The Ocular Surface 24 (2022) 40–50

Contents lists available at ScienceDirect

The Ocular Surface

journal homepage: www.elsevier.com/locate/jtos

The Link module of human TSG-6 (Link_TSG6) promotes wound healing, suppresses inflammation and improves glandular function in mouse models of Dry Eye Disease

Joo Youn Oh [a,b,*], Jin Suk Ryu [b], Hyeon Ji Kim [b], Nikolaos Kouvatsos [c,d], Rebecca J. Dodd [c,d], Se Hyun Choi [b], Yu Jeong Kim [b], Caroline M. Milner [d,e], Anthony J. Day [c,d,e,**]

[a] Department of Ophthalmology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul, 03080, South Korea
[b] Laboratory of Ocular Regenerative Medicine and Immunology, Biomedical Research Institute, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul, 03080, South Korea
[c] Wellcome Centre for Cell-Matrix Research, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Sciences Centre, Manchester M13 9PT, United Kingdom
[d] Division of Cell Matrix Biology and Regenerative Medicine, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Sciences Centre, Manchester M13 9PT, United Kingdom
[e] Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, M13 9PT, United Kingdom

ARTICLE INFO

Keywords:
Dry eye disease
Link_TSG6
Link module
TNF-Stimulated gene/protein-6
TSG-6

ABSTRACT

Purpose: To investigate the potential of the Link_TSG6 polypeptide comprising the Link module of human TSG-6 (TNF-stimulated gene/protein-6) as a novel treatment for dry eye disease (DED).
Methods: We analyzed the therapeutic effects of topical application of Link_TSG6 in two murine models of DED, the NOD.B10.H2^b mouse model and the desiccating stress model. The effects of Link_TSG6 on the ocular surface and DED were compared with those of full-length TSG-6 (FL_TSG6) and of 0.05% cyclosporine (Restasis®). Additionally, the direct effect of Link_TSG6 on wound healing of the corneal epithelium was evaluated in a mouse model of corneal epithelial debridement.
Results: Topical Link_TSG6 administration dose-dependently reduced corneal epithelial defects in DED mice while increasing tear production and conjunctival goblet cell density. At the highest dose, no corneal lesions remained in ~50% of eyes treated. Also, Link_TSG6 significantly suppressed the levels of inflammatory cytokines at the ocular surface and inhibited the infiltration of T cells in the lacrimal glands and draining lymph nodes. Link_TSG6 was more effective in decreasing corneal epithelial defects than an equimolar concentration of FL_TSG6. Link_TSG6 was significantly more potent than Restasis® at ameliorating clinical signs and reducing inflammation. Link_TSG6 markedly and rapidly facilitated epithelial healing in mice with corneal epithelial debridement wounds.
Conclusion: Link_TSG6 holds promise as a novel therapeutic agent for DED through its effects on the promotion of corneal epithelial healing and tear secretion, the preservation of conjunctival goblet cells and the suppression of inflammation.

1. Introduction

Dry eye disease (DED) is the most prevalent ocular surface disorder, affecting up to 50% of some populations [1], and is characterized by a loss of homeostasis of the tear film [2]. Multiple mechanisms are involved in the maintenance of tear film homeostasis at the ocular surface, which is constantly exposed to desiccating stress. In particular, ocular surface inflammation plays a critical role in disrupting

* Corresponding author. Department of Ophthalmology, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, South Korea.
** Corresponding author. Wellcome Centre for Cell-Matrix Research, Division of Cell Matrix Biology and Regenerative Medicine, School of Biological Sciences and Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Sciences Centre, Manchester M13 9PT, United Kingdom.
E-mail addresses: jooyounoh77@gmail.com, bonzoo1@snu.ac.kr (J.Y. Oh), anthony.day@manchester.ac.uk (A.J. Day).

https://doi.org/10.1016/j.jtos.2021.12.012
Received 31 July 2021; Received in revised form 30 October 2021; Accepted 23 December 2021
Available online 27 December 2021
1542-0124/© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

homeostatic mechanisms and perpetuating DED [3]. Thus, in recent years, strategies targeted at controlling ocular surface inflammation have been the focus of the development of novel DED therapeutics.

In previous studies, we found that topical administration of TSG-6, the secreted protein product of the TNFAIP6 gene, had profound effects on the treatment of DED by suppressing inflammation and promoting corneal epithelial cell migration [4,5]. TSG-6 is an endogenous multifunctional protein that is constitutively expressed in some tissues, especially those that provide a barrier to the environment such as amniotic membrane, lung and skin [6]; TSG-6 immunoreactivity has been observed in the extracellular matrix of the human corneal epithelium [7]. During inflammation, TSG-6 is upregulated in most cells and protects tissues by modulating inflammation and enhancing repair [6]. Moreover, it has been shown that TSG-6 mediates many of the immunomodulatory and reparative functions of mesenchymal stem/stromal cells (MSCs) in various disease models of the eye and other tissues [6,8]. Given its homeostatic role as an intrinsic protective protein, TSG-6 has the potential to be developed as a novel biological drug for DED.

However, there are several problems with translating the full-length TSG-6 protein (FL_TSG6) into clinical use. Firstly, FL_TSG6 is difficult to make in significant amounts. Secondly, the protein has poor solubility and stability due to aggregation, giving rise to inconsistent efficacies of the recombinant FL_TSG6 in experimental models. Importantly, these disadvantages are not associated with the recombinant Link module from human TSG-6 (Link_TSG6); this independently folded domain [9] is easier to make than FL_TSG6 and is highly soluble and stable in solution. Furthermore, we have previously shown that Link_TSG6 is as potent as the full-length protein at inhibiting neutrophil migration in both *in vivo* and *in vitro* assay systems [10,11] and is more potent than FL_TSG6 at suppressing inflammatory pathways in models of osteoarthritis (https://doi.org/10.1101/2021.03.23.21254102).

In this study, we investigated the therapeutic potential of topical Link_TSG6 administration in two murine models of DED, 1) the NOD.B10.H2^b mouse model exhibiting ocular Sjögren's syndrome (SjS)-like phenotype and 2) the desiccating environmental stress model induced by exposure to low humidity and increased airflow along with muscarinic blockade. Furthermore, we compared the therapeutic effects of Link_TSG6 on DED with those of FL_TSG6 and of 0.05% cyclosporine (Restasis®). The direct effects of Link_TSG6 on corneal epithelial wound healing were also evaluated in a murine corneal epithelial debridement model.

2. Materials and methods

2.1. Animals and animal models

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (IACUC No.15-0051, 16–0142, 17–0067, 18–0042, 19–0068) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

C57BL/6 and BALB/c mice were purchased from OrientBio Inc. (Seongnam, Korea), and breeding pairs of NOD.B10.H2^b mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained at the facility in the Biomedical Research Institute of Seoul National University Hospital accredited by AAALAC.

For an autoimmune-mediated DED model, 12-week-old NOD.B10.H2^b mice were used because this strain spontaneously develops an ocular phenotype similar to human SjS such as dacryoadenitis and aqueous deficient dry eye (ADDE) [12,13]. In experiments using NOD.B10.H2^b mice, 12-week-old C57BL/6 mice were used as negative controls.

For induction of desiccating stress, 7-week-old C57BL/6 mice were housed in a perforated cage in a dry cabinet with an electric fan and dehumidifier. The airflow from the fan was continuously allowed into the cage for 24 h a day for the entire study period, and the humidity was maintained at 30–35% inside the cage. In addition, mice received intraperitoneal (IP) injections of scopolamine hydrobromide (0.5 mg/0.2 mL, Sigma-Aldrich, St. Louis, MO) TID. This evaporative dry eye (EDE) model was modified from a previously described method [14].

For creation of corneal epithelial wounding, 8-week-old BALB/c mice were anesthetized with IP injection of zolazepam-tiletamine (Zoletil®, Virbac, Carros, France) and topical administration of proparacaine ophthalmic solution; then a central corneal area was demarcated with a 2-mm-diameter trephine, and the epithelium in the area was removed by scraping with a No.13 surgical blade.

2.2. Reagents and treatment

The recombinant human Link_TSG6 protein was prepared essentially as described previously [15,16], lyophilized and stored at -20 °C. Endotoxin levels for the protein were determined to be below 0.1 EU/mg using a Pierce LAL assay (Thermofisher, Altrincham, UK). Residual trifluoracetic acid (TFA) arising from the final HPLC purification was removed by treatment with endotoxin free AG 1-X2 resin (BioRad, Watford, UK) and quantified by ^{19}F NMR spectroscopy relative to a TFA calibration curve (data not shown; S.E. Powell, R.J. Dodd, G.A. Hassall, N. Kouvatsos, C.M. Milner and A.J. Day, unpublished method).

The freeze-dried Link_TSG6 was dissolved in sterile phosphate-buffered saline (PBS) at desired concentrations (0.01, 0.1, 1.0 or 10 μg in 5 μL PBS corresponding to concentrations of 0.18, 1.8, 18 and 180 μM, respectively) prior to topical administration to the eye.

The lyophilized form of recombinant human FL_TSG6 was purchased from R&D Systems (Cat No. 2104-TS, Minneapolis, MN) and was reconstituted in sterile PBS at desired concentrations (0.0327–3.27 μg in 5 μL PBS) prior to *in vivo* use.

Restasis® (cyclosporine ophthalmic emulsion 0.05%) was purchased from Allergan (Irvine, CA).

Link_TSG6, FL_TSG6, Restasis® or PBS were topically applied to the ocular surface of mice BID or QID for the pre-determined treatment period.

2.3. Phenol red thread test and corneal vital staining

The volume of tear secretion was quantified by the phenol red thread test (Zone-Quick™, Showa Yakuhin Kako Co. Ltd, Tokyo, Japan). The folded end of a phenol red-impregnated cotton thread was hooked over the temporal one-third of the lower eyelid margin. After 15 s, the length of tear wetting (red portion) was measured.

Punctate epithelial erosions were observed in DED mice after corneal vital staining with 3% (v/v) lissamine green dye (NOD.B10.H2^b mice) or 0.25% (v/v) fluorescein dye (C57BL/6 mice with desiccating stress; with fluorescein being used because of the dark eye color), before and after treatment, and the extent of staining was graded independently by two individuals (J.Y.O. and Y.J.K.) in a blinded manner using the standardized scale system (score 0: no staining; score 0.5: trace; score 1: less than one-third; score 2: less than two-thirds; and score 3: more than two-thirds staining of the cornea) [17].

The area of the corneal epithelial defect was measured in a corneal epithelial debridement wound model after staining with 0.25% (v/v) fluorescein dye. Photographs of each cornea were taken at the same magnification under a cobalt blue light, and the proportion of the stained area to the total corneal surface was calculated using ImageJ software (NIH).

2.4. Histopathology

After mice were humanely sacrificed by cervical dislocation under general anesthesia, the whole eyeball (including the forniceal conjunctiva and the cornea) and the lacrimal glands (both extraorbital and intraorbital glands) were excised and subjected to histologic or molecular assay. For histologic examination, the tissues were fixed in 10% (v/

v) formaldehyde. Then, 4-μm-thick sections were cut and subjected to hematoxylin-eosin staining, Periodic Acid-Schiff (PAS) staining (ab150680, Abcam, Cambridge, MA) or CD3 immunostaining (ab5690, Abcam).

For evaluation of conjunctival goblet cells, the number of PAS-stained cells was counted in 4 different sections through the superior and inferior conjunctival fornices in each eye by two independent researchers (J.S.R. and H.J.K.); the average number per section in each eye was determined as the goblet cell count. The numbers of inflammatory foci in the extraorbital glands, defined as independent areas of lymphocytic infiltration with >50 cells [12], were also counted in hematoxylin-eosin- or CD3-stained sections in a similar manner.

2.5. Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The excised tissues were lysed in RNA isolation reagent (RNA-Bee, Tel-Test Inc., Friendswood, TX) and homogenized with an ultrasound sonicator (Cole Parmer Instruments, Vernon Hills, IL). Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA) and converted to first-strand cDNA by reverse transcription (High Capacity RNA-to-cDNA Kit, Applied Biosystems, Carlsbad, CA). Then reverse transcription quantitative PCR (RT-qPCR) amplification was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in an ABI 7500 Real-Time PCR System (Applied Biosystems). All PCR probe sets were

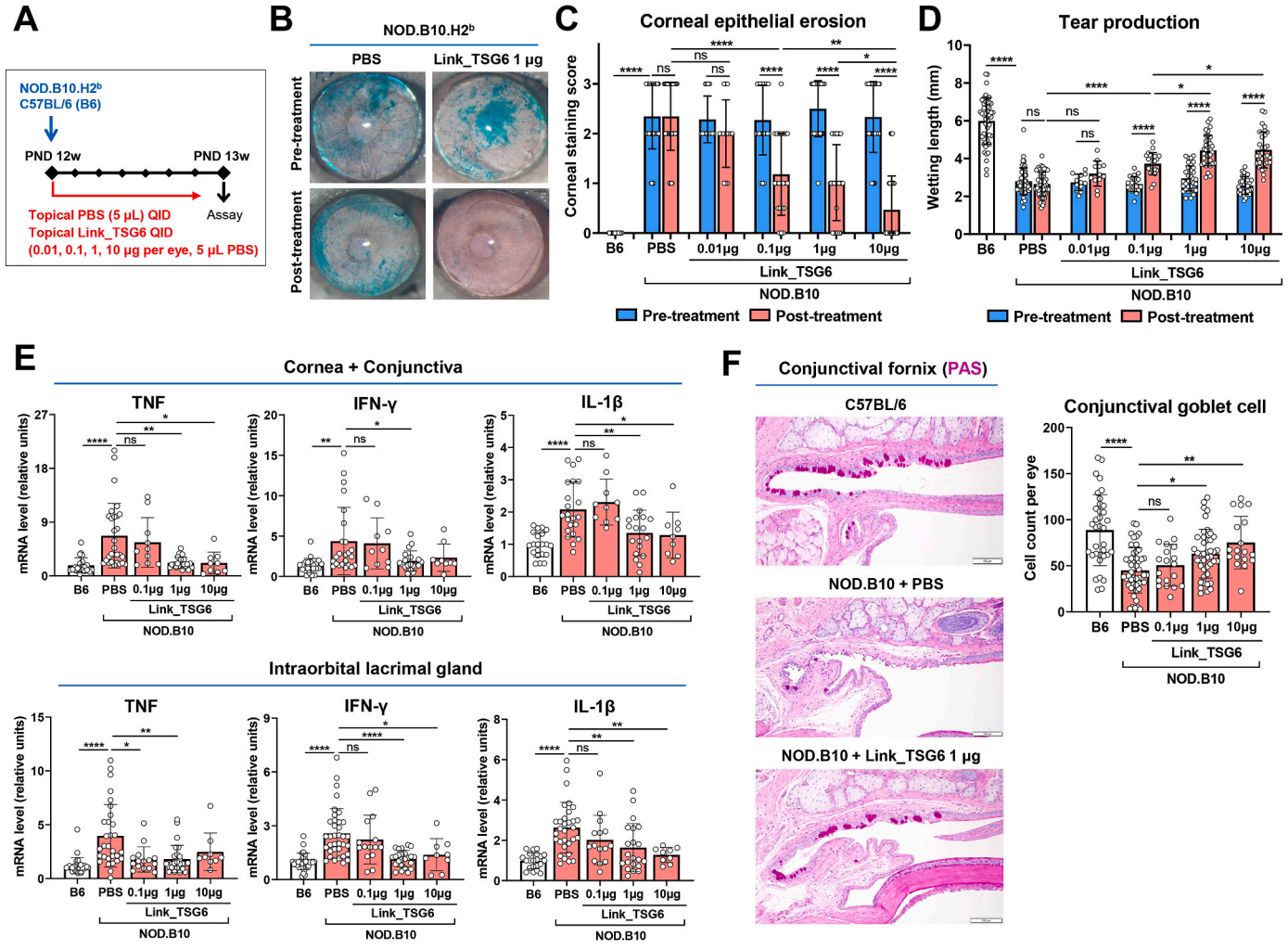

Fig. 1. Topical Link_TSG6 dose-dependently alleviates DED in NOD.B10.H2^b mice.

A. Treatment protocol. 12-week-old NOD.B10.H2^b mice were treated topically with Link_TSG6 (0.01–10 μg in 5 μL PBS) or vehicle (5 μL PBS) QID for 7 days. C57BL/6 (B6) mice were used as a negative control.

B. Representative photographs of corneas with lissamine green vital dye staining pre-treatment (post-natal day (PND) 12 weeks) and post-treatment (after 7 days of treatment; PND 13 weeks). The green-stained area reflects the parts of the cornea with epithelial damage.

C. Quantification of corneal epithelial defects as graded by the standardized scoring system (0–3) pre- and post-treatment (n = 40 eyes for C57BL/6 (B6) control group; n = 52 eyes for PBS group; n = 12 eyes for Link_TSG6 0.01 μg group; n = 22 eyes for Link_TSG6 0.1 μg group; n = 34 eyes for Link_TSG6 1 μg group; n = 30 eyes for Link_TSG6 10 μg group).

D. The amount of tear secretion as measured by a phenol red thread test pre- and post-treatment.

E. The levels of pro-inflammatory cytokine mRNAs in the ocular surface (both cornea and conjunctiva) and the intraorbital lacrimal gland as analyzed by RT-qPCR. Values are shown relative to those in C57BL/6 (B6) control eyes.

F. Representative microphotographs of the forniceal conjunctiva with PAS staining of mucin-secreting conjunctival goblet cells. The number of conjunctival goblet cells was quantified in PAS-stained slides; cell counts per eye are shown. Scale bar: 100 μm.

In C–F mean values ± SD are shown, where each circle depicts the data from an individual eye. Data are pooled from 3 to 6 independent experiments each containing 3–5 animals (6–10 eyes) per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant, as analyzed by one-way ANOVA and Tukey's test (C, D, F, IFN-γ and IL-1β in E), Kruskal–Wallis test and Dunn's multiple-comparison test (TNF in E) or by paired *t*-test (for comparison between pre- and post-treatment eyes in C, D).

purchased from Applied Biosystems (TaqMan Gene Expression Assay kits). The assays were performed in dual technical replicates for each biological sample. Data were normalized to GAPDH and expressed as fold changes relative to controls.

2.6. Flow cytometry

Single-cell suspensions were prepared by mincing cervical lymph nodes (CLNs) between two frosted ends of glass slides and filtering through a cell strainer; cells were stained with fluorescent dye-conjugated antibodies against CD4 (Cat No. 25–0041, eBioscience, Waltham, MA), IFN-γ (Cat No. 11–7311, eBioscience) and IL-17 (Cat No. 17–7177, eBioscience). The stained cells were assayed using a flow cytometer (S1000EXi Flow Cytometer, Stratedigm, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

2.7. Statistical analysis

Prism software (GraphPad, San Diego, CA) was used for statistical tests and generation of graphs. D'Agostino and Pearson test or Shapiro-Wilk test were used to test for a normal distribution of data in each group. One-way ANOVA with Tukey's test or Kruskal–Wallis test with Dunn's multiple-comparisons test were applied for comparison of mean values from more than two groups. Mann–Whitney *U* test was used for comparison of the means of two groups. A paired *t*-test was used to compare the means of the pre- and post-treatment values in the same animal. Data are presented as mean ± SD. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Therapeutic effects of Link_TSG6 in the NOD.B10 mouse model of DED

First, we evaluated the dose-dependent effects of Link_TSG6 in the NOD.B10.H2^b mouse model. Twelve-week-old NOD.B10.H2^b mice were treated with either topical administration of Link_TSG6 (0.01, 0.1, 1, 10 μg in 5 μL PBS) or PBS (5 μL) QID for 7 days (Fig. 1A). C57BL/6 mice without any treatment were used as negative controls. After 7 days of treatment, the cornea was clinically observed, and tear volume was measured. The ocular surface (including the cornea and the conjunctiva) and the lacrimal glands (both the extraorbital and intraorbital glands) were subjected to molecular and histologic assays.

Link_TSG6 treatment significantly reduced punctate epithelial erosions in the cornea and increased tear production, as compared to pre-treatment, whereas PBS had no effects on corneal epithelial erosions or tear secretion (Fig. 1B–D). The effects of Link_TSG6 on corneal epithelial defects and tear production were dose-dependent (Fig. 1C and D) and at the highest dose, ~50% of treated eyes had no corneal lesions remaining (Fig. 1C). Link_TSG6 also significantly suppressed mRNA levels of TNF, IFN-γ and IL-1β in the ocular surface and in intraorbital lacrimal glands in a dose-dependent manner (Fig. 1E); eyes treated with 1 or 10 μg doses of Link_TSG6 had similar levels of inflammatory cytokines to those seen in control C57BL/6 mice. In the extraorbital glands

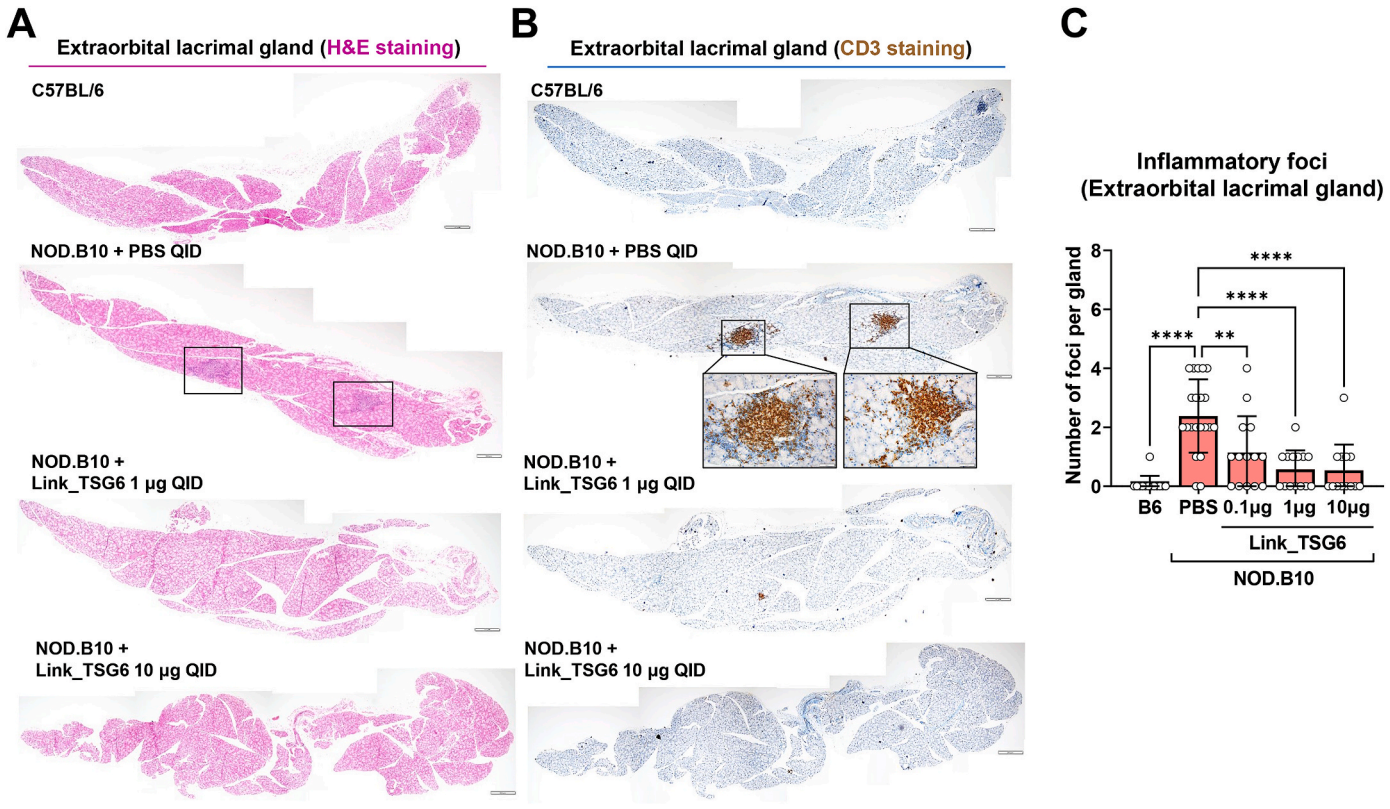

Fig. 2. Link_TSG6 suppresses CD3$^+$ T cell infiltration in the lacrimal glands.
A. Hematoxylin-eosin (H&E) staining of the extraorbital lacrimal glands in C57BL/6 control mice and NOD.B10.H2^b mice receiving 7 days of PBS or Link_TSG6 treatment (QID). Scale bar: 200 μm.
B. CD3 immunostaining of the extraorbital lacrimal glands in C57BL/6 control mice and NOD.B10.H2^b mice receiving PBS or Link_TSG6 for 7 days (QID). Scale bar: 200 μm.
C. Quantification of the number of inflammatory foci (an independent area of lymphocytic infiltration with >50 cells) in the extraorbital lacrimal glands. Each circle depicts the data from a single eye. Data (mean ± SD) were pooled from 3 independent experiments containing 3–5 animals per group in each experiment. $^{**}p < 0.01$, $^{****}p < 0.0001$, ns: not significant, as analyzed by Kruskal-Wallis test and Dunn's multiple-comparison test.

the expression was not significantly up-regulated for any of the cytokine genes (data not shown) meaning that it was not possible to come to any conclusions regarding the effect of Link_TSG6.

Furthermore, PAS staining showed that conjunctival goblet cell numbers were preserved by 1 or 10 μg Link_TSG6 treatment, while PBS or 0.1 μg Link_TSG6 did not affect the goblet cell density (Fig. 1F). CD3 immunostaining revealed that T cell infiltration, which was markedly increased in the extraorbital lacrimal glands of NOD.B10.H2^b mice, was significantly suppressed by Link_TSG6 treatment (Fig. 2).

Therefore, these data demonstrated that topical Link_TSG6 administration dose-dependently alleviated corneal epithelial erosions, improved tear secretion and conjunctival goblet cell numbers, and repressed inflammation in the ocular surface and the lacrimal glands of NOD.B10.H2^b mice, a model of primary ocular SjS.

3.2. Comparison of the effects of Link_TSG6 and FL_TSG6 in the NOD.B10 model

We next compared the therapeutic effects of Link_TSG6 with those of equivalent molar doses of FL_TSG6, where the proteins were administered BID. Twelve-week-old NOD.B10.H2^b mice were topically treated with either Link_TSG6 (0.01, 0.1, 1 μg in 5 μL PBS) or FL_TSG6 (0.0327, 0.327, 3.27 μg in 5 μL PBS) or PBS (5 μL) BID for 7 days (Fig. 3A). The molecular weight of recombinant human TSG-6 (R&D Systems) was determined to be 35.7 kDa by mass spectrometry, based on which we calculated equimolar doses of 0.0327, 0.327, 3.27 μg of FL_TSG6 corresponding to 0.01, 0.1, 1 μg of Link_TSG6, respectively. It should be noted that given FL_TSG6's poor solubility [18] (N. Kouvatsos, C.M. Milner and A.J. Day, unpublished data), it was not possible to compare the full-length protein directly with the 10 μg dose of Link_TSG6.

Before treatment, there were similar levels of corneal epithelial erosions and impaired tear production in all NOD.B10.H2^b mice. Seven days after treatment, both Link_TSG6 and FL_TSG6 induced significant reductions in the epithelial erosions and improvements in tear production (Fig. 3B–D). Notably, Link_TSG6 was significantly more potent than equimolar concentrations of FL_TSG6 at attenuating corneal epithelial erosions (Fig. 3C).

Link_TSG6 1 μg and FL_TSG6 3.27 μg were equally effective at increasing the number of conjunctival goblet cells, while lower doses of Link_TSG6 and FL_TSG6 failed to impact the goblet cell density (Fig. 3E). Similarly, the number of CD3-stained inflammatory foci (areas with >50 lymphocytes) in the extraorbital lacrimal glands was significantly decreased by Link_TSG6 1 μg and FL_TSG6 3.27 μg (Fig. 3F).

These results indicated that Link_TSG6 was more effective than FL_TSG6 at promoting healing of corneal epithelial lesions in the NOD.B10 mouse. Moreover, 1 μg Link_TSG6 was found to have similar therapeutic effects when given 2-times per day (Fig. 3) compared to Link_TSG6 administered 4-times per day (Figs. 1 and 2), so BID dosing was used in all subsequent experiments.

3.3. Comparison of the therapeutic effects of Link_TSG6 with Restasis®

We compared the therapeutic effects of Link_TSG6 with those of 0.05% cyclosporine (Restasis®) in the NOD.B10.H2^b mouse model.

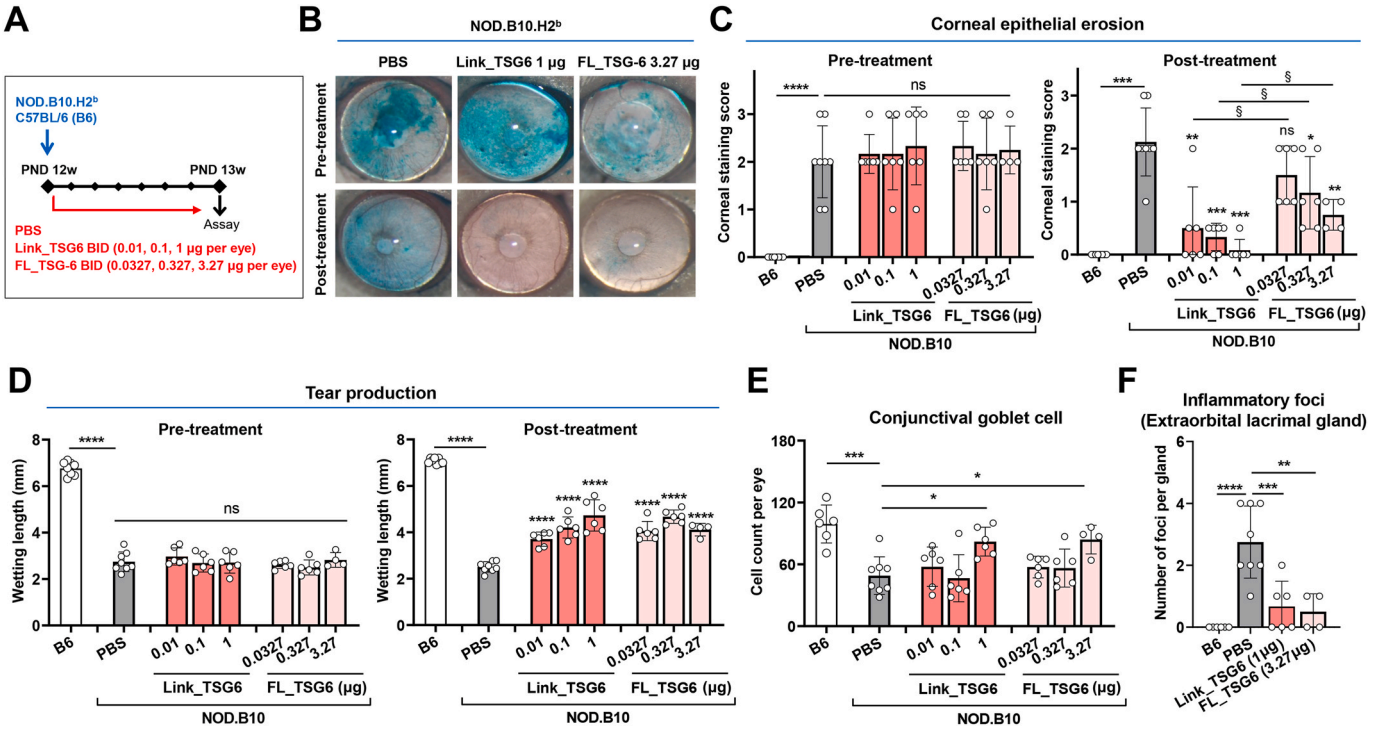

Fig. 3. Comparison of Link_TSG6 and FL_TSG6 in NOD.B10.H2^b model of DED.

A. Experimental protocol. 12-week-old NOD.B10.H2^b mice were treated topically with equimolar doses of Link_TSG6 (0.01–1 μg in 5 μL PBS) and FL_TSG6 (0.0327–3.27 μg in 5 μL PBS) or with PBS (5 μL) BID for 7 days; C57BL/6 (B6) mice served as a negative control. 4 animals (8 eyes) were used per group except for 2 animals (4 eyes) in the FL_TSG6 3.27 μg group. After 7 days of treatment assays were performed.

B. Representative photographs of corneas following lissamine green staining pre-treatment and after 7 days of treatment (post-treatment).

C. Corneal staining scores as an indicator of the amount of corneal epithelial damage pre- and post-treatment.

D. Quantification of tear production using the phenol red thread test pre- and post-treatment.

E. The number of conjunctival goblet cells per eye as counted in PAS-stained sections.

F. The number of inflammatory foci in the extraorbital lacrimal gland per eye as determined in CD3-stained sections.

In C–F mean values ± SD are shown, where each circle indicates the data from an individual eye. $^{*/\S}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns: not significant, Mann–Whitney *U* test (C) or by one-way ANOVA and Tukey's multiple-comparison test (D–F). The asterisks in C and D indicate the values relative to PBS-treated group.

Either Link_TSG6 (0.1, 1, 10 μg in 5 μL PBS) or the same volume of Restasis® were instilled BID onto the eyes of 12-week-old NOD.B10.H2^b mice for 7 days (Fig. 4A).

Results revealed that Link_TSG6 (1 and 10 μg) was considerably more effective in reducing corneal epithelial erosions than Restasis® (Fig. 4B and C), although the effects of Link_TSG6 and Restasis® on tear production were similar (Fig. 4D).

Likewise, Link_TSG6 (1 and 10 μg) caused a significant increase in the number of conjunctival goblet cells, whereas Restasis® did not have any effect on the goblet cell counts (Fig. 4E). Both Link_TSG6 (0.1, 1 and 10 μg) and Restasis® significantly decreased the number of CD3$^+$ inflammatory foci in the extraorbital lacrimal glands (Fig. 4F).

Hence, Link_TSG6 was more effective in attenuating corneal epithelial defects and preserving conjunctival goblet cells, and was equally effective in inhibiting lacrimal gland inflammation when compared directly with Restasis®.

3.4. *Preventive and therapeutic effects of Link_TSG6 in the desiccating stress model and comparison with Restasis®*

Having demonstrated the efficacy of Link_TSG6 in the NOD.B10.H2^b mouse model of ocular SjS, we went on to test whether Link_TSG6 might also be effective in the treatment of a more prevalent form of DED, i.e. EDE. For this purpose, we utilized the desiccating environmental stress model that emulates EDE, in which 7-week-old C57BL/6 mice were kept in a dry chamber with forced airflow and low humidity while receiving muscarinic blockade for 10 days. Simultaneously with desiccating injury, Link_TSG6 (0.1, 1, 10 μg in 5 μL PBS) or Restasis® were topically administered to the eyes of the C57BL/6 mice BID for 10 days (Fig. 5A). The same volume of PBS was applied in the positive control group, and C57BL/6 mice without desiccating injury served as negative controls.

Consistent with previous observations [14,19–21], desiccating injury induced corneal epithelial defects, impaired tear production, and increased the percentages of IFN-γ$^+$CD4$^+$ Th1 cells and IL-17$^+$CD4$^+$ Th17 cells in draining CLNs (Fig. 5B–D). Treatment with Link_TSG6 (1 or 10 μg) significantly prevented the development of DED as reflected by decreased corneal epithelial erosions and/or enhanced tear production, as compared to Link_TSG6 0.1 μg- or PBS-treated mice (Fig. 5B and C). Similarly, the percentages of IFN-γ$^+$CD4$^+$ Th1 cells and IL-17$^+$CD4$^+$ Th17 cells in CLNs were significantly lowered by 1 or 10 μg Link_TSG6 (Fig. 5D). Link_TSG6 (1 μg) was more effective than Restasis® in reducing corneal epithelial erosions, preserving tear production and suppressing Th1 cells (Fig. 5B and C); in this model, Restasis had no significant effect on corneal epithelial lesions or infiltration of Th1 cells into CLNs.

In addition, we investigated the therapeutic effects of Link_TSG6 on the ocular surface that had already been exposed to desiccating injury. To this end, 7-week-old C57BL/6 mice were subjected to desiccating stress (induced by dry chamber housing and muscarinic blockade) for 14 days; from day 7 to day 14 the mice were treated topically with Link_TSG6 (0.1, 1, 10 μg in 5 μL PBS) or PBS (5 μL) BID (Fig. 5E). Assays showed that Link_TSG6 (0.1, 1 or 10 μg) significantly reversed corneal

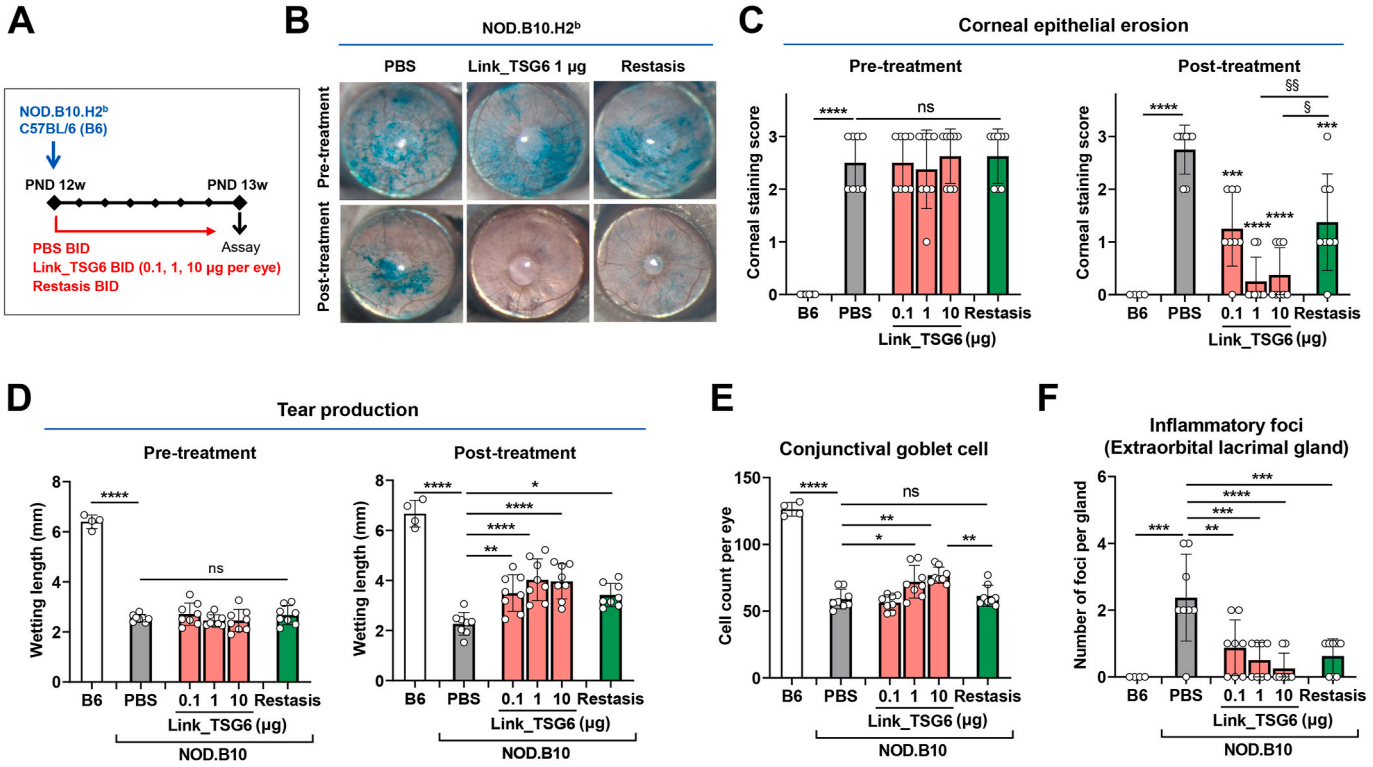

Fig. 4. Topical Link_TSG6 is more effective in treating DED than Restasis® in NOD.B10.H2^b mice.

A. Treatment protocol. 12-week-old NOD.B10.H2^b mice were treated with topical administration of Link_TSG6 (0.01–10 μg in 5 μL PBS), Restasis® (5 μL) or PBS (5 μL) BID for 7 days; C57BL/6 (B6) mice served as a negative control. 4 animals (8 eyes) were used per group except for 2 animals (4 eyes) in C57BL/6 (B6) negative control.

B. Representative photographs of corneal lissamine green staining pre-treatment and after 7 days of treatment (post-treatment).

C. Corneal staining scores following lissamine green staining pre- and post-treatment.

D. Measurement of tear production pre- and post-treatment.

E. Conjunctival goblet cell count after 7 days of treatment.

F. The number of inflammatory foci in the extraorbital lacrimal gland after 7 days of treatment.

In C–F mean values ± SD are shown, where each circle indicates the data from an individual eye. */$^{§}p < 0.05$, **/ $^{§§}p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant, as analyzed by one-way ANOVA and Tukey's multiple-comparison test. The asterisks in C indicate the values relative to PBS-treated group.

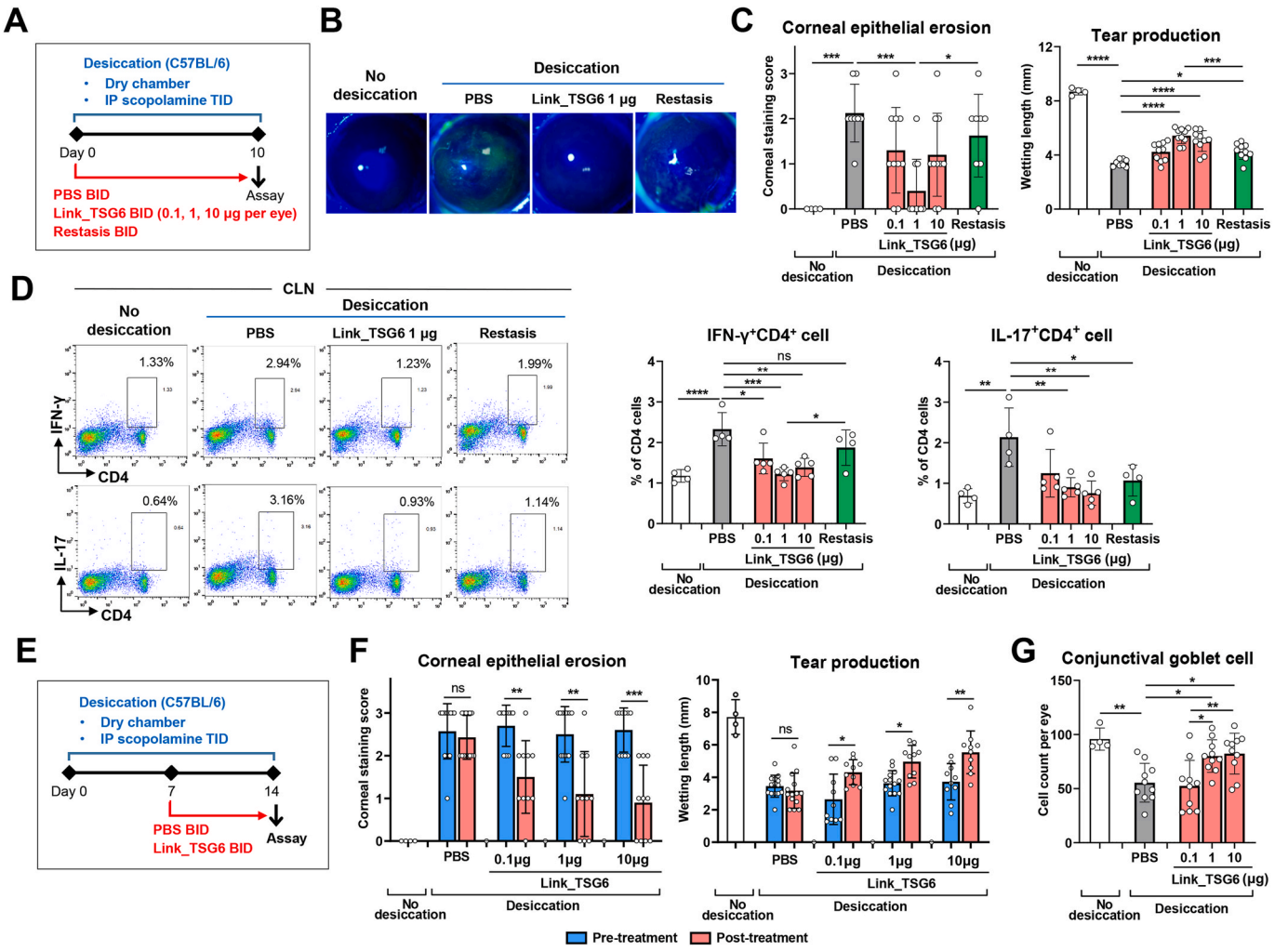

Fig. 5. Link_TSG6 has preventive and therapeutic effects on DED in an environmental desiccating stress model.

A. Experimental protocol for evaluation of preventive effect. To induce desiccating injury, 7-week-old C57BL/6 mice were placed in a dry chamber and injected intraperitoneally with scopolamine TID for 10 days, during which time topical administration of Link_TSG6 (0.1–10 μg in 5 μL PBS), Restasis® (5 μL) or PBS (5 μL) BID was performed. C57BL/6 (B6) mice that were not subjected to desiccating injury served as a negative control. 4–5 animals (8–10 eyes) were used per group except for 2 animals (4 eyes) in B6 negative control.

B. Representative photographs of corneal fluorescein staining after 10 days of treatment.

C. Quantitative analysis of corneal epithelial defects (left panel) and tear production (right panel).

D. Representative flow cytometry cytograms (left) and quantitation of IFN-γ^+CD4$^+$ cells (middle) and IL-17$^+$CD4$^+$ cells (right) in draining cervical lymph nodes (CLN).

E. Experimental protocol for evaluation of therapeutic effect. 7-week-old C57BL/6 mice were subjected to desiccating injury for 14 days (from day 0–14). Starting from day 7, the mice received topical administration of Link_TSG6 (0.1–10 μg in 5 μL PBS) or PBS (5 μL) BID for 7 days. At day 14, assays were carried out.

F. Quantitation of corneal epithelial defects (left) and tear production (right) at day 7 (before treatment start; pre-treatment) and at day 14 (7 days of treatment; post-treatment).

G. Measurement of conjunctival goblet cell number post-treatment.

In C, D, F and G mean values ± SD are shown, where each circle represents the data from an individual eye. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant, as analyzed either by one-way ANOVA and Tukey's multiple-comparison test, or by paired *t*-test for comparison between pre- and post-treatment eyes in F.

epithelial defects and restored tear production (Fig. 5F), and that Link_TSG6 (1 or 10 μg) significantly increased the number of conjunctival goblet cells (Fig. 5G).

These results collectively indicate that Link_TSG6 had both preventive and therapeutic effects in the environmental desiccating stress model.

3.5. *Effects of Link_TSG6 on corneal epithelial healing in the epithelial debridement wound model*

To investigate the direct effect of Link_TSG6 on corneal epithelial wound healing, we generated a 2-mm-diameter epithelial defect in the central cornea of 8-week-old BALB/c mice by gentle scraping with a No.13 surgical blade. Immediately after wounding, Link_TSG6 (0.1 or 1 μg in 5 μL PBS) or PBS (5 μL) was topically applied to the eyes BID for one day (Fig. 6A).

Twenty-four hours later, observation of the cornea under fluorescein vital dye staining revealed that Link_TSG6 dramatically reduced the epithelial defect area (Fig. 6B and C). A similar observation was made with regard to IL-6 mRNA transcript levels in the cornea (Fig. 6D).

4. Discussion

Decades of research have revealed that DED is a multifactorial

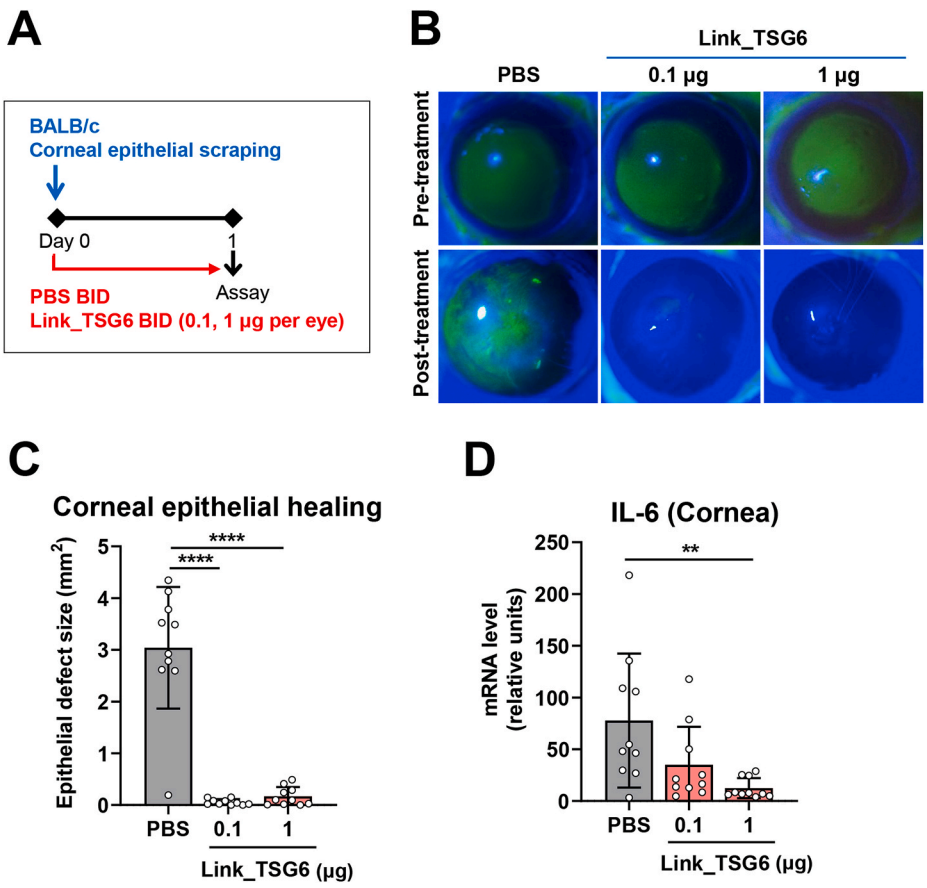

Fig. 6. Link_TSG6 accelerates healing in mice following corneal epithelial debridement wounding.
A. Experimental scheme. A 2-mm-diameter region of epithelium in the central cornea was removed by scraping in 8-week-old BALB/c mice, and the eyes were treated topically with Link_TSG6 (0.1 or 1 μg in 5 μL PBS) or PBS (5 μL) BID. 5 animals (10 eyes) were used per group. 24 h later, the corneas were extracted for assays.
B. Representative corneal photographs under a cobalt blue filter following fluorescein staining before treatment (pre-treatment) and after 24 h of treatment (post-treatment). Green-stained areas correspond to corneal epithelial defects.
C. Measurement of the corneal epithelial defect areas.
D. Real-time RT-qPCR for pro-inflammatory cytokine IL-6. The mRNA levels are presented as fold changes relative to the levels in normal corneas without injury.
In C and D mean values ± SD are shown, where each circle indicates the data from an individual eye. $^{**}p < 0.01$, $^{****}p < 0.0001$ as analyzed by one-way ANOVA and Tukey's multiple-comparison test.

disease in which an unstable and hyperosmolar tear film and inflammation play etiological roles, causing damage to the ocular surface epithelium [3]. These epithelial changes and subsequent loss of barrier function further destabilize the tear film and amplify inflammation, thereby making DED a self-perpetuating, chronic disorder [3,22]. Thus, strategies aimed both at the inhibition of inflammation and the protection (or restoration) of the epithelium are key to the development of disease-modifying DED therapies.

Currently, there are three U.S. Food and Drug Administration (FDA)-approved DED treatments: 1) cyclosporine 0.05% (Restasis®, Allergan, Irvine, CA) and 0.09% (Cequa™, Sun Pharma, Cranbury, NJ), 2) lifitegrast 5% (Xiidra®, Novartis) and 3) loteprednol etabonate 0.25% (Eysuvis®, Kala Pharmaceuticals, Watertown, MA).

Cyclosporine is a calcineurin inhibitor that blocks IL-2 activation of lymphocytes. Lifitegrast is a small molecule integrin antagonist, which blocks binding between lymphocyte function-associated antigen (LFA)-1 (CD11a, CD18) and intercellular adhesion molecule (ICAM)-1 (CD54). Both cyclosporine and lifitegrast act on T cells, suppressing activation and migration of the cells. Since its first FDA approval in 2003, topical cyclosporine has been widely used for DED treatment; however, a recent comprehensive review has revealed that there is low-to-moderate evidence for the efficacy of topical cyclosporine in the treatment of DED, mainly due to inconsistent results across studies [23]. Moreover, topical cyclosporine is frequently associated with treatment-related adverse events, leading to discontinued use in up to 29% of patients [23]. Lifitegrast 5% (Xiidra®) was approved for treatment of the signs and symptoms of DED by the FDA in 2016 [24]; however, the European Medicines Agency (EMA) recently concluded that the effectiveness of Xiidra was not demonstrated across different symptoms of DED (https://www.ema.europa.eu/en/medicines/human/withdrawn-applications/xiidra).

Although T cells are likely important players in the pathophysiology of chronic DED (e.g., based on adoptive transfer experiments in mouse desiccation-injury models [25]), other factors, including the pro-inflammatory death of cells in the ocular surface epithelium and innate immune cell activation upon desiccating stress, play a critical role in the initiation and perpetuation of DED. Therefore, it is perhaps not surprising that therapies only targeting T cells, such as cyclosporine and lifitegrast, have limited efficacy in the treatment of DED. Another FDA-approved drug Eysuvis® is a topical corticosteroid (loteprednol etabonate 0.25%). Corticosteroids rapidly suppress cells of both the innate and adaptive immune systems through induction of apoptosis and transcriptional repression of inflammatory cytokines [26–28]. However, topical corticosteroids also induce apoptosis of corneal epithelial cells [29,30], and are often associated with cataract formation and elevation of intraocular pressure, precluding their long-term use in chronic conditions like DED.

Over the past decade, we have investigated the therapeutic potential and mechanism of mesenchymal stem/stromal cells (MSCs) in the eye [8,31]. MSCs, a heterogeneous population of spindle-shaped, plastic-adherent stromal cells isolated from various connective tissues, exhibit prominent abilities to modulate both innate and adaptive immune responses and to facilitate tissue regeneration. Multiple studies by our group and others have indicated that MSCs have beneficial effects in models of DED by suppressing inflammation and promoting regeneration in the lacrimal gland and ocular surface [32–41]. In line with these preclinical data, a recently published clinical study by Moller-Hansen et al. (2021) demonstrated a rapid, dramatic, and persistent improvement in symptoms and signs of DED after an intra-lacrimal gland injection of MSCs in SjS-related DED patients [42]. Another clinical study by Liang et al. (2021) showed the safety and efficacy of subconjunctival injection of MSCs in corneal epithelial healing in patients with acute ocular burns [43]. It is well-known that MSCs exert their therapeutic effects by secreting paracrine factors in response to inflammatory stimuli [44–46]. Among the protective factors that are

produced by activated MSCs, TSG-6 has been identified to mediate the anti-inflammatory action of MSCs in the cornea [47–52] as well as in many other organ/disease systems [6].

TSG-6 is an endogenous protein that is not constitutively expressed in most adult tissues, being upregulated by inflammatory mediators or upon injury [6,53]. However, it has been found to be present in tissues which are metabolically active or subject to challenges from the environment [6], including the cornea [7]. TSG-6 provides protection and maintains homeostasis through multiple anti-inflammatory and tissue-protective properties [6]. Indeed, in previous studies, we found that topical application of recombinant human FL_TSG6 was effective in the treatment of DED by alleviating inflammation and corneal epithelial defects and improving tear production in both the NOD.B10.H2^b mouse model and the concanavalin A-induced lacrimal gland inflammation model [4,5]. The anti-inflammatory mechanism of TSG-6 involves inhibition of the migration and/or function of innate immune cells such as neutrophils [10,11,54–57], macrophages [58–65] and dendritic cells [66,67], all of which are involved in DED pathophysiology [68]. In addition, TSG-6 has been shown to influence adaptive immunity, inhibiting T cell migration and activation [66,69], e.g., by increasing the numbers of regulatory T cells and tolerogenic antigen-presenting cells [66].

As noted above, FL_TSG6 has problems that prevent successful clinical translation: instability and tendency to aggregate, poor solubility and difficulty of manufacturing at scale. By contrast, Link_TSG6, the recombinant Link module from the human TSG-6 protein, is not associated with these disadvantages; it is easier to make, is highly soluble and stable in solution, and it retains many of the activities and ligand-binding properties of the full-length protein [6].

In the present study, we demonstrated both preventive and therapeutic effects of topical Link_TSG6 administration in two DED models, the NOD.B10.H2^b mouse model and an environmental desiccating stress model. Link_TSG6 decreased corneal epithelial defects, with no lesions present in about 50% of treated eyes (e.g., after 7 days of treatment), while increasing tear production and goblet cell density. Link_TSG6 also suppressed inflammation in the ocular surface and lacrimal glands, and reduced the number of Th1 and Th17 cells in ocular draining lymph nodes. In addition, topical Link_TSG6 directly accelerated corneal epithelial wound healing in mice following epithelial debridement. Importantly, Link_TSG6 was more effective than Restasis in ameliorating clinical signs of DED and repressing inflammation of the ocular surface; moreover it was more efficacious than FL_TSG6 at reducing the extent of corneal lesions.

Link_TSG6 has been found previously to be a potent inhibitor of neutrophil migration [10,55], mediated by its interaction with CXCL8 that blocks the binding of this chemokine to heparan sulfate (e.g., on endothelial cells) and thereby modulates its bioavailability and activity [11]. Link_TSG6 also interacts with other chemokines, including CCL2, CCL5, CCL19, CCL21 and CXCL12 [11,70], which are responsible for the migration of monocytes, macrophages, dendritic cells and T lymphocytes within eye tissues in the context of DED [71,72]. From the present study it is apparent that Link_TSG6 inhibits the infiltration of CD3$^+$ T cells into lacrimal glands during DED. Link_TSG6's suppression of Th1 and Th17 cell numbers in CLNs is likely through its inhibition of the migration/function of antigen presenting dendritic cells (e.g., in a CCL19-dependent manner [72]). Link_TSG6 also reduced the expression of pro-inflammatory cytokines at the ocular surface and in lacrimal glands, as has been observed previously in an air-pouch model of acute inflammation [10], indicating that this protein has multiple anti-inflammatory effects.

In previous studies TSG-6 has been shown to directly promote the migration of corneal epithelial cells *in vitro* [4,73] and to enhance wound healing in the cornea and skin *in vivo* [6,73–75]. From the data presented here, it is apparent that Link_TSG6 retains these properties of the full-length protein, i.e., with rapid wound closure in the corneal debridement model. The mechanism underlying this is currently unclear. However, integrins have a well-established role in corneal wound healing [76] and previously it has been found that Link_TSG6 enhances the binding of fibronectin to cell surface $\alpha_5\beta_1$ [77], an interaction that is central to epithelial cell migration.

In some contexts, TSG-6's anti-inflammatory activities have been attributed to its enzymatic activity that leads to the covalent modification of the polysaccharide hyaluronan with heavy chains of the inter-alpha-inhibitor family [6]. This mechanism of action can be ruled out here since this function relies on a metal ion binding site within the TSG-6 CUB module [78] and the isolated Link module domain (Link_TSG6) lacks this activity [10].

DED is one of the most common reasons for individuals seeking medical eye care [79,80]. Nevertheless, most treatment options consist of over-the-counter preparations of artificial tears and ocular lubricants, with a limited number of prescription drugs that primarily target T cells. Given the fact that DED develops by a vicious cycle of epithelial damage, innate immune activation and subsequent adaptive immune activation, new drugs, targeting multiple steps of the pathophysiology are needed. In this regard endogenous proteins with anti-inflammatory and tissue-protective properties, such as thymosin β4 [81,82] and pigment epithelium-derived factor [83–85], are currently in development or under investigation. We herein propose that Link_TSG6 is a promising candidate as a novel DED therapeutic that can both suppress inflammation and promote epithelial regeneration/repair.

Funding

This work was supported by the National Research Foundation of Korea (2014R1A2A1A11050895, 2018R1A2B2004108, 2021R1A2C3004532 to J.Y.O.), Biotechnology and Biological Sciences Research Council (1792538 to A.J.D. and C.M.M.) and Versus Arthritis (21946 to A.J.D. and C.M.M.).

Declaration of competing interest

A.J.D., C.M.M. and J.Y.O. are co-inventors on a published patent (WO/2021/013452) and along with R.J.D. and N.K. are shareholders in Link Biologics Limited, which is developing Link_TSG6 as a treatment for DED.

Acknowledgement

We would also like to thank Giles A. Hassall for technical assistance and Sophie E Powell for her work on the development of the TFA removal/quantification method.

References

[1] Stapleton F, Alves M, Bunya VY, Jalbert I, Lekhanont K, Malet F, et al. TFOS DEWS II epidemiology report. Ocul Surf 2017;15:334–65. https://doi.org/10.1016/j.jtos.2017.05.003.

[2] Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo CK, et al. TFOS DEWS II definition and classification report. Ocul Surf 2017;15:276–83. https://doi.org/10.1016/j.jtos.2017.05.008.

[3] Bron AJ, de Paiva CS, Chauhan SK, Bonini S, Gabison EE, Jain S, et al. TFOS DEWS II pathophysiology report. Ocul Surf 2017 Jul;15(3):438–510. https://doi.org/10.1016/j.jtos.2017.05.011.

[4] Lee MJ, Kim DH, Ryu JS, Ko AY, Ko JH, Kim MK, et al. Topical TSG-6 administration protects the ocular surface in two mouse models of inflammation-related dry eye. Invest Ophthalmol Vis Sci 2015;56:5175–81. https://doi.org/10.1167/iovs.14-16307.

[5] Kim YJ, Ryu JS, Park SY, Lee HJ, Ko JH, Kim MK, et al. Comparison of topical application of TSG-6, cyclosporine, and prednisolone for treating dry eye. Cornea 2016;35:536–42. https://doi.org/10.1097/ICO.0000000000000756.

[6] Day AJ, Milner CM. TSG-6: a multifunctional protein with anti-inflammatory and tissue-protective properties. Matrix Biol 2019;78–79:60–83. https://doi.org/10.1016/j.matbio.2018.01.011.

[7] Lardner E, van Setten GB. Detection of TSG-6-like protein in human corneal epithelium. Simultaneous presence with CD44 and hyaluronic acid. J Fr Ophtalmol 2020;S0181–5512(20):30303-X. https://doi.org/10.1016/j.jfo.2020.03.005.

[8] Oh JY, Lee RH. Mesenchymal stromal cells for the treatment of ocular autoimmune diseases. Prog Retin Eye Res 2021 Mar 26:100967. https://doi.org/10.1016/j.preteyeres.2021.100967.

[9] Blundell CD, Mahoney DJ, Almond A, DeAngelis PL, Kahmann JD, Teriete P, et al. The link module from ovulation- and inflammation-associated protein TSG-6 changes conformation on hyaluronan binding. J Biol Chem 2003;278:49261–70. https://doi.org/10.1074/jbc.M309623200.
[10] Getting SJ, Mahoney DJ, Cao T, Rugg MS, Fries E, Milner CM, et al. The link module from human TSG-6 inhibits neutrophil migration in a hyaluronan- and inter-alpha -inhibitor-independent manner. J Biol Chem 2002;277:51068–76. https://doi.org/10.1074/jbc.M205121200.
[11] Dyer DP, Thomson JM, Hermant A, Jowitt TA, Handel TM, Proudfoot AE, et al. TSG-6 inhibits neutrophil migration via direct interaction with the chemokine CXCL8. J Immunol 2014;192:2177–85. https://doi.org/10.4049/jimmunol.1300194.
[12] Doyle ME, Boggs L, Attia R, Cooper LR, Saban DR, Nguyen CQ, et al. Autoimmune dacryoadenitis of NOD/LtJ mice and its subsequent effects on tear protein composition. Am J Pathol 2007;171:1224–36. https://doi.org/10.2353/ajpath.2007.070388.
[13] Barabino S, Dana MR. Animal models of dry eye: a critical assessment of opportunities and limitations. Invest Ophthalmol Vis Sci 2004;45:1641–6. https://doi.org/10.1167/iovs.03-1055.
[14] Dursun D, Wang M, Monroy D, Li DQ, Lokeshwar BL, Stern ME, et al. A mouse model of keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci 2002;43:632–8.
[15] Day AJ, Aplin RT, Willis AC. Overexpression, purification, and refolding of link module from human TSG-6 in Escherichia coli: effect of temperature, media, and mutagenesis on lysine misincorporation at arginine AGA codons. Protein Expr Purif 1996;8:1–16. https://doi.org/10.1006/prep.1996.0068.
[16] Higman VA, Blundell CD, Mahoney DJ, Redfield C, Noble ME, Day AJ. Plasticity of the TSG-6 HA-binding loop and mobility in the TSG-6-HA complex revealed by NMR and X-ray crystallography. J Mol Biol 2007;371:669–84. https://doi.org/10.1016/j.jmb.2007.05.073.
[17] Horwath-Winter J, Schneider MR, Wackernagel W, Rabensteiner D, Boldin I, Haller-Schober EM, et al. Influence of single-fraction Gamma-Knife radiosurgery on ocular surface and tear function in choroidal melanoma patients. Br J Ophthalmol 2013;97:466–70. https://doi.org/10.1136/bjophthalmol-2012-302402.
[18] Kim SJ, Lee HJ, Yun JH, Ko JH, Choi DY, Oh JY. Intravitreal TSG-6 suppresses laser-induced choroidal neovascularization by inhibiting CCR2+ monocyte recruitment. Sci Rep 2015;5:11872. https://doi.org/10.1038/srep11872.
[19] Chen Y, Chauhan SK, Lee HS, Stevenson W, Schaumburg CS, Sadrai Z, et al. Effect of desiccating environmental stress versus systemic muscarinic AChR blockade on dry eye immunopathogenesis. Invest Ophthalmol Vis Sci 2013;54:2457–64. https://doi.org/10.1167/iovs.12-11121.
[20] Guzmán M, Keitelman I, Sabbione F, Trevani AS, Giordano MN, Galletti JG. Desiccating stress-induced disruption of ocular surface immune tolerance drives dry eye disease. Clin Exp Immunol 2016;184:248–56. https://doi.org/10.1111/cei.12759.
[21] Kim KW, Ryu JS, Ko JH, Kim JY, Kim HJ, Lee HJ, et al. FUT1 deficiency elicits immune dysregulation and corneal opacity in steady state and under stress. Cell Death Dis 2020;11:285. https://doi.org/10.1038/s41419-020-2489-x.
[22] Pflugfelder SC, de Paiva CS. The pathophysiology of dry eye disease: what we know and future directions for research. Ophthalmology 2017;124:S4–13. https://doi.org/10.1016/j.ophtha.2017.07.010.
[23] de Paiva CS, Pflugfelder SC, Ng SM, Akpek EK. Topical cyclosporine A therapy for dry eye syndrome. Cochrane Database Syst Rev 2019;9:CD010051. https://doi.org/10.1002/14651858.CD010051.pub2.
[24] Lifitegrast (Xiidra) for dry eye disease. JAMA 2017;317:1473–4. https://doi.org/10.1001/jama.2016.12872.
[25] El Annan J, Chauhan SK, Ecoiffier T, Zhang Q, Saban DR, Dana R. Characterization of effector T cells in dry eye disease. Invest Ophthalmol Vis Sci 2009;50:3802–7. https://doi.org/10.1167/iovs.08-2417.
[26] Schmidt S, Rainer J, Ploner C, Presul E, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ 2004;11(Suppl 1):S45–55. https://doi.org/10.1038/sj.cdd.4401456.
[27] Stahn C, Löwenberg M, Hommes DW, Buttgereit F. Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. Mol Cell Endocrinol 2007;275:71–8. https://doi.org/10.1016/j.mce.2007.05.019.
[28] Schweingruber N, Reichardt SD, Lühder F, Reichardt HM. Mechanisms of glucocorticoids in the control of neuroinflammation. J Neuroendocrinol 2012;24:174–82. https://doi.org/10.1111/j.1365-2826.2011.02161.x.
[29] Ryu JS, Ko JH, Kim MK, Wee WR, Oh JY. Prednisolone induces apoptosis in corneal epithelial cells through the intrinsic pathway. Sci Rep 2017;7:4135. https://doi.org/10.1038/s41598-017-04509-8.
[30] Bourcier T, Forgez P, Borderie V, Scheer S, Rostène W, Laroche L. Regulation of human corneal epithelial cell proliferation and apoptosis by dexamethasone. Invest Ophthalmol Vis Sci 2000;41:4133–41.
[31] Oh JY, Kim E, Yun YI, Lee RH. Mesenchymal stromal cells for corneal transplantation: literature review and suggestions for successful clinical trials. Ocul Surf 2021;20:185–94. https://doi.org/10.1016/j.jtos.2021.02.002.
[32] Lee MJ, Ko AY, Ko JH, Lee HJ, Kim MK, Wee WR, et al. Mesenchymal stem/stromal cells protect the ocular surface by suppressing inflammation in an experimental dry eye. Mol Ther 2015;23:139–46. https://doi.org/10.1038/mt.2014.159.
[33] Aluri HS, Samizadeh M, Edman MC, Hawley DR, Armaos HL, Janga SR, et al. Delivery of bone marrow-derived mesenchymal stem cells improves tear production in a mouse model of Sjögren's syndrome. Stem Cell Int 2017;2017:3134543. https://doi.org/10.1155/2017/3134543.
[34] Abughanam G, Elkashty OA, Liu Y, Bakkar MO, Tran SD. Mesenchymal stem cells extract (MSCsE)-Based therapy alleviates xerostomia and keratoconjunctivitis sicca in Sjogren's syndrome-like disease. Int J Mol Sci 2019;20:4750. https://doi.org/10.3390/ijms20194750.
[35] Li X, Lu X, Sun D, Wang X, Yang L, Zhao S, et al. Adipose-derived mesenchymal stem cells reduce lymphocytic infiltration in a rabbit model of induced autoimmune dacryoadenitis. Invest Ophthalmol Vis Sci 2016;57:5161–70. https://doi.org/10.1167/iovs.15-17824.
[36] Lu X, Li N, Zhao L, Guo D, Yi H, Yang L, et al. Human umbilical cord mesenchymal stem cells alleviate ongoing autoimmune dacryoadenitis in rabbits via polarizing macrophages into an anti-inflammatory phenotype. Exp Eye Res 2020;191:107905. https://doi.org/10.1016/j.exer.2019.107905.
[37] Dietrich J, Ott L, Roth M, Witt J, Geerling G, Mertsch S, et al. MSC transplantation improves lacrimal gland regeneration after surgically induced dry eye disease in mice. Sci Rep 2019;9:18299. https://doi.org/10.1038/s41598-019-54840-5.
[38] Park SA, Reilly CM, Wood JA, Chung DJ, Carrade DD, Deremer SL, et al. Safety and immunomodulatory effects of allogeneic canine adipose-derived mesenchymal stromal cells transplanted into the region of the lacrimal gland, the gland of the third eyelid and the knee joint. Cytotherapy 2013;15:1498–510. https://doi.org/10.1016/j.jcyt.2013.06.009.
[39] Villatoro AJ, Fernández V, Claros S, Rico-Llanos GA, Becerra J, Andrades JA. Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model. BioMed Res Int 2015;2015:527926. https://doi.org/10.1155/2015/527926.
[40] Bittencourt MK, Barros MA, Martins JF, Vasconcellos JP, Morais BP, Pompeia C, et al. Allogeneic mesenchymal stem cell transplantation in dogs with keratoconjunctivitis sicca. Cell Med 2016;8:63–77. https://doi.org/10.3727/215517916X693366.
[41] Sgrignoli MR, Silva DA, Nascimento FF, Sgrignoli DAM, Nai GA, da Silva MG, et al. Reduction in the inflammatory markers CD4, IL-1, IL-6 and TNFα in dogs with keratoconjunctivitis sicca treated topically with mesenchymal stem cells. Stem Cell Res 2019;39:101525. https://doi.org/10.1016/j.scr.2019.101525.
[42] Møller-Hansen M, Larsen AC, Toft PB, Lynggaard CD, Schwartz C, Bruunsgaard H, et al. Safety and feasibility of mesenchymal stem cell therapy in patients with aqueous deficient dry eye disease. Ocul Surf 2021;19:43–52. https://doi.org/10.1016/j.jtos.2020.11.013.
[43] Liang L, Luo X, Zhang J, Su W, Zhu W, Xie Y, et al. Safety and feasibility of subconjunctival injection of mesenchymal stem cells for acute severe ocular burns: a single-arm study. Ocul Surf 2021;22:103–9. https://doi.org/10.1016/j.jtos.2021.07.008.
[44] Prockop DJ, Oh JY. Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. J Cell Biochem 2012;113:1460–9. https://doi.org/10.1002/jcb.24046.
[45] Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol Ther 2012;20:14–20. https://doi.org/10.1038/mt.2011.211.
[46] Lee RH, Oh JY, Choi H, Bazhanov N. Therapeutic factors secreted by mesenchymal stromal cells and tissue repair. J Cell Biochem 2011;112:3073–8. https://doi.org/10.1002/jcb.23250.
[47] Roddy GW, Oh JY, Lee RH, Bartosh TJ, Ylostalo J, Coble K, et al. Action at a distance: systemically administered adult stem/progenitor cells (MSCs) reduce inflammatory damage to the cornea without engraftment and primarily by secretion of TNF-α stimulated gene/protein 6. Stem Cell 2011;29:1572–9. https://doi.org/10.1002/stem.708.
[48] Yun YI, Park SY, Lee HJ, Ko JH, Kim MK, Wee WR, et al. Comparison of the anti-inflammatory effects of induced pluripotent stem cell-derived and bone marrow-derived mesenchymal stromal cells in a murine model of corneal injury. Cytotherapy 2017;19:28–35. https://doi.org/10.1016/j.jcyt.2016.10.007.
[49] Lee RH, Yu JM, Foskett AM, Peltier G, Reneau JC, Bazhanov N, et al. TSG-6 as a biomarker to predict efficacy of human mesenchymal stem/progenitor cells (hMSCs) in modulating sterile inflammation in vivo. Proc Natl Acad Sci U S A 2014;111:16766–71. https://doi.org/10.1073/pnas.1416121111.
[50] Oh JY, Lee RH, Yu JM, Ko JH, Lee HJ, Ko AY, et al. Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. Mol Ther 2012;20:2143–52. https://doi.org/10.1038/mt.2012.165.
[51] Song HB, Park SY, Ko JH, Park JW, Yoon CH, Kim DH, et al. Mesenchymal stromal cells inhibit inflammatory lymphangiogenesis in the cornea by suppressing macrophage in a TSG-6-dependent manner. Mol Ther 2018;26:162–72. https://doi.org/10.1016/j.ymthe.2017.09.026.
[52] Zhang N, Luo X, Zhang S, Liu R, Liang L, Su W, et al. Subconjunctival injection of tumor necrosis factor-α pre-stimulated bone marrow-derived mesenchymal stem cells enhances anti-inflammation and anti-fibrosis in ocular alkali burns. Graefes Arch Clin Exp Ophthalmol 2021;259:929–40. https://doi.org/10.1007/s00417-020-05017-8.
[53] Milner CM, Day AJ. TSG-6: a multifunctional protein associated with inflammation. J Cell Sci 2003;116:1863–73. https://doi.org/10.1242/jcs.00407.
[54] Wisniewski HG, Naime D, Hua JC, Vilcek J, Cronstein BN. TSG-6, a glycoprotein associated with arthritis, and its ligand hyaluronan exert opposite effects in a murine model of inflammation. Pflügers Archiv 1996;431:R225–6. https://doi.org/10.1007/BF02346350.
[55] Cao TV, La M, Getting SJ, Day AJ, Perretti M. Inhibitory effects of TSG-6 Link module on leukocyte-endothelial cell interactions in vitro and in vivo. Microcirculation 2004;11:615–24. https://doi.org/10.1080/10739680490503438.
[56] Szántó S, Bárdos T, Gál I, Glant TT, Mikecz K. Enhanced neutrophil extravasation and rapid progression of proteoglycan-induced arthritis in TSG-6-knockout mice. Arthritis Rheum 2004;50:3012–22. https://doi.org/10.1002/art.20655.

[57] Magaña-Guerrero FS, Domínguez-López A, Martínez-Aboytes P, Buentello-Volante B, Garfias Y. Human amniotic membrane mesenchymal stem cells inhibit neutrophil extracellular traps through TSG-6. Sci Rep 2017;7:12426. https://doi.org/10.1038/s41598-017-10962-2.
[58] Choi H, Lee RH, Bazhanov N, Oh JY, Prockop DJ. Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. Blood 2011;118:330–8. https://doi.org/10.1182/blood-2010-12-327353.
[59] Oh JY, Choi H, Lee RH, Roddy GW, Ylöstalo JH, Wawrousek E, et al. Identification of the HSPB4/TLR2/NF-κB axis in macrophage as a therapeutic target for sterile inflammation of the cornea. EMBO Mol Med 2012;4:435–48. https://doi.org/10.1002/emmm.201200221.
[60] Mittal M, Tiruppathi C, Nepal S, Zhao YY, Grzych D, Soni D, et al. TNFα-stimulated gene-6 (TSG6) activates macrophage phenotype transition to prevent inflammatory lung injury. Proc Natl Acad Sci U S A 2016;113:E8151–8. https://doi.org/10.1073/pnas.1614935113.
[61] Watanabe R, Sato Y, Ozawa N, Takahashi Y, Koba S, Watanabe T. Emerging roles of tumor necrosis factor-stimulated gene-6 in the pathophysiology and treatment of atherosclerosis. Int J Mol Sci 2018;19:465. https://doi.org/10.3390/ijms19020465.
[62] Zhao Y, Zhu XY, Song T, Zhang L, Eirin A, Conley S, et al. Mesenchymal stem cells protect renal tubular cells via TSG-6 regulating macrophage function and phenotype switching. Am J Physiol Ren Physiol 2021;320:F454–63. https://doi.org/10.1152/ajprenal.00426.2020.
[63] Liu Y, Zeng R, Wang Y, Huang W, Hu B, Zhu G, et al. Mesenchymal stem cells enhance microglia M2 polarization and attenuate neuroinflammation through TSG-6. Brain Res 2019;1724:146422. https://doi.org/10.1016/j.brainres.2019.146422.
[64] Song WJ, Li Q, Ryu MO, Ahn JO, Bhang DH, Jung YC, et al. TSG-6 secreted by human adipose tissue-derived mesenchymal stem cells ameliorates DSS-induced colitis by inducing M2 macrophage polarization in mice. Sci Rep 2017;7:5187. https://doi.org/10.1038/s41598-017-04766-7.
[65] Galipeau J. Macrophages at the nexus of mesenchymal stromal cell potency: the emerging role of chemokine cooperativity. Stem Cell 202;36:1145-1154. doi: 10.1002/stem.3380.
[66] Kota DJ, Wiggins LL, Yoon N, Lee RH. TSG-6 produced by hMSCs delays the onset of autoimmune diabetes by suppressing Th1 development and enhancing tolerogenicity. Diabetes 2013;62:2048–58. https://doi.org/10.2337/db12-0931.
[67] Kui L, Chan GC, Lee PP. TSG-6 downregulates IFN-alpha and TNF-alpha expression by suppressing IRF7 phosphorylation in human plasmacytoid dendritic cells. Mediat Inflamm 2017;2017:7462945. https://doi.org/10.1155/2017/7462945.
[68] Perez VL, Stern ME, Pflugfelder SC. Inflammatory basis for dry eye disease flares. Exp Eye Res 2020;201:108294. https://doi.org/10.1016/j.exer.2020.108294.
[69] Kato T, Okumi M, Tanemura M, Yazawa K, Kakuta Y, Yamanaka K, et al. Adipose tissue-derived stem cells suppress acute cellular rejection by TSG-6 and CD44 interaction in rat kidney transplantation. Transplantation 2014;98:277–84. https://doi.org/10.1097/TP.0000000000000230.
[70] Dyer DP, Salanga CL, Johns SC, Valdambrini E, Fuster MM, Milner CM, et al. The anti-inflammatory protein TSG-6 regulates chemokine function by inhibiting chemokine/glycosaminoglycan interactions. J Biol Chem 2016;291:12627–40. https://doi.org/10.1074/jbc.M116.720953.
[71] Nicolle P, Liang H, Reboussin E, Rabut G, Warcoin E, Brignole-Baudouin F, et al. Proinflammatory markers, chemokines, and enkephalin in patients suffering from dry eye disease. Int J Mol Sci 2018;19:1221. https://doi.org/10.3390/ijms19041221.
[72] Wang T, Li W, Cheng H, Zhong L, Deng J, Ling S. The important role of the chemokine Axis CCR7-CCL19 and CCR7-CCL21 in the pathophysiology of the immuno-inflammatory response in dry eye disease. Ocul Immunol Inflamm 2021;29:266–77. https://doi.org/10.1080/09273948.2019.1674891.
[73] Di G, Du X, Qi X, Zhao X, Duan H, Li S, et al. Mesenchymal stem cells promote diabetic corneal epithelial wound healing through TSG-6-dependent stem cell activation and macrophage switch. Invest Ophthalmol Vis Sci 2017;58:4344–54. https://doi.org/10.1167/iovs.17-21506.
[74] Shakya S, Mack JA, Alipour M, Maytin EV. Cutaneous wounds in mice lacking TSG-6 exhibit delayed closure and an abnormal inflammatory response. J Invest Dermatol 2020;140:2505–14. https://doi.org/10.1016/j.jid.2020.04.015.
[75] Qi Y, Jiang D, Sindrilaru A, Stegemann A, Schatz S, Treiber N, et al. TSG-6 released from intradermally injected mesenchymal stem cells accelerates wound healing and reduces tissue fibrosis in murine full-thickness skin wounds. J Invest Dermatol 2014;134:526–37. https://doi.org/10.1038/jid.2013.328.
[76] Carter RT. The role of integrins in corneal wound healing. Vet Ophthalmol 2009;12 (Suppl 1):2–9. https://doi.org/10.1111/j.1463-5224.2009.00726.x.
[77] Kuznetsova SA, Mahoney DJ, Martin-Manso G, Ali T, Nentwich HA, Sipes JM, et al. TSG-6 binds via its CUB_C domain to the cell-binding domain of fibronectin and increases fibronectin matrix assembly. Matrix Biol 2008;27:201–10. https://doi.org/10.1016/j.matbio.2007.10.003.
[78] Briggs DC, Birchenough HL, Ali T, Rugg MS, Waltho JP, Ievoli E, et al. Metal ion-dependent heavy chain transfer activity of TSG-6 mediates assembly of the cumulus-oocyte matrix. J Biol Chem 2015;290:28708–23. https://doi.org/10.1074/jbc.M115.669838.
[79] Khan RS, Rizvi S, Syed BA, Bielory L. Current market trends in anterior ocular inflammatory disease landscape. Curr Opin Allergy Clin Immunol 2019;19:503–9. https://doi.org/10.1097/ACI.0000000000000562.
[80] Bradley JL, Özer Stillman I, Pivneva I, Guerin A, Evans AM, Dana R. Dry eye disease ranking among common reasons for seeking eye care in a large US claims database. Clin Ophthalmol 2019;13:225–32. https://doi.org/10.2147/OPTH.S188314.
[81] Sosne G, Ousler GW. Thymosin beta 4 ophthalmic solution for dry eye: a randomized, placebo-controlled, Phase II clinical trial conducted using the controlled adverse environment (CAE™) model. Clin Ophthalmol 2015;9:877–84. https://doi.org/10.2147/OPTH.S80954.
[82] Sosne G. Thymosin beta 4 and the eye: the journey from bench to bedside. Expet Opin Biol Ther 2018;18:99–104. https://doi.org/10.1080/14712598.2018.1486818.
[83] Ma B, Zhou Y, Liu R, Zhang K, Yang T, Hu C, et al. Pigment epithelium-derived factor (PEDF) plays anti-inflammatory roles in the pathogenesis of dry eye disease. Ocul Surf 2021;20:70–85. https://doi.org/10.1016/j.jtos.2020.12.007.
[84] Singh RB, Blanco T, Mittal SK, Alemi H, Chauhan SK, Chen Y, et al. Pigment epithelium-derived factor enhances the suppressive phenotype of regulatory T cells in a murine model of dry eye disease. Am J Pathol 2021;191:720–9. https://doi.org/10.1016/j.ajpath.2021.01.003.
[85] Singh RB, Blanco T, Mittal SK, Taketani Y, Chauhan SK, Chen Y, et al. Pigment Epithelium-derived Factor secreted by corneal epithelial cells regulates dendritic cell maturation in dry eye disease. Ocul Surf 2020;18:460–9. https://doi.org/10.1016/j.jtos.2020.05.002.