, such as lymphocytic infiltration of lacrimal glands and reduced secretory function.[22,23] These changes usually initiate in the mice at 8 weeks of age and continue throughout 40 to 46 weeks.[22] In this study, we commenced TSG-6 application at the postnatal 12 weeks and found that after TSG-6 treatment, the inflammation and epithelial damage in the ocular surface were decreased, and lacrimal production increased, compared to before treatment. These findings indicate the therapeutic effects of TSG-6 on the ocular surface inflammation and DES. Given that DES is a multifactorial disease, further studies to evaluate the effects of TSG-6 in other models of DES would help substantiate the preventive and therapeutic potential of TSG-6 for human patients with DES.

In conclusion, our results demonstrate that topical TSG-6 application protected the ocular surface by suppressing inflammation and enhancing corneal epithelial wound healing in two mouse models of inflammation-mediated DES. The results provide a rationale to further explore TSG-6 eye drops as a novel therapy for patients with DES and other inflammatory ocular surface disorders.

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