Dry Eye–Induced Conjunctival Epithelial Squamous Metaplasia Is Modulated by Interferon-γ

Cintia S. De Paiva,1 Arturo L. Villarreal,1 Rosa M. Corrales,1 Hassan T. Rahman,1 Victor Y. Chang,1 William J. Farley,1 Michael E. Stern,2 Jerry Y. Niederkorn,3 De-Quan Li,1 and Stephen C. Pflugfelder1

PURPOSE. To investigate the role of interferon (IFN)-γ in the pathogenesis of conjunctival squamous metaplasia in dry eye.

METHODS. Experimental dry eye was created by subjecting C57BL/6 and IFN-γ-knockout mice to desiccating environmental stress for 5 or 10 days. T-cell antigens and IFN-γ were detected by immunohistochemistry. Goblet cells were counted in periodic acid Schiff (PAS)-stained sections. Expression of small, proline-rich protein (SPRR)-2 was evaluated by confocal microscopy. Tear IFN-γ was measured by immunobead assay.

RESULTS. Dry eye promoted migration of CD4+ T cells and IFN-γ+ cells into goblet cell zones of the conjunctiva and increased the concentration of IFN-γ in tears. This migration was accompanied by progressive goblet cell loss and an increase in SPRR-2 expression in the conjunctival epithelium. A significant inverse correlation was observed between the density of infiltrating CD4+ T cells and goblet cells. Dry eye had no effect on conjunctival goblet cell density in IFN-γ-knockout mice; however, exogenous administration of IFN-γ significantly decreased goblet cell density after 5 days.

CONCLUSIONS. Conjunctival epithelial response to experimental dryness is related to the degree of CD4+ T-cell infiltration and the level of IFN-γ production. These findings suggest that IFN-γ plays a pivotal role in promoting conjunctival squamous metaplasia in dry eye, and they provide insight into the immune pathogenesis of keratoconjunctivitis sicca. (Invest Ophthalmol Vis Sci. 2007;48:2553–2560) DOI:10.1167/iovs.07-0069

Dry eye is one of the most prevalent medical conditions, affecting 7% to 20% of the adult population older than 40 years.1 Dry eye commonly causes eye irritation and blurred vision symptoms and in severe cases, such as Sjögren’s syndrome, may lead to blindness from corneal opacification or ulceration.2–4 Reduced aqueous tear production is known to promote squamous metaplasia with goblet cell (GC) loss in the conjunctival epithelium. The mechanism by which these pathologic changes occur has not been established, but there are several lines of evidence that inflammation is involved in the process. First, activated T cells have been detected in the conjunctival epithelium of patients with dry eye.5–10 Second, the greatest goblet loss has been observed in dry eye conditions that develop in conjunction with systemic inflammatory diseases, such as Sjögren’s syndrome, graft-versus-host disease, and Stevens-Johnson syndrome.11–13 CD4+ T cells from mice with experimental dry eye (EDE), when adoptively transferred to T-cell–deficient nude mice that had not been exposed to desiccating stress, produced severe inflammation in the lacrimal glands, cornea, and conjunctiva, resulting in decreased tear production and conjunctival GC loss.14 Finally, topically applied cyclosporine, a T-cell immunomodulatory agent, was found to have a salutary effect on conjunctival GC density in a murine dry eye model.15 This agent increased conjunctival GC density by nearly 200% in dry eyes after 6 months of treatment.16 Activated T lymphocytes and NK cells are the main source of the Th-1 cytokine, interferon (IFN)-γ. It is a pleiotropic cytokine involved in the regulation of nearly all phases of immune and inflammatory responses. IFN-γ has the potential to upregulate conjunctival epithelial differentiation-related proteins.17 IFN-γ has been reported to increase levels of RNA transcripts encoding the cornified envelope precursor involucrin in normal human cultured keratinocytes.18

The purpose of this study was to evaluate the role of the Th-1 cytokine IFN-γ in the conjunctival epithelial squamous metaplasia that develops in response to dry eye.

MATERIALS AND METHODS

Mouse Model of Dry Eye

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental dry eye (EDE) was induced in C57BL6 mice (n = 40), 6 to 8 weeks of age, by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) into alternating hindquarters administered four times a day (8 AM, 11 AM, 2 PM, and 5 PM) with exposure to an air draft and <40% ambient humidity for 18 hours per day, as previously reported.14,15,19–20 Mice were euthanatized after 5 or 10 days of treatment. A group of age- and gender-matched mice that did not receive any treatment to induce dry eye served as untreated (UT) control subjects.

Exogenous Administration of IFN-γ

To evaluate the role of IFN-γ in conjunctival GC loss, we subjected C57BL/6 and C57BL/6 IFN-γ knockout (B6yKO; B6.129s7-Ifngtm1Tfj;
Adoptive Cell Transfer of CD4+ T cells
Spleens were collected from mice subjected to EDE and one donor-equivalent of spleen was transferred intraperitoneally (IP) to six different syngeneic nude mice. One donor-equivalent is defined as the number of cells remaining after the respective in vitro manipulation (CD4+ T-cell enrichment) of a single spleen from a single donor. The remaining cells represent the total lymphocyte population for that spleen cell category for a single donor. One splenic equivalent of T cells was equal to approximately 5 × 10^6 cells. Spleen cells were enriched for CD4+ T cells by positive selection using rat anti-mouse CD4-conjugated magnetic microbeads (MACS system; Miltenyi Biotec Inc., Auburn, CA), as described elsewhere. The CD4+ enriched cell suspensions contained >87% CD4+ T cells, as determined by flow cytometry. Cells were washed with RPMI medium before being adoptively transferred in 0.1 mL of RPMI medium.

Histology and Periodic Acid Schiff Staining
Enucleated eyes were fixed in 10% formalin and embedded in paraffin. Six-μm sections were stained with either hematoxylin and eosin or periodic acid-Schiff (PAS) reagent. Sections from five left eyes in each group were examined and photographed with a microscope (Eclipse E400; Nikon, Garden City, NY) equipped with a digital camera (DXM 1200; Nikon). Goblet cell density in the superior and inferior conjunctiva was measured in three eyes of each group (three slides per eye) by image-analysis software (Metavue 6.24r; Molecular Devices, Sunnyvale, CA), and the results are expressed as the number of GCs per 100 μm.

Immunohistochemistry
The eyes and lids of mice in each group were excised, embedded in optimal cutting temperature (OCT) compound (VWR, Suwanee, GA), and flash-frozen in liquid nitrogen. Sagittal 8-μm sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at −80°C. Tissue sections were used for immunohistochemistry, as previously described.

Tissue sections were fixed with either cold 95% ethanol for detection of CD45, CD11b, or cold acetone at −20°C for 5 minutes for detection of CD4, CD8, myeloperoxidase, interleukin (IL)-4, and IFN-γ. After fixation, endogenous peroxidases were quenched with 0.3% H2O2 for 10 minutes. The sections were sequentially blocked with avidin-biotin (Vector Laboratories, Burlingame, CA) for 10 minutes each. After the reaction was blocked with 20% normal goat serum (NGS) in PBS for 45 minutes, primary rat monoclonal antibodies against CD45 (clone 30-F11, 10 μg/mL), CD11b (clone M1/70, 6.25 μg/mL), CD4 (clone H129.9, 10 μg/mL), or CD8 (clone 53-6.7, 3.125 μg/mL) (all from BD Pharmingen, San Jose, CA); IL-4 (clone 11B11, 20 μg/mL) or IFN-γ (clone R4-6A2, 20 μg/mL) (all from Biolegend, San Diego, CA); or rabbit polyclonal sera against myeloperoxidase (1:400; Fremont, NeoMarkers, Fremont, CA) were applied and incubated for 1 hour at room temperature. After they were extensively washed, the sections were incubated with appropriate biotinylated secondary antibodies (all from BD Pharmingen), using streptavidin biotin (Vectastain Elite ABC Kit; Vector Laboratories), according to the manufacturer’s protocol. The samples were finally incubated with diaminobenzidine (DAB; NovaRed; Vector Laboratories) peroxidase substrate to give a red stain for 5 to 9 minutes (optimized for each antibody) and counterstained with Mayer’s hematoxylin. Secondary antibody alone and rat anti-mouse isotype (BD Pharmingen) controls were performed. Sections from six different right eyes per group per time point were examined and photographed with a microscope equipped with a digital camera (DXM 1200; Nikon).

The number of cells staining positively for each antigen was counted in tissue sections. Three sections (at least 100 μm apart) from six different animals of each strain and at each time point were evaluated. Positively stained cells were counted in the GC-rich area of the conjunctiva, over a length of at least 500 μm in the epithelium and to a depth of 75 μm below the epithelial basement membrane in the stroma, for a distance of 500 μm (Metave 6.2r; Molecular Devices). Results were expressed as the number of positive cells per 100 μm. The CD4/CD8 ratio was calculated using the mean values obtained for both markers.

Immunofluorescent Staining and Laser Scanning Confocal Microscopy
Small, proline-rich protein (SPRR)-2 expression was evaluated by laser scanning confocal microscopy in tissue sections, as previously described. Cryosections from UT and EDE control mice and each experimental group (two slides per group from three different animals/time point) were fixed with acetone at −20°C for 5 minutes and permeabilized with PBS containing 0.1% Triton-X for 10 minutes. After blocking with 20% NGS in PBS for 45 to 60 minutes, polyclonal rabbit serum against SPRR-2 (1:100 dilution of neat serum; Alexis Biochmicals, San Diego, CA) was applied and incubated for 1 hour at RT. Secondary antibody, Alexa-Fluor 488-conjugated goat anti-rabbit IgG was then applied and incubated in a dark chamber for 1 hour, followed by counterstaining with propidium iodide (2 μg/mL in PBS) for 30 minutes. Cryosection digital images (512 × 512 pixels) were captured with a laser scanning confocal microscope (LSM 510 with krypton-argon and He-Ne laser; Carl Zeiss Meditec, Inc., Thornwood, NY) with 488-excitation and 543-nm emission filters (LP505 and LP560, respectively; Carl Zeiss Meditec, Inc.). They were acquired with a 40×/1.3 oil-immersion objective. Images from treatment and control corneas were captured with identical photomultiplier tube gain settings and processed using the microscope software (LSM-PC; Carl Zeiss Meditec, Inc.) and image-analysis software (Photoshop 6.0; Adobe Inc., San Jose, CA). The intensity of the staining was graded in six conjunctival sections, by a masked observer, and the results were averaged. Briefly, in each digital picture, seven elliptical regions (of 1076 pixels2) were drawn by a masked observer with image-analysis software (Metavue 6.24r; Molecular Devices) and the integrated intensity calculated by the software was recorded on a spread sheet (Excel; Microsoft, Redmond, WA). The results within each image were summed and are presented as the mean of all images within a group, in fluorescence units ×103.

Tear Fluid Collection and Immunobead Assay
Tear fluid washings were collected by a previously reported method. Briefly, 1.5 μL of mouse cytokine assay buffer (Beadlyte; Upstate Biotechnology, Lake Placid, NY) was instilled into the conjunctival sac. The tear fluid and buffer were collected with a 1-μL volume glass capillary tube (Drummond Scientific Co., Broomhall, PA) by capillary action from the tear meniscus in the lateral canthus. The tear washings from both eyes of two mice were pooled (4 μL) in mouse cytokine assay buffer (6 μL; Beadlyte; Upstate Biotechnology) and were stored at −80°C until the immunobead assay was performed. One experiment consisted of two mice per time point, and the results are the mean ± SD of four independent experiments.

Tear washings were added to wells containing 10 μL of 1× beads that were tagged with anti-mouse IFN-γ monoclonal antibodies (Upstate Biotechnology). Serial dilutions of IFN-γ were added to wells in the same plate as the mouse tear samples, to generate a standard curve.
The plate was incubated overnight at 4°C to capture IFN-γ by the antibody conjugated fluorescent beads. After three washes with assay buffer, 25 μl of biotinylated secondary antibody mixture was applied for 1.5 hours in the dark at room temperature. The reactions were detected with streptavidin-phycocerythrin (100 IS 2.3 system; Luminex, Austin, TX). The limit of detection of this assay was 8 pg/mL.

Statistical Analysis
The unpaired t-test was used to compare the effect of EDE (UT versus 5 days or UT versus 10 days, or 5 days versus 10 days) in C57BL/6 mice or the effect of IFN-γ (EDE5 days+BSA versus EDE5 days+IFN-γ) on GC density, tear IFN-γ concentration, and SPRR-2 intensity in each group (C57BL/6 and B6yKO). One-way analysis of variance (ANOVA) with Tukey post hoc testing was used for statistical comparisons of the inflammatory infiltration of conjunctiva. Nonparametric Spearman correlation analysis was used to evaluate the correlation between GC density and the number of CD4+ T cells. P ≤ 0.05 was considered statistically significant. These tests were performed with commercial software (Prism 3.0 software; GraphPad Software Inc., San Diego, CA).

RESULTS
Effect of Desiccating Stress on CD4+ T-Cell Migration into the Conjunctival Epithelium in C57BL/6 Mice
Conjunctival biopsy samples obtained from C57BL/6 mice (n = 6 for each time point) were evaluated for the presence of inflammatory/immune cell markers (CD4, CD8, CD11b, CD45, and myeloperoxidase) by immunohistochemistry. The density of cells positive for these antigens in the conjunctival epithelium and stroma is noted in Table 1. Resident CD4+ and CD8+ T cells were found in the epithelium and stroma of UT control animals. We noted a significant increase in CD4+ cells in the epithelium at 5 and 10 days of EDE (Fig. 1A; P < 0.001). In contrast, a decrease of CD4+ cells was noted in the conjunctival stroma at 5 and 10 days. Desiccating stress caused a profound decrease in the number of CD8+ cells in the conjunctival epithelium after 5 and 10 days of EDE (Fig. 1B; P < 0.001). In the conjunctival stroma, the number of CD8+ decreased at 5 days, with a significant increase at 10 days, when compared with 5 days (P < 0.05). The CD4/CD8 ratio in the conjunctiva increased in the epithelium (UT, 0.93; 5 days, 3.9; 10 days, 3.0) and stroma (UT, 4.6; 5 days, 7.4; 10 days, 2.4) in response to EDE.

Effect of Desiccating Stress on Production of IFN-γ in C57BL/6 Mice
No IFN-γ staining was present in UT control mice and after 5 days of EDE in C57BL/6 mice (Fig. 1Ca). However, IFN-γ+ cells were observed in the epithelium in the GC-rich area of the conjunctiva in C57BL/6 mice after 10 days of EDE (Fig. 1Cb, arrows). IFN-γ also stained some mucin strands after 10 days of EDE (Fig. 1Cb). In contrast, no IL-4+ cells were noted in the conjunctiva at any time point (data not shown). Using an immunobead assay, we observed that tear IFN-γ progressively increased to significantly higher concentrations after 10 days of EDE in C57BL/6 mice (Fig. 2C), with a greater increase noted at 10 days than at 5 days (P < 0.05). To confirm that IFN-γ-producing CD4+ T cells caused conjunctival GC loss, we stained frozen conjunctival sections from six allogenic nude mice that adoptively received CD4+ T selected cells from EDE donors for IFN-γ. We have reported that these adoptively transferred CD4+ T cells home to the ocular surface tissues.14 As noted on Figure 1Cc, a great number of IFN-γ positive cells were found in the basal epithelium and stroma in the GC area of the conjunctiva of CD4+ recipient nude mice.

Table 1. Comparison of Inflammatory Infiltration of the Conjunctiva in UTs and after 5 and 10 Days of EDE in B6 Mice

<table>
<thead>
<tr>
<th>Area</th>
<th>UT</th>
<th>EDE 5 Days</th>
<th>EDE 10 Days</th>
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<tbody>
<tr>
<td>CD4</td>
<td></td>
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</tr>
<tr>
<td>Epithelium</td>
<td>1.35 ± 0.47</td>
<td>2.01 ± 0.78***</td>
<td>2.04 ± 0.63***</td>
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<tr>
<td>Struma</td>
<td>2.81 ± 1.09</td>
<td>1.93 ± 0.63***</td>
<td>2.03 ± 0.80***</td>
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<tr>
<td>CD8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>1.45 ± 1.12</td>
<td>0.44 ± 0.54***</td>
<td>0.67 ± 0.53***</td>
</tr>
<tr>
<td>Struma</td>
<td>0.66 ± 0.62</td>
<td>0.19 ± 0.32</td>
<td>0.86 ± 0.86†</td>
</tr>
<tr>
<td>CD11b</td>
<td></td>
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<td>Epithelium</td>
<td>1.60 ± 0.85</td>
<td>1.50 ± 0.82</td>
<td>1.39 ± 0.65</td>
</tr>
<tr>
<td>Struma</td>
<td>7.23 ± 1.70</td>
<td>7.12 ± 2.37</td>
<td>7.03 ± 1.78</td>
</tr>
<tr>
<td>CD45</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Epithelium</td>
<td>4.17 ± 1.48</td>
<td>2.70 ± 1.37</td>
<td>3.40 ± 1.81</td>
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<tr>
<td>Struma</td>
<td>6.06 ± 1.97</td>
<td>6.15 ± 1.54</td>
<td>5.67 ± 0.94</td>
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<tr>
<td>Myelo</td>
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<tr>
<td>Epithelium</td>
<td>0.88 ± 0.63</td>
<td>0.27 ± 0.43</td>
<td>0.56 ± 0.70</td>
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<tr>
<td>Struma</td>
<td>1.02 ± 0.58</td>
<td>0.25 ± 0.43*</td>
<td>0.54 ± 0.47</td>
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</table>

Data are shown as the mean ± SD; n = 6/time point (cells/100 μm). Myelo, myeloperoxidase.

*p < 0.05, **p < 0.01, ***p < 0.001 versus C57BL/6 UT.
†P < 0.05 versus B6 5 days.

Effect of Desiccating Stress on Conjunctival GC Density in C57BL/6 Mice
A significant decrease in PAS-positive GCs was noted in the conjunctiva after 5 and 10 days of EDE in C57BL/6 mice (mean ± SD; 5.17 ± 0.66 and 3.90 ± 0.91, respectively) compared with UT control samples (6.88 ± 1.05 GC/100 μm; P < 0.0001 for both). The GC density at 10 days was significantly lower than 5 days (P < 0.001; Fig. 2A, n = 3 for each time point).

Correlation of GC Density with CD4+ Cell Density
To determine whether the number of CD4+ cells corresponded to the decrease in GCs in EDE, we evaluated the correlation between GC and CD4+ cell density in the conjunctival epithelium. A significant inverse correlation was observed between the number of GCs and CD4+ cells, with a coefficient of determination of 0.26 (P = 0.016; Fig. 2B).

Effect of Desiccating Stress on Production of IFN-γ in C57BL/6 Mice
No IFN-γ staining was present in UT control mice and after 5 days of EDE in C57BL/6 mice (Fig. 1Ca). However, IFN-γ+ cells were observed in the epithelium in the GC-rich area of the conjunctiva in C57BL/6 mice after 10 days of EDE (Fig. 1Cb, arrows). IFN-γ also stained some mucin strands after 10 days of EDE (Fig. 1Cb). In contrast, no IL-4+ cells were noted in the conjunctiva at any time point (data not shown). Using an immunobead assay, we observed that tear IFN-γ progressively increased to significantly higher concentrations after 10 days of EDE in C57BL/6 mice (Fig. 2C), with a greater increase noted at 10 days than at 5 days (P < 0.05). To confirm that IFN-γ-producing CD4+ T cells caused conjunctival GC loss, we stained frozen conjunctival sections from six allogenic nude mice that adoptively received CD4+ T selected cells from EDE donors for IFN-γ. We have reported that these adoptively transferred CD4+ T cells home to the ocular surface tissues.14 As noted on Figure 1Cc, a great number of IFN-γ positive cells were found in the basal epithelium and stroma in the GC area of the conjunctiva of CD4+ recipient nude mice.

Effects of Interferon-γ Deficiency and Exogenous IFN-γ Administration on Conjunctival GC Density
To determine whether IFN-γ production contributes to GC loss in EDE, we evaluated the effects of desiccating stress with or without exogenous administration of IFN-γ on conjunctival GC density in C57BL/6 and B6yKO mice in three different samples of each strain per time point.

In C57BL/6 mice, subconjunctival administration of IFN-γ after 5 days of EDE significantly decreased the number of GCs (P < 0.0002, Fig. 2A). Compared to un.injected and BSA-injected eyes, the conjunctival GCs in IFN-γ-injected eyes appeared smaller and were buried within the middle layers of the conjunctival epithelium (Fig. 3A, arrows in insets of top right column). Certain areas were devoid of GCs (Fig. 3A; asterisks in top right column). GC density after subconjunctival administration of IFN-γ in UT C57BL/6 mice was not different from the BSA-injected UT C57BL/6 group (5.38 ± 1.23 vs. 5.10 ± 0.72 GCS per 100 μm; P = 0.11).

B6yKO mice had a GC density comparable to C57BL/6 mice at baseline (Fig. 2D; P > 0.05), with large, plump GCs located at the conjunctival surface (Fig. 3A, inset in bottom left column). Desiccating stress had no effect on the number of GCs in this strain (6.70 ± 0.96 vs. 6.65 ± 0.90 GC per 100 μm in UT...
and 5 days of EDE; Fig. 2D, $P = 0.79$); however, subconjunctival injection of IFN-γ produced a marked decrease in GC density compared with vehicle-injected eyes (4.66 ± 0.78 vs. 6.08 ± 0.59 GCs per 100 μm; Fig. 2D, $P < 0.0001$) and the same changes in GC morphology that were seen in IFN-γ-injected C57BL/6 mice were noted (Fig. 3A, inset in bottom).

**FIGURE 1.** (A, B) Immunohistochemical staining of CD4$^+$ (A) and CD8$^+$ (B) T cells in the conjunctivae of C57BL/6 (B6; $n = 6$ per time point). (C) Immunohistochemical staining of IFN-γ$^+$ cells (solid arrows) in the conjunctiva of C57BL/6 (B6; Ca, Cb) mouse and nude mouse recipients of CD4$^+$ T-selected cells from EDE donors (CD4$^+$; (Cc) higher magnification of epithelium of CD4$^+$ mice; $n = 6$ per time point). Solid arrows: positively stained cell; dashed arrow: positively stained mucus strands (Cb). Inset: higher magnification of epithelium of C57BL6 mouse after 10 days of EDE. UT, untreated; CN, cornea; CJ, conjunctiva. Scale bar, 25 μm.

**FIGURE 2.** (A) GC density in UT, after 5 and 10 days of experimental dry eye and after subconjunctival injection of BSA or IFN-γ in C57BL/6 mice. (B) Correlation of CD4$^+$ T cells with GC density. $r^2 = 0.26$, $r = 0.509$, $P = 0.016$. ($r^2$, coefficient of determination; $r$, coefficient of correlation). (C) IFN-γ concentration in tear fluid samples obtained from C57BL/6 mice. (D) Goblet cell density in UT, after 5 and 10 days of experimental dry eye and after subconjunctival injection of BSA or IFN-γ in B6-KO mice. Data are the mean ± SD (error bars) for three animals per strain per time point, but the IFN-γ measurement in tears (C) which represent the mean of four independent experiments.
The GC density after subconjunctival administration of IFN-γ/H9253 in UT B6/H9253 KO mice was not different from the UT B6/H9253 KO vehicle-injected group (5.72 ± 0.75 vs. 5.72 ± 0.83 GCs per 100 μm; P = 0.82).  

**Effects of Interferon-γ Deficiency and Exogenous Administration of IFN-γ on SPRR-2 Expression**

To determine whether IFN-γ affects production of cornified envelope differentiation proteins by the epithelium, we evaluated the effects of desiccating stress and exogenous administration IFN-γ on expression of the cornified envelope precursor, SPRR-2 (Fig. 3B) in three different samples of each strain and time point. The mean integrated intensity of SPRR-2 staining in all groups is provided in Table 2.

Diffuse cytoplasmic staining for SPRR-2 (Fig. 3B) was found in confocal images of the apical conjunctival epithelium of UT C57BL/6 mice. EDE produced a significant increase of SPRR-2 intensity at 5 and 10 days in C57BL/6 mice (2.45 ± 0.48 and 3.21 ± 0.64 fluorescence units [FU] × 10⁵, respectively; P = 0.02 and P = 0.01 compared with UT mice). There was no difference in the intensity of SPRR-2 staining between 5 and 10 days of EDE in C57BL/6 mice.
The effects of exogenous administration of BSA or IFN-γ injected subconjunctivally were evaluated after 5 days of EDE in B6 and B6KO mice and in UT B6yKO and B6 mice. Data are shown as the mean ± SD of three different animals/time point (fluorescence units/UT 10 days 3.21 of three different animals/time point (fluorescence units).

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<thead>
<tr>
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<th>C57BL/6</th>
<th>B6yKO</th>
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<tbody>
<tr>
<td>UT</td>
<td>1.67 ± 0.28*</td>
<td>1.72 ± 0.20†</td>
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<tr>
<td>UT + BSA</td>
<td>1.81 ± 0.23</td>
<td>1.71 ± 0.24</td>
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<tr>
<td>UT + IFN-γ</td>
<td>2.48 ± 0.89††</td>
<td>1.91 ± 0.23††</td>
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<tr>
<td>5 days</td>
<td>2.45 ± 0.48</td>
<td>2.91 ± 0.12</td>
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<tr>
<td>5 days + BSA</td>
<td>2.10 ± 0.54</td>
<td>2.35 ± 0.40</td>
</tr>
<tr>
<td>5 days + IFN-γ</td>
<td>3.17 ± 0.42***</td>
<td>2.96 ± 0.10***</td>
</tr>
<tr>
<td>10 days</td>
<td>3.21 ± 0.64†††</td>
<td>NA</td>
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</table>

Within-strain comparisons: *P = 0.02 vs. 5 days and P = 0.01 vs. 10 days; †P = NS vs. UT + BSA; ††P = 0.03 vs. 5 days + BSA; †††P = NS vs. 5 days; †‡P = NS vs. 5 days; †‡‡P = NS vs. UT + BSA.

Subconjunctival injection of IFN-γ in C57BL/6 and B6yKO mice after 5 days of EDE increased SPPR-2 immunoreactivity, notably around many apical epithelial cells and some GCs (Fig. 3B), compared with vehicle-injected mice (3.17 ± 0.42 vs. 2.10 ± 0.54 FU × 10³; †P = 0.03 in C57BL/6; 2.96 ± 0.10 vs. 2.55 ± 0.40 FU × 10³, †P = 0.03 in B6yKO). SPPR-2 staining after subconjunctival administration of IFN-γ in UT C57BL/6 and B6yKO mice was higher than in UT vehicle-injected eyes, although the differences were not statistically significant (2.48 ± 0.89 vs. 1.81 ± 0.23 FU × 10³; and 1.91 ± 0.230 vs. 1.71 ± 0.24 FU × 10³, P > 0.05 for both mouse strains).

**DISCUSSION**

This study evaluated the relationship between the immunologic and epithelial response of the conjunctiva to experimental ocular surface dryness in the C57BL/6 mouse strain. The C57BL/6 strain, which has a predisposition to a Th1 inflammatory response, 30–32 showed a significant increase in CD4⁺ T-cell and CD4⁺/CD8⁺ cell ratio in the GC-rich zone of the conjunctival epithelium. This was accompanied by a significant decrease in conjunctival GC density and increased expression of the cornified envelope precursor protein SPPR-2 in the conjunctival epithelium.

We performed several experiments to determine whether cytokines produced by the infiltrating CD4⁺ T cells contribute to the loss of conjunctival GCs and the increased expression of SPPR-2. Cells staining positively for the Th-1 cytokine IFN-γ were observed in the conjunctiva of C57BL/6 mice after 10 days of EDE. The identity of these IFN-γ-producing cells was not identified; however, many IFN-γ cells were noted in the conjunctiva of nude mice after adoptive transfer of CD4⁺ T cells from mice with EDE for 5 days. To determine whether IFN-γ may be responsible for the decrease in conjunctival GC density in these mice, we evaluated the effects of EDE on the conjunctiva in B6yKO mice. In contrast to wild-type mice, there was no change in GC density in response to desiccating stress in the IFN-γ KO strain. Reconstitution of these mice with subconjunctival injection of IFN-γ resulted in a significant decrease in GCs after 5 days and also produced a change in the appearance of the remaining GCs, many of which appeared smaller and submerged in the epithelium, rather than at their normal surface location. Furthermore, the effects of subconjunctival administration of IFN-γ were evaluated in wild-type C57BL/6 mice that were subjected to EDE. This cytokine caused a significant decrease in GCs in this strain compared with the BSA-treated control. It also induced morphologic changes in the GCs that were similar to those seen in B6yKO mice that were treated with IFN-γ. Of note, GC densities in UT B6yKO mice and UT C57BL/6 that received subconjunctival IFN-γ were not different from those in the vehicle-injected mice. This indicates that both desiccating stress and IFN-γ are necessary to produce this phenotypic change.

We also found that EDE caused an increase in expression of SPPR-2, one of the cornified envelope precursor proteins in C57BL6 mice. Reconstitution of 5 days B6yKO mice with IFN-γ or subconjunctival administration of IFN-γ in UT C57BL/6 mice significantly increased SPPR-2 expression in the conjunctival epithelium.

These findings are significant for several reasons. First, they showed that desiccating ocular surface stress attracts CD4⁺ T cells into the conjunctival epithelium in C57BL/6 mice. The migration was accompanied by a significant decrease in CD8⁺ T cells and suggests that the stressed ocular surface epithelia may liberate chemotactic factors that attract these CD4⁺ T cells and that there may be antigens exposed by dryness that initiates an antigen-specific T-cell response. Some CD8⁺ T cells have been shown to function as suppressor cells during the secondary and memory phases of immunity. 33–36 These concepts are consistent with our previous finding that CD4⁺ T cells from immunocompetent mice with EDE produce inflammation of the cornea, conjunctiva, and lacrimal gland when adoptively transferred to T-cell-deficient nude mice. 14 Our present study did not attempt to determine whether the T cells infiltrating the conjunctival epithelium were antigen specific. Second, it appears that there may be factors in addition to dryness, such as cytokines liberated by the infiltrating T cells, that contribute to the disappearance of conjunctival GCs in EDE. The inverse correlation between the number of CD4⁺ T cells and GCs in the conjunctivae of mice with dry eye supports this hypothesis. Finally, we have observed that C57BL/6 mice develop greater GC loss and CD4⁺ T cell infiltration in their conjunctiva than do BALB/c mice, which have a Th-2 predilection. 14 These observed differences in the magnitude of GC loss and CD4⁺ T-cell infiltration suggests that immunogenetic factors may be responsible in part for the severity of the conjunctival epithelial disease that develops in response to desiccating stress.

IFN-γ may affect conjunctival epithelial homeostasis by a variety of potential mechanisms. One mechanism is a direct effect on production of differentiation-related proteins. Cornified envelope precursor proteins are normally expressed in low levels in the wet conjunctival mucosa, as opposed to differentiated skin epidermal cells, which have a thick cornified envelope that plays an important role in maintaining barrier function. 17 Among the SPPR-2 genes, only the SPPR-2a promoter was found to have an IFN-γ-stimulated response element. 57 Another mechanism may be stimulating apoptosis. 38–40 IFN-γ alone or in combination with TNF-α has been shown to have a proapoptotic effect on other mucosal epithelia. 41–43 We have reported that there is an increase in conjunctival epithelial apoptosis in EDE and that this is greatest in the GC-rich zones where the T-cell infiltration is observed. 26

These findings support a role for T-cell-produced factors in the pathogenesis of the severe squamous metaplasia that occurs in immune-inflammatory cell conditions where marked CD4⁺ T cell infiltration of the conjunctiva has been observed (e.g., Sjögren’s syndrome, mucus membrane pemphigoid, Stevens-Johnson syndrome, and graft-versus-host disease). 34–46 The results suggest that strategies that limit CD4⁺ T-cell migration into the conjunctival epithelia or those that decrease IFN-γ production may prevent the shift toward cornification and GC loss in dry eye. It is possible that the increase in conjunctival goblet density has been observed in patients with dry eye treated topically with the T-cell immunomodulatory
agent cyclosporine may be due to its ability to inhibit production of IFN-γ by activated T cells.

References


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