

# Topical Administration of Lacritin Is a Novel Therapy for Aqueous-Deficient Dry Eye Disease

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**PURPOSE.** Lacritin is a tear glycoprotein with prosecretory, prosurvival, and mitogenic properties. We examined lacritin levels in the tears of Sjögren's syndrome (SS) patients and explored the therapeutic potential of topical lacritin for the treatment of keratoconjunctivitis sicca.

**METHODS.** Tears from healthy controls ( $n = 14$ ) and SS patients ( $n = 15$ ) were assayed for lacritin using a C-terminal antibody. In a paired-eye study, autoimmune regulator (Aire) knockout (KO) mice ( $n = 7$ ) were treated three times daily for 21 days with 10  $\mu$ L of 4  $\mu$ M lacritin (left eye) or vehicle (PBS) control (right eye). Tear secretion and ocular surface integrity were assessed at baseline and after treatment. Immunohistochemical staining of CD4+ T cells, cytokeratin-10 (K10), and cytokeratin-12 (K12) expression in the cornea and CD4+ T cell infiltration in the lacrimal glands were assessed.

**RESULTS.** Lacritin monomer ( $421.8 \pm 65.3$  ng [SS] vs.  $655.8 \pm 118.9$  ng [controls];  $P = 0.05$ ) and C-terminal fragment protein ( $125 \pm 34.1$  ng [SS] vs.  $399.5 \pm 84.3$  ng [controls];  $P = 0.008$ ) per 100  $\mu$ L of tear eluate were significantly lower in SS patients. In Aire KO mice treated with lacritin, tear secretion increased by 46% ( $13.0 \pm 3.5$  mm vs.  $8.9 \pm 2.9$  mm;  $P = 0.01$ ) and lissamine green staining score significantly decreased relative to baseline ( $-0.417 \pm 0.06$  vs.  $0.125 \pm 0.07$ ;  $P = 0.02$ ). Expression of K10 but not K12 in the cornea was significantly decreased in lacritin-treated eyes. Focal CD4+ T cell infiltration of the lacrimal glands was significantly reduced on the lacritin-treated side versus the untreated side.

**CONCLUSIONS.** Lacritin is significantly reduced in the tears of SS patients. Topically administered lacritin has therapeutic potential for the treatment of aqueous-deficient dry eye disease.

**Keywords:** aqueous-deficient dry eye, topical therapeutics, Sjögren's syndrome, tears protein, lacritin

Dry Eye Disease (DED) affects nearly 5 million Americans aged 50 years and older. The Dry Eye WorkShop (DEWS) committee proposed two types of DED: evaporative and aqueous-deficient.<sup>1</sup> In autoimmune diseases like Sjögren's syndrome (SS), lymphocytic targeting of the tear-producing lacrimal glands is thought to depress basal secretory capacity, giving rise to aqueous-deficient dry eye, known clinically as keratoconjunctivitis sicca (KCS). Keratoconjunctivitis sicca is among the most common and debilitating manifestations of SS. As KCS progresses, the ocular surface can undergo a process of transdifferentiation from a nonkeratinized, mucosal epithelium to one that is pathologically keratinized and deficient in mucus-secreting goblet cells. This process, known as squamous metaplasia, is a devastating, end-stage consequence of dry eye disease that causes considerable morbidity.

While the DEWS committee clearly established the essential role of inflammation in the pathogenesis of dry eye, the inflammatory response is likely, in part, the downstream consequence of desiccating stress resulting from reduced tear secretion. In the clinic, dry eye disease is often treated using a

combination of artificial tears and anti-inflammatory agents such as cyclosporine A and corticosteroids. While these agents have been shown to improve the signs and symptoms of dry eye disease, long-term use of corticosteroids carries considerable risk and the effectiveness of aggressive lubrication is limited. Thus, the development of alternative therapeutic approaches to enhance tear secretion and minimize cellular stress in dry eye patients is worth consideration.

In recent years, the therapeutic potential of endogenous tear glycoprotein lacritin has been gaining attention for its prosecretory and mitogenic properties.<sup>2,3</sup> Lacritin has been shown to promote basal tearing when topically added to eyes of normal rabbits<sup>4</sup> and rescue cultured human corneal epithelial cells from inflammatory cytokine stress, including stress-induced by tears from dry eye patients.<sup>5</sup> Lacritin is one of a few eye-selective glycoproteins secreted by components of the lacrimal functional unit into the tears and its discovery emerged indirectly from a screen for novel factors capable of addressing dry eye.<sup>6</sup> Although readily detected in basal tears,<sup>7</sup>

expression is greatest by acinar cells of the lacrimal gland from which it is released upon carbachol stimulation.<sup>8</sup>

As a tear protein that promotes basal secretion and maintains ocular surface integrity, it is perhaps not surprising that lacritin has been reported to be decreased in patients with dry eye disease.<sup>9,10</sup> Lacritin is detected in tears as an active monomer of ~25 kD and as inactive, tissue transglutaminase generated dimers, trimers, and larger polymers. The ~12- to 15-kD C-terminal fragment with activity retained is also apparent in normal tears.<sup>11</sup> To our knowledge, the level of lacritin has never been reported in patients with aqueous-deficient dry eye resulting from SS. We hypothesized that if a lacritin monomer and/or bioactive C-terminal fragment were deficient in tears of SS patients then, restoring active lacritin might offer therapeutic benefit.

Here, we explored lacritin levels in the tears of SS patients and the potential therapeutic benefits of lacritin as a topical intervention using a murine model of autoimmune-mediated aqueous-deficient dry eye. Mice deficient in the autoimmune regulator (*Aire*) gene spontaneously develop a CD4<sup>+</sup> T cell-mediated disease of the lacrimal glands and eyes that mimics the clinical characteristics of SS,<sup>12,13</sup> including reduced tear secretion, loss of mucosal epithelial integrity, and lymphocytic infiltration of the ocular surface and lacrimal gland. Our studies show significantly reduced levels of lacritin protein in the tears of SS patients compared with healthy controls. Moreover, topical lacritin promoted tear secretion, restored ocular surface integrity, and reduced focal infiltration of CD4<sup>+</sup> T cells to the lacrimal glands of *Aire*-deficient mice. These results provide the first in vivo evidence supporting lacritin's potential utility in the treatment of aqueous-deficient dry eye.

## MATERIALS AND METHODS

### Materials

Rat anti-mouse CD4 (550280) was from BD Pharmingen (Franklin Lakes, NJ, USA), antibodies against cytokeratin-10 (K10) (sc-31770) and K12 (sc-17101) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); AlexaFluor 488-conjugated donkey anti-goat was from Invitrogen (Carlsbad, CA, USA); Horseradish peroxidase (HRP)-conjugated donkey anti-Rat IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Triton X-100, Tween 20, and pilocarpine were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Diaminobenzidine (DAB) kit and antibody diluent were from DAKO North America (Carpinteria, CA, USA). Antigen retrieval citrate buffer (pH 6.0) was from Invitrogen. Mounting medium (FluorSave) was from Millipore Corp. (Billerica, MA, USA). Lissamine green stain was from Leiter's Pharmacy and Compounding Center (San Jose, CA, USA). Diagnostic tear test (Zone-Quick Thread Tears) were from Showa Yakuhin Kako Co., Ltd. (Tokyo, Japan), distributed by Menicon American, Inc. (San Mateo, CA, USA).

Nitrocellulose membrane and ECL reagents were from Amersham, GE Healthcare (Little Chalfont, UK). Horseradish peroxidase-conjugated donkey anti-rabbit antibody was from Jackson ImmunoResearch Laboratories, Inc. Lacritin was generated in *E. coli* from the pLAC plasmid as an intein fusion protein. Expressed lacritin was purified on chitin columns with mercaptoethanol elution that cleaves off the intein tag. Further purification was performed on diethylaminoethanol columns, all as previously described.<sup>4</sup> The recombinant lacritin is approximately 18 kD in size. Purified lacritin is stable, without cleavage or degradation, for 6 months when lyophilized and stored frozen. When reconstituted, it is stable at either 4°C or room temperature for a 24-hour period. A new vial of

lyophilized lacritin was reconstituted on each day of the treatment period. Lacritin truncation mutant lacking 25 amino acids from its C-terminus (C-25) was synthesized by previously described methods.<sup>14</sup> Lacritin antibody referred to as *anti-N-65 Lac C-term* was produced in rabbits against the C-terminal end of human recombinant lacritin using the N-65 truncation mutant.<sup>11</sup>

### Animals

Nonobese diabetic (NOD) and Balb/c mice with targeted mutation in the *Aire* gene were gifts from Mark Anderson, MD, University of California San Francisco. *Aire* gene-deficient mice on the NOD and BALB/c background share a similar ocular phenotype but differ slightly in rate of disease development. We considered both backgrounds for our initial evaluation of lacritin's therapeutic potential and found the results to be nearly identical. All mice were housed in a pathogen-free barrier facility at UCSF Offspring were genotyped for the *Aire* mutation by PCR of genomic DNA from tail clippings. All experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Treatment Regimen

*Aire* knockout (KO) mice on the NOD background, aged 5 to 6 weeks old and on the BALB/c background, aged 6 to 7 weeks old, were used for the study. All mice ( $n = 7$ ) received 10  $\mu$ L of 50  $\mu$ g/mL or 4  $\mu$ M lacritin in the left eye and 10  $\mu$ L of PBS in the right eye. Dosing was three times daily for 21 days. Lissamine green staining assessment and tear secretion assays were performed on each eye before treatment at baseline, 1 and 3 weeks after treatment.

### Lissamine Green Staining and Quantification

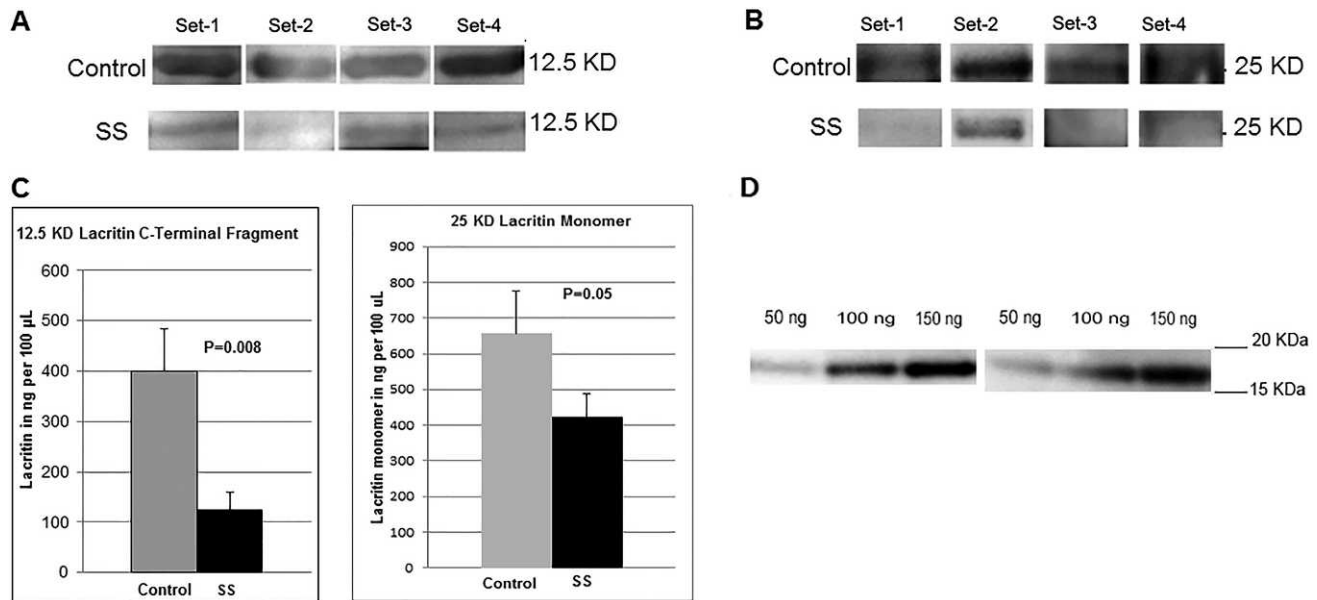
After anesthesia with isoflurane, 5  $\mu$ L of 1% lissamine green was applied to the ocular surface. Photographs of the eye were taken using a digital camera (Nikon Corp., Tokyo, Japan) fitted to a stereo microscope (Olympus Zoom Stereo; Olympus America, Inc., Center Valley, PA, USA). Images were scored on a 4-point scale by four independent, trained, and masked observers. A score of 1 represented less than 25% of the ocular surface stained positive with lissamine dye; a score of 2 represented 25% to 50% stained positive; 3 represented 50% to 75% stained positive; and 4 represented greater than 75% of the ocular surface with positive staining. The average score from all four observers was used for each eye. The difference in the staining score at the end of weeks 1 and 3 with respect to the pretreatment baseline was reported.

### Tear Secretion Assay

Tear secretion was quantified following intraperitoneal injection of 4.5 mg/kg pilocarpine. Ten minutes after injection, mice were anesthetized with isoflurane and tear secretion measured by millimeters of wetting on a phenol red thread (Showa Yakuhin Kako Co., Ltd.). The difference in tear secretion between lacritin and PBS-treated eyes was reported at multiple time points.

### Immunohistochemical Staining

We detected CD4-expressing T cells and K10- and K12-expressing corneal epithelial cells by immunofluorescence on frozen tissue sections fixed in acetone. Briefly, the sections were fixed in ice-cold acetone at 20°C for 10 minutes followed by washing with PBS. Tissues were permeabilized with 0.2%



**FIGURE 1.** Active lacritin monomer and C-terminal fragment are reduced in the tears of SS patients. Western blot results for (A) ~12.5-kD C-terminal fragment and (B) 25-kD lacritin monomer in control and SS patient tears. Four representative sets of tear samples from SS and control patients are shown. (C) Estimated concentration of 12.5-kD C-terminal fragment and 25-kD lacritin monomer for control and SS patient tears. (D) Two representative examples of standard curves generated using increasing concentrations of 18-kD recombinant lacritin.

Triton X-100 for 10 minutes and after washing in PBS, were blocked for 1 to 2 hours in 5% BSA plus 3% serum derived from the species in which the secondary antibody was made. Sections were incubated at 4°C overnight with primary antibodies anti-CD4 (BD Pharmingen); anti-K10 and anti-K12 (Santa Cruz Biotechnology, Inc.), each diluted 1/50 in Dako antibody diluent. After washing with PBS-Tween, fluorescein (Invitrogen) or peroxidase-labeled (Jackson ImmunoResearch Laboratories, Inc.) secondary antibody (1:400 in Dako antibody diluent) was added for 1 hour at room temperature. Sections for immunofluorescence staining were counterstained with DAPI and then cover-slipped with aqueous fluorescent preserving mounting medium (Millipore Corp.). Sections with peroxidase labeled anti-CD4 immunconjugates were developed with the chromogen DAB in the presence of substrate H<sub>2</sub>O<sub>2</sub>, per manufacturer's instructions. After rinsing in PBS, the sections were dehydrated and cover-slipped in nonaqueous mounting medium.

### Human Tear Samples

All human tear samples were collected and handled in accordance to human study guidelines approved by the Committee for Human Research at the University of California, San Francisco and followed the tenets of the Declaration of Helsinki. Details of the study were explained and informed consent was obtained. Tear samples from SS patients and age-matched healthy controls were collected using Schirmer strips at the UCSF Proctor Foundation (three controls and five SS samples) or provided through the Sjögren's International Collaborative Clinical Alliance tissue repository (11 controls and 10 SS samples). Diagnostic criteria for Sjögren's syndrome were consistent with established American College of Rheumatology (ACR) criteria.<sup>15</sup> One of the 29 participants had an associated autoimmune disease. Schirmer strips were stored in a -80°C freezer until analysis. At the time of analysis, the wetted portion of the Schirmer strip was cut into smaller pieces and immersed in 100  $\mu$ L of PBS for 3 hours at room temperature. Eluted tear proteins were assessed by Western blot.

### Western Blotting

Eluted tear samples and known quantities of recombinant lacritin (18 kD) were loaded onto 4% to 12% or 12% protein gels (Novex Nupage Bis-Tris; Life Technologies, Inc.), electrophoresed at 150 volts, and transferred to nitrocellulose membranes (Amersham Hybond ECL; GE Healthcare). Blots were blocked with 5% BSA, incubated with anti-N-65 Lac C-term antibody (1:1000 in blocking buffer) overnight at 4°C. After washing with PBS-Tween (0.05%), blots were incubated for 1 hour at room temperature with HRP-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:20,000. Blots were developed via chemiluminescence with Western blotting substrate (Amersham ECL; GE Healthcare) and imaged using a commercial imaging system (Fuji LAS400; Fuji Medical Systems, Stamford, CT, USA).

### Estimation of Lacritin Tear Protein

Along with tear eluate, known quantities (25–200 ng) of 18 kD recombinant lacritin were loaded onto each gel to generate a standard curve. The signal intensity of lacritin bands was determined by multiplying the area of the band with the average pixel intensity, which provided volume-sum intensity for each band. The volume-sum intensity of the recombinant lacritin bands was used to plot an internal standard curve for each experiment. The amount of lacritin protein in tear samples was estimated using the regression equation generated by the standard curve.

### Image Quantification

Trained and masked observers quantified immunofluorescent images of K10 and K12 staining. The percentage of corneal epithelium stained positive by anti-K10 or anti-K12 antibody was determined for each image. The average percentage staining was used for further analysis. CD4+ cells in the cornea and limbus were assessed by manual counting and expressed per 100,000 square pixel area of the limbus and per 1000 pixel units of length of the cornea. In the lacrimal glands, there was



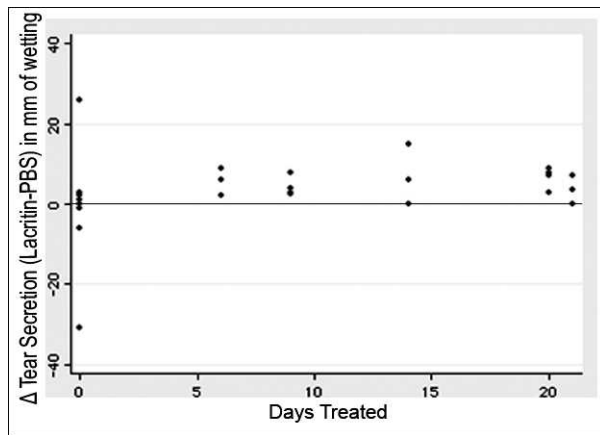


FIGURE 2. Topical lacritin increases tear secretion. The difference in tear secretion between lacritin and PBS-treated eyes of Aire KO mice at multiple time points during treatment is shown. Data points above the zero line indicate improved tear secretion.

noticeable focal infiltration of the CD4<sup>+</sup> T cells; CD4<sup>+</sup> T cell foci were manually counted by seven knowledgeable masked graders and number of foci per millimeter square was reported. Tissue area was defined by the region of interest (ROI) tool of the imaging software (Nikon NIS-Elements; Nikon Instruments, Inc., Tokyo, Japan).

### Statistical Analysis

The Shapiro-Wilk test was used to test whether datasets were normally distributed. We used a *t*-test or one-way ANOVA to examine statistical significance for normally distributed data, and the Kruskal-Wallis test for nonparametric analysis. The specific *P* values for each test are reported. Each experiment was performed at least twice.

## RESULTS

### Lacritin Protein Is Reduced in the Tears of SS Patients

Lacritin is detected in normal tears as an active monomer of ~25 kD, an active ~12.5 kD C-terminal fragment and as inactive, tissue transglutaminase-generated dimers, trimers, and larger polymers. We evaluated levels of C-terminal fragment (Fig. 1A) and active lacritin monomer (Fig. 1B) in human tears from 14 healthy controls and 15 SS patients by Western blot and estimated the levels by densitometry (Fig. 1C). Estimated levels of the C-terminal fragment protein ( $125 \pm 34.1$  ng in SS versus  $399.5 \pm 84.3$  ng per 100  $\mu$ L tear eluate in controls;  $P < 0.01$ ) were decreased in SS tears. Similarly, lacritin monomer ( $421.8 \pm 65.3$  ng in SS versus  $655.8 \pm 118.9$  ng per 100  $\mu$ L tear eluate in controls;  $P = 0.05$ ) was significantly lower in SS tears compared with healthy controls.

### Topical Lacritin Promotes Tear Secretion

Next, we assessed the therapeutic potential of lacritin as a secretagogue for basal tearing in aqueous-deficient dry eye. Aire KO mice suffer from reduced basal- and pilocarpine-stimulated tear secretion as early as 4 weeks. When left untreated, tear secretion progressively decreases to less than approximately 50% to 80% of initial tearing.<sup>16</sup> However, within 1 week of initiating topical therapy, Aire KO eyes ( $n = 7$ ) treated with lacritin benefited from a 32% increase in tearing

( $11.2 \pm 2.0$  mm of wetting with lacritin versus  $8.5 \pm 2.1$  mm with PBS;  $P < 0.001$ ). By 3 weeks, tearing had further increased to 46% ( $13.0 \pm 3.5$  mm with lacritin versus  $8.9 \pm 2.9$  mm with PBS;  $P = 0.01$ ). The difference in tear secretion between lacritin and PBS-treated eyes of Aire KO mice at multiple time points during treatment is shown in Figure 2. Data points above the zero line indicate improved tear secretion. Thus, lacritin appeared to elicit a prosecretory function in the eyes of Aire KO mice.

### Topical Lacritin Maintains Ocular Surface Integrity

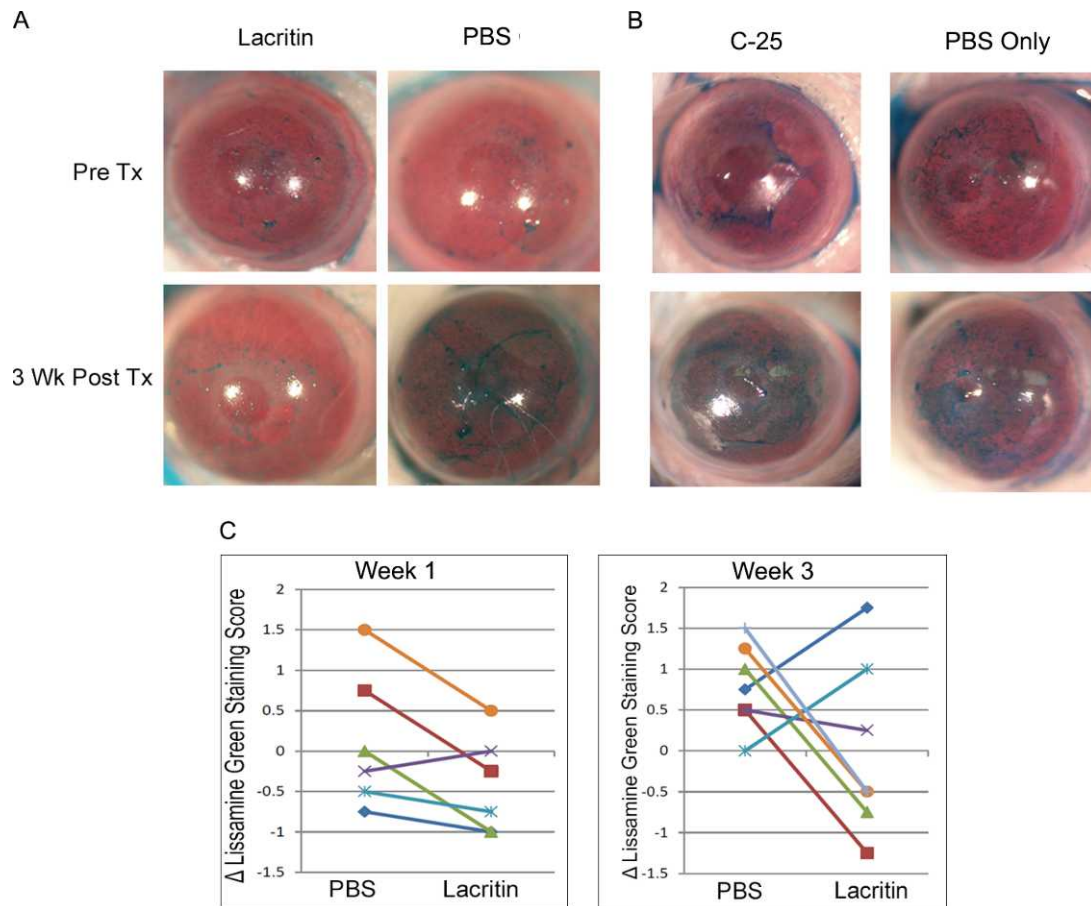
Lissamine green stains damaged epithelial cells lining the ocular mucosal surface to provide a read out of ocular surface damage. When left untreated, Aire KO mice have progressive epithelial damage to the ocular surface and increased lissamine green staining starting as early as 4 weeks of age.<sup>12,17</sup> To assess whether lacritin's survival activity<sup>5</sup> could protect the ocular surface epithelium from damage induced by aqueous tear deficiency in Aire KO mice, we quantified lissamine green staining in eyes treated with lacritin or PBS using a 4-point scale. Whereas staining of PBS-treated eyes increased with time relative to baseline, topical lacritin led to decreased lissamine green staining (Fig. 3A). To rule out the possibility of a protein protection or vehicle effect, we showed that neither inactive lacritin truncation mutant "C-25" nor PBS was beneficial (Fig. 3B). Change in lissamine green staining score relative to pretreatment levels in PBS and lacritin-treated eyes 1 and 3 weeks post treatment indicated significant benefit by 3 weeks ( $-0.417 \pm 0.06$  lacritin versus  $0.125 \pm 0.07$  PBS;  $P = 0.02$ ), but not at 1 week ( $0.0 \pm 0.06$  lacritin versus  $0.78 \pm 0.06$  PBS;  $P = 0.08$ ). Thus, the restoration of cellular integrity in lacritin-treated eyes occurred after the significant increase in tear secretion noted at 1 week.

### Topical Lacritin Restores Ocular Surface Mucosal Phenotype

Corneal-specific cytokeratin K12 is abundant in the healthy ocular mucosal epithelium. Chronic inflammation of the ocular surface provokes a shift from K12 to skin-specific cytokeratin, K10, as the cornea transforms from a transparent, nonkeratinized corneal epithelium to an opaque, keratinized, skin-like epithelium. The percentage of corneal surface staining positive with anti-K12 or anti-K10 antibody was quantified by image analysis, as well as graded by masked observers. Whereas K12 expression remained essentially stable ( $80.1\% \pm 4.8\%$  lacritin versus  $85.6\% \pm 1.8\%$  PBS;  $P > 0.10$ ), we noted a significant reduction in K10 staining in eyes treated with lacritin ( $19.0 \pm 7.5$  lacritin versus  $29.8 \pm 9.7$  PBS;  $P = 0.03$ ; Fig. 4). These results suggested that corneal lineage was maintained in lacritin-treated eyes with decreased transformation to K10-expressing cells of epidermal lineage.

### Topical Lacritin Alters CD4<sup>+</sup> T Cell-Mediated Exocrinopathy but Not CD4<sup>+</sup> T Cell Infiltration of the Ocular Surface

Lymphocytic infiltration of the lacrimal gland and ocular surface are classic disease manifestations of SS.<sup>12</sup> Autoreactive CD4<sup>+</sup> T cells most heavily infiltrate the limbal area of the ocular surface and lacrimal glands in Aire KO mice. Topical lacritin had no apparent effect on the pattern or distribution of CD4<sup>+</sup> T cells infiltrating either the corneal stroma ( $12.4 \pm 2.1$  with lacritin versus  $14.6 \pm 1.6$  with PBS) or the limbus ( $29.6 \pm 2.5$  with lacritin versus  $34.6 \pm 2.9$  with PBS; Figs. 5A, 5B). However, CD4<sup>+</sup> T cell infiltration of the lacrimal gland,



**FIGURE 3.** Topical lacritin improved corneal epithelial integrity. (A) Representative images of ocular surface lissamine green staining in Aire KO mice before (*top panels*) and after (*bottom panels*) 3 weeks of 3 times daily treatment with topical lacritin (*left*) or PBS control (*right*). (B) Representative images of eyes treated with inactive lacritin truncation mutant C-25 or PBS alone. (C) Change in lissamine green staining score relative to pretreatment levels in PBS and lacritin-treated eyes 1 week (*left*;  $n=6$ ) and 3 weeks (*right*;  $n=7$ ) post treatment. Negative slope indicates improvement due to lacritin.

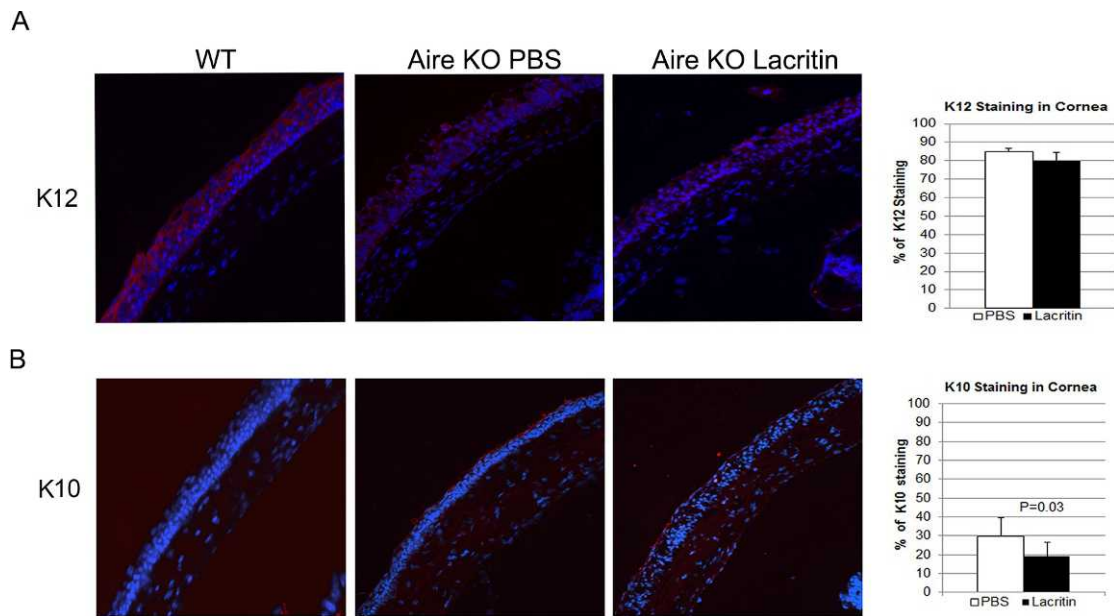
measured as the number of lymphocytic foci/per millimeter square area of lacrimal gland tissue, was significantly reduced ( $3.68 \pm 0.65/\text{mm}^2$  with lacritin versus  $9.7 \pm 1.5/\text{mm}^2$  with PBS;  $P=0.01$ ; Figs. 5D, 5E). Interestingly, while the number of lymphocytic foci was markedly affected by lacritin treatment, there was no difference in the total number of CD4+ T cell infiltrating the lacrimal glands. Taken together, topical lacritin causes a significant restoration of tear secretion, prevents loss of ocular surface integrity, and suppresses the formation of lymphocytic foci in the lacrimal glands of Aire KO mice.

## DISCUSSION

At present, the management of dry eye disease is predominantly accomplished through aggressive lubrication with artificial tears and topical anti-inflammatory agents. Although cyclosporine A has been shown to marginally increase tear secretion (~23%) in a small percentage of dry eye patients (~15%), the effectiveness of this broad acting T cell inhibitor is limited and discomfort upon instillation is a common side effect that leads to discontinuation.<sup>18</sup> With an increased understanding of the molecular events that modulate inflammation and tear secretion in dry eye disease, there is a push to develop targeted therapeutics that reduce inflammation and induce tear secretion with minimal side effects.

Lacritin is a tear protein that, in its monomeric form, autonomously promotes tearing<sup>4</sup> and ocular surface survival.<sup>5</sup> It is the only identified growth-like factor decreased in tears from patients with ocular surface inflammation resulting from blepharitis,<sup>19</sup> and it is downregulated in contact lens-related dry eye.<sup>10</sup> A recent study involving 129 dry eye patients and 73 healthy controls, revealed significantly less tear lacritin monomer in 95% of dry eye patients; representing one of only seven tear proteins downregulated in dry eye.<sup>9</sup>

With evidence of reduced lacritin monomer in the setting of inflammatory ocular surface disease, we explored lacritin levels in tears of SS patients where true aqueous deficiency is associated with T cell-mediated exocrinopathy. Accurate determination of specific peptide levels in tears is generally difficult as is the case with any complex biological fluid. Seifert et al.<sup>7</sup> estimated that lacritin represented ~4% of total tear proteins (~18–22  $\mu\text{M}$ ).<sup>20</sup> However, the ELISA method employed did not distinguish lacritin monomer from its variably polymerized forms and the ~12.5 KD C-terminal fragment. Since only the monomer and ~12.5 kD fragment are active, our approach using Western blot is likely to be more indicative of functionally active lacritin in tears. However, one weakness in both approaches is the utilization of nonglycosylated recombinant lacritin to generate the standard curve. Although antibody reactivity may be affected by glycosylation, epitope blocking by glycosylation was not apparent for lacritin in the context of tears.<sup>7</sup> Also, SS patients, like other dry eye patients,



**FIGURE 4.** Topical lacritin reduced aberrant K10 expression of the corneal epithelium but did not affect K12. Representative image of (A) K12 and (B) K10 staining (both red) along the corneal surface of WT mice (left), PBS-treated (middle), and lacritin-treated eyes (right) of Aire KO mice after 3 weeks. Adjacent graphs provide quantitative assessment based on the percentage of corneal surface that showed K12 and K10 expression in PBS- and lacritin-treated eyes. Nuclei stained blue with DAPI.

generally have reduced tear output and reduced total tear protein, thus, making quantitative comparisons normalized to total proteins or tear volume less informative of the underlying biological phenomenon. To standardize our measurements, we adopted a uniform tear collection and elution procedure. Our estimates indicated the total amount of lacritin available from a Schirmer strip following a 5-minute collection procedure and a uniform elution into 100  $\mu$ L of PBS. This approach emphasized the bioavailability of active lacritin on the ocular surface over a set period of time. Apparent from our data, was the substantial deficiency in tear levels of both the monomeric and the C-terminal forms of lacritin in SS patient tears compared with healthy controls. How such a difference translates physiologically in the context of lacritin's logarithmic biphasic dose response<sup>7,14</sup> remains to be determined. Moreover, reduced bioavailability of lacritin monomer in aqueous-deficient dry eye requires further study. Interestingly, lacritin monomer is subject to intermolecular cross-linking via a tear tissue transglutaminase-catalyzed reaction between lysines 82 or 85 as amine donor and glutamine 106 as amine acceptor. By interfering with ligation of the lacritin coreceptor "syndecan-1,"<sup>21</sup> crosslinking inactivates lacritin,<sup>11</sup> (Romano JA, Laurie GW, unpublished observations, 2013). Hyperosmolar stress increases tissue transglutaminase expression.<sup>22</sup> Thus, hyperosmolarity of the tear film resulting from aqueous tear deficiency<sup>22-24</sup> may provoke the crosslinking and inactivation of lacritin by tissue transglutaminase.

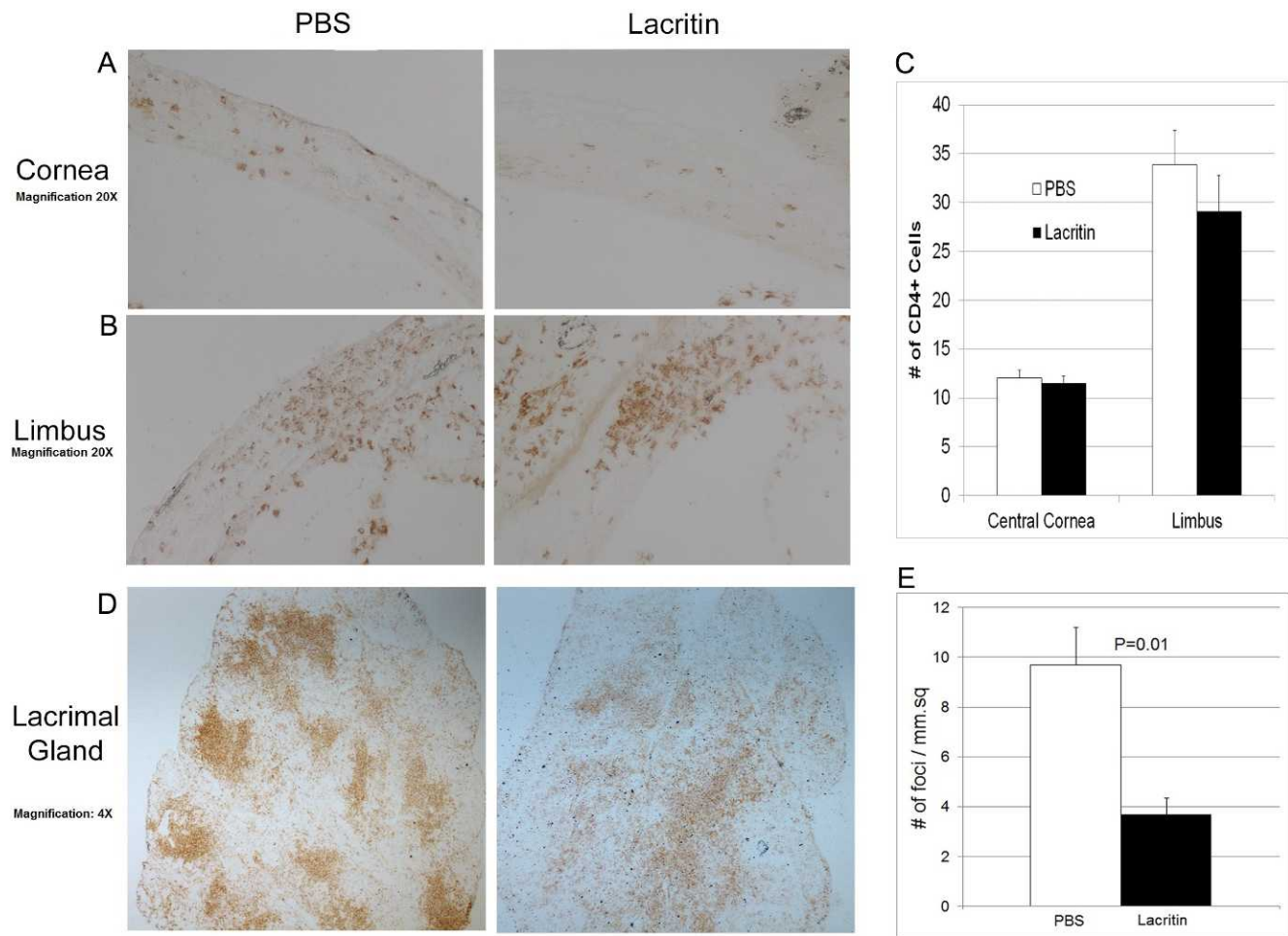
In conjunction with reduced lacritin levels in the tears of SS patients, our data provide the first in vivo evidence for lacritin's therapeutic benefit in ocular surface inflammation associated with aqueous-deficient dry eye. Three weeks of 3-times daily topical lacritin significantly improved stimulated tear secretion and decreased corneal epithelial damage. All lacritin-treated eyes at all time points demonstrated improved tear secretion compared with PBS-treated eyes. Maintenance of epithelial integrity was equally profound and was noted at the histological level where the characteristic switch from mucosal to epidermal lineage was reduced. In addition to lacritin's profound effect on tear secretion and ocular surface charac-

teristics, lacritin also significantly reduced focal infiltration of CD4+ T cells to the lacrimal glands. Similar to previous studies in normal rabbits,<sup>4</sup> repeated topical application of lacritin or lacritin truncation mutant C-25 caused no adverse effects to the ocular mucosal surface. Currently we are investigating whether topical human recombinant lacritin triggers an immune response in treated mice. While negative control lacritin C-25 displayed no benefit, it was equally capable of triggering such a response. In human dry eye, human topical lacritin would be immunologically inert.

Although the exact mechanism by which topical lacritin promotes tearing is not understood, preliminary data suggest that corneal sensory nerves might be directly or indirectly involved (Hirata H, Laurie GW, unpublished observations, 2013). Lacrimal tear secretion is tightly regulated through corneal innervation.<sup>25</sup> Corneal sensory afferents transmit stimuli from the cornea to the lacrimal nucleus in the brain, where efferent signals that modulate aqueous production by the lacrimal glands are sent. Recent evidence suggests a functional role for reduced corneal innervation in the pathogenesis of DED. We believe lacritin plays a functional role in maintaining corneal innervation during homeostasis, and that loss of lacritin in the setting of chronic inflammation has detrimental effects on sensory innervation that results in reduced tear secretion and loss of epithelial integrity. While tight junctions lining the ocular surface form a barrier to lacritin permeability during homeostasis, growth factors such as the platelet-derived growth factor and VEGF released in response to cellular stress are capable of permeabilizing the epithelium,<sup>26</sup> thus providing lacritin direct access to syndecan-1-expressing neuronal cells within the corneal epithelium and subbasal nerve plexus.<sup>4</sup> While syndecan-1 is known to regulate neuron growth in *C. elegans*,<sup>26</sup> further studies of lacritin's effects on corneal innervation and neuronal stimulation of lacrimation in the setting of dry eye are of significant interest and the topic of ongoing investigation.

In addition to its prosecretory properties, lacritin is an established mitogen. Lacritin promotes tear secretion and cell survival by targeting the cell surface heparan sulfate proteo-





**FIGURE 5.** Lacritin alters CD4+ T cell infiltration of the lacrimal gland but not the ocular surface. Pattern and density of CD4+ T cell infiltrates (stained brown with DAB) in the (A) cornea and (B) limbus following 3 weeks of 3 times daily topical treatment with PBS or lacritin. (C) Quantitative assessment of CD4+ T cells per 1000-pixel length of the central cornea and 100,000 square pixel area of the limbus in PBS- and lacritin-treated eyes. (D) Pattern and density of CD4+ T cell infiltrates (stained brown with DAB) in the lacrimal gland of Aire KO mice treated with PBS or lacritin. (E) Quantitative assessment of focal CD4+ T cell infiltration of the lacrimal glands, expressed as number of foci per millimeter squared of lacrimal gland tissue ipsilateral to PBS- and lacritin-treated eyes.

glycan syndecan-1 (SDC1) via a heparanase-dependent mechanism.<sup>21,27</sup> Syndecan-1 is widely expressed on both corneal and conjunctival epithelia.<sup>28</sup> Heparanase cleavage of the heparan sulfate moiety exposes and generates a binding site for the C-terminal  $\alpha$ -helix domain of lacritin between the heparan sulfate chains within SDC1's N-terminus.<sup>27</sup> In subconfluent cells not stressed by inflammatory cytokines, ligation of lacritin to SDC1 sets off a cascade of signaling that includes activation of phospholipase D, a key mediator of cell secretion and proliferation.<sup>14</sup> In contrast, stressed cells respond to lacritin by rapidly acetylating the longevity factor forkhead box O3 (FOXO3) to thereby ligate autophagy-related protein (ATG) 101 and transiently accelerate autophagy, which also stimulates the binding of family member FOXO1 with autophagic mediator ATG7.<sup>5</sup> This work offers a potential mechanism by which lacritin rescues ocular surface integrity and restores tear production—even under intense inflammatory conditions as observed in Aire KO mice. Lacritin also diminished the transformation of corneal-specific, K12-expressing epithelium to the pathologically keratinized K10-expressing epidermal-like phenotype, thus offering promise for a therapeutic modality that prevents the onset and devastating consequences of squamous metaplasia in severe dry eye disease.

An especially intriguing result was the altered pattern of lymphocytic infiltration in the lacrimal glands of Aire KO mice treated with topical lacritin. The complex nature of the T cell's role in SS-associated autoimmunity is well recognized.<sup>29</sup> In fact, expanded lymphocytic foci characterize autoimmune exocrinopathy and serve as the single most important diagnostic criteria for patients with SS. The mechanism whereby topical lacritin reduced the number of lymphocytic foci, but not the total number of infiltrating T cells, is compelling and may reflect changes in the cellular structure that permits easier movement of leukocytes through the tissue, thereby altering the composition of infiltrating immune cells and changing the cytokine profile in a manner not fully understood. Interestingly, the reduced number of lymphocytic foci in lacrimal glands of Aire KO mice was not paralleled in the eye where the presence and pattern of infiltrating T cells remained unchanged. The effects of topical lacritin on the lacrimal gland may also reflect changes in the specialized eye-associated lymphoid tissue (EALT), that is continuous from the corneal surface through the conjunctival-associated lymphoid tissue to the lacrimal drainage-associated lymphoid tissue.<sup>30,31</sup> The flux of immune-information across the EALT is mediated by recognition and presentation of antigens to lymphocytes, which then proliferate and differentiate into effector T cells.

Lacritin's cytoprotective effect on the ocular mucosal epithelium could be hypothesized to interrupt or mitigate the inflammatory cycle, thus protecting the lacrimal gland from focal infiltration of auto-antigen primed CD4+ T cells.

Finally, in an effort to control for inherent variability in the dry eye phenotype between Aire KO mice, we used a paired-eye comparison to examine lacritin's therapeutic potential. We observed a noticeable increase in tear secretion in eyes treated with PBS between the first and third week. While repeated treatment with PBS may help to restore epithelial integrity of the aqueous-deficient ocular surface, we cannot rule out a potential crossover effect from lacritin treatment to the contralateral eye. Such an effect has also been noted by our collaborators although definitive evidence does not yet exist. Although a crossover effect would confound the interpretation, it would only underestimate lacritin's true therapeutic potential as a tear secretagogue.

## CONCLUSIONS

Our work revealed reduced levels of active lacritin monomer and C-terminal fragment in tears of SS patients compared to healthy patients with no history of ocular disease. We used an established model of autoimmune mediated aqueous-deficient dry eye that mimics the clinical characteristics of SS to demonstrate the therapeutic potential of topical lacritin as a prosecretory and prosurvival tear supplement. Lacritin treatment was well tolerated and significantly reduced the pathological effects of chronic inflammation associated with dry eye. Thus, additional studies to examine its use as a standalone or adjunct therapy to treat the signs and symptoms of dry eye disease are warranted and provide promise for the millions of patients who suffer from dry eye.

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