Desiccating Stress Promotion of Th17 Differentiation by Ocular Surface Tissues through a Dendritic Cell-Mediated Pathway

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PURPOSE. To explore the phenomenon that corneal and conjunctival tissues subjected to desiccating stress (DS) promote Th17 differentiation by stimulating the production of Th17-inducing cytokines through a dendritic cell (DC)-mediated pathway.

METHODS. Experimental dry eye was created by subjecting C57BL/6 mice to desiccating environmental stress. Corneal and conjunctival explants from dry eye or control mice were cocultured with DCs for 24 hours before CD4⁺ T cells were added for an additional 4 to 7 days. Expression of Th17-associated genes in the cornea, conjunctiva, DCs, and CD4⁺ T cells was evaluated by real-time PCR. Cytokine concentrations in coculture supernatants were measured by immunobead assay. IL-17-producing T cells were identified by ELISPOT bioassay.

RESULTS. Higher levels of IL-17A, TGF- β 1, TGF- β 2, IL-6, IL-23, and IL-1 β mRNA transcripts and TGF- β 1, IL-6, and IL-1 β protein were observed in corneal epithelium and conjunctiva from dry eye mice. DCs cocultured with epithelial explants from dry eye mice for 2 days produced higher levels of TGF- β 1, IL-6, IL-23, and IL-1 β mRNA transcripts and of TGF- β 1, IL-6, and IL-1 β protein. CD4⁺ T cells cocultured with DCs and epithelial explants from dry eye mice expressed increased levels of IL-17A, IL-17F, IL-22, CCL20, and retinoic acid receptor-related orphan receptor- γ t mRNA transcripts and increased IL-17A protein and number of IL-17-producing T cells (Th17 cells).

CONCLUSIONS. These findings demonstrate that DS creates an environment on the ocular surface that stimulates the production of Th17-inducing cytokines by corneal and conjunctival epithelia that promote Th17 differentiation through a dendritic cell-mediated pathway. (*Invest Ophthalmol Vis Sci.* 2010;51:3083-3091) DOI:10.1167/iovs.09-3838

Considered major players in the immune response to infectious organisms, CD4⁺ T helper (Th) cells also promote systemic and organ-specific autoimmune diseases. To convey

their full function, Th cells secrete a variety of cytokines and can be subdivided into three different types based on their cytokine signature: interferon (IFN)- γ -secreting Th1; interleukin (IL)-4-, IL-5-, and IL-13-secreting Th2,¹ and IL-17-producing Th17 cells.^{2,3} Th17 has recently been identified as a T helper cell subset distinct from Th1 and Th2 cells. IL-17-producing T cells have been identified as key effector cells in a variety of human autoimmune diseases and experimental autoimmune diseases in mouse models, including multiple sclerosis, rheumatoid arthritis, respiratory disease, systemic lupus erythematosus, psoriasis, systemic sclerosis, chronic inflammatory bowel disease, experimental autoimmune encephalomyelitis, and autoimmune uveitis.^{4,5} IL-17A (also known as IL-17) and IL-17F are the founding members of the IL-17 cytokine family. IL-17 activates nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways.^{6,7} The genes encoding IL-17A and IL-17F are localized in the same chromosomal region in mice and humans. Similar to IL-17, IL-17F expression has been associated with a number of inflammatory diseases, but IL-17F was noted to have significantly weaker activity than IL-17.^{8,9} IL-22, a member of the IL-10 family, was recently shown to be expressed by Th17 cells.¹⁰⁻¹² Furthermore, Th17 cells in mice and humans have been reported to produce CC-chemokine attractant ligand 20 (CCL20).^{13,14} Therefore, Th17 cells express a unique and expanding array of proinflammatory products.

Th17 cell differentiation is regulated by cytokines. Transforming growth factor (TGF)- β and IL-6 seem to be necessary for the initiation of Th17 cell differentiation¹⁵⁻¹⁷ and are broadly expressed by many cell types in the body, including epithelial and dendritic cells. IL-23 and IL-1, both of which are products of activated myeloid cells, including dendritic cells, macrophages,^{18,19} and inflamed epithelial cells, expand and maintain partially differentiated Th17 cells in the presence of IL-6 and TGF- β .¹⁷ During Th-cell differentiation, regulatory cytokines act through selective members of the signal transducer and activator of transcription (STAT) family to regulate gene transcription.²⁰ STAT proteins regulate Th-cell differentiation at least in part through the induction of lineage-specific transcription factors; the expression of T-bet protein is induced by STAT1 in developing Th1 cells,²¹ and the expression of GATA-binding protein 3 (GATA3) is regulated by STAT6 in Th2 cells.²² The differentiation of Th17 cells is initiated by STAT3, downstream of IL-6-induced signaling.^{23,24} Activation of STAT3 induces the expression of retinoic acid receptor-related orphan receptor- α (ROR α) and ROR γ t,²⁵ two transcription factors that promote the Th17 cell-associated gene expression program, leading to the production of IL-17, IL-17F, and IL-22.

Dendritic cells (DCs) have an important function in Th17-cell differentiation. They are antigen-presenting cells specialized to activate CD4⁺ T cells and, through their interaction with CD4⁺ T cells, to initiate primary immune responses.

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Furthermore, when primed, certain DCs express a high-level of Th17-inducing cytokines, including IL-6, TGF- β , IL-23 and IL-1.^{17,26–28}

Dry eye has been demonstrated to cause inflammation on the ocular surface, evidenced by increased levels of inflammatory cytokines (IL-1, IL-6, TNF- α) in the tear fluid and corneal and conjunctival epithelia and infiltration of the conjunctiva with CD4⁺ T cells.^{29–35} Recently, increased levels of IL-17, IL-23, and IL-6 were found in saliva and salivary gland biopsy specimens obtained from patients with the severe autoimmune condition Sjögren syndrome.^{36,37} Our group and others have also found increased expression of Th17-associated cytokines and IL-17-producing cells in the ocular surface epithelium of patients with dry eye and in the ocular surface epithelium and draining lymph nodes in experimental murine dry eye.^{38,39} These findings indicate that the ocular surface epithelium is an enriched site for Th17-inducing cytokines, including TGF- β 1, IL-6, IL-23, and IL-1. Based on these findings, we hypothesized that ocular surface epithelia subjected to desiccating stress (DS) are capable of stimulating DCs to produce Th17-inducing cytokines (i.e., IL-6, IL-23, TGF- β 1) and to promote the Th17 differentiation of CD4⁺ T cells. The purpose of this study was to evaluate the ability of ocular surface tissues exposed to DS to activate Th17-inducing DCs and to modulate Th17 differentiation of CD4⁺ T cells in a coculture system.

MATERIALS AND METHODS

Mouse Model of Dry Eye

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

DS was induced in 6- to 8-week-old C57BL/6 mice of both sexes (Jackson Laboratories, Bar Harbor, ME), by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) in alternating hindquarters four times a day (8 am, 11 am, 2 pm, 5 pm) with exposure to an air draft and <40% ambient humidity for 18 hours per day, as previously reported.^{40,41} Mice were euthanized after 5 or 10 days (DS5 and DS10, respectively) of this treatment. A group of age- and sex-matched mice maintained in a nonstressed (NS) environment without exposure to air drafts served as control subjects.

Immunohistochemistry

Mouse eyes and adnexa ($n = 3$) were excised, embedded in optimal cutting temperature (OCT) compound (VWR, Suwanee, GA), and flash frozen in liquid nitrogen. Sagittal 8- μ m sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at -80°C . Immunohistochemistry was performed to detect dendritic cells in the conjunctiva using hamster anti-CD11c-specific antibody (clone HL3, 12.5 $\mu\text{g}/\text{mL}$; BD Biosciences, San Diego, CA).

Cryosections were stained with primary antibody followed by mouse anti-hamster biotinylated secondary antibody (BD Biosciences) and an avidin-biotin complex kit (Vectastain Elite; Vector Laboratories, Burlingame, CA) using reagents (NovaRed; Vector Laboratories), as previously described.²⁹ Secondary antibody alone and appropriate anti-mouse isotype (BD Biosciences) controls were also performed. Three sections from each animal were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon, Melville, NY).

Flow Cytometry Analysis

Mouse eyes and lids ($n = 10$) were excised, pooled, and incubated in 10 mL of 5 mg/mL protease (Dispase II; Roche Molecular Biochemicals, Indianapolis, IN) in a shaker at 37°C for 1 hour, followed by neutral-

ization with Hanks balanced solution (Invitrogen-Gibco, Grand Island, NY) supplemented with 3% fetal bovine serum (HyClone, Logan, UT). Corneal epithelium was scraped with a disposable blade, and the bulbar and tarsal conjunctival epithelia were removed with cytology brushes under a dissecting microscope. Corneal and conjunctival cells were pooled, centrifuged at 2000 rpm for 5 minutes, filtered, and resuspended.

Single-cell suspensions of cornea and conjunctiva were stained with anti-CD16/32 (to block Fc receptors; BD PharMingen, San Diego, CA), followed by cell surface staining with FITC-anti-CD11c (clone HL3; BD Biosciences). Negative controls consisted of cells stained with FITC-isotype antibody (BD PharMingen). Cells were then resuspended in fixation-permeabilization solution (Cytofix/Cytoperm; BD PharMingen). A cytometer (LSRII Benchtop; BD PharMingen) was used for flow cytometry, and data were analyzed with BD software (Diva; BD PharMingen).

Generation and Treatment of Bone Marrow-Derived Dendritic Cells

Bone marrow-derived DCs were generated as described by Lutz et al.⁴² Briefly, 6- to 8-week-old C57BL/6 normal mice were killed, and femurs were removed. Bone marrow cells (5×10^6) collected from femurs were seeded in 100-mm dishes in 10 mL medium (RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 $\mu\text{g}/\text{mL}$ gentamicin, 1.25 $\mu\text{g}/\text{mL}$ amphotericin B, 50 μM 2-mercaptoethanol) containing 20 ng/mL recombinant mouse granulocyte macrophage-colony-stimulating factor (rmGM-CSF; R&D Systems, Minneapolis, MN; catalog no. 415-ML) and 10 ng/mL recombinant mouse IL-4 (R&D Systems; catalog no. 404-ML). Cultures were fed on days 3, 6, and 8. On day 9, nonadherent and loosely adherent cells were harvested as bone marrow-derived DCs. The purity of the DCs, determined by flow analysis of surface CD11c staining, was greater than 60% (data not shown), similar to that previously reported study by Lutz et al.⁴²

Isolation of Murine CD4⁺ T Cells

Murine CD4⁺ T cells were isolated from the spleens and cervical lymph nodes of 6- to 8-week-old C57BL/6 normal mice, as described previously.⁴³ Spleens and cervical lymph nodes of mice were surgically excised, crushed between two sterile frosted glass slides, and made into a single-cell suspension. Red blood cells in the suspension were lysed ammonium chloride, and the suspension was centrifuged at 1000 rpm for 5 minutes, filtered, and resuspended with rat anti-mouse CD4-conjugated magnetic micro beads (MACS system; Miltenyi Biotec) diluted with cold 0.5% BSA in PBS (1:10 dilution, 90 μL buffer, and 10 μL beads for every 1×10^7 cells). After 15 minutes' incubation at 4°C and washing, the cells were resuspended in 500 μL buffer per 1×10^8 cells and loaded onto a minicolumn. Positive cells attached to the column were removed with a plunger, and the CD4⁺ T cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. The CD4-enriched cell suspensions contained >80% (data not shown) CD4⁺ T cells, as determined by flow cytometry.

In Vitro Th17 Differentiation of CD4⁺ T Cells Cocultured with DCs, Epithelial Explants, or Both from DS and NS Mice

Corneas and conjunctivas from NS, DS5, and DS10 mice were removed aseptically and cultured at 37°C in serum-free RPMI medium (20 corneas and conjunctivas/1 mL/well). Supernatants were collected after 48 hours, and cytokine concentrations were measured by immunobead assay (Luminex; Upstate-Millipore, Lake Placid, NY).

DCs (1×10^6) were cultured ex vivo in 24-well plates in serum-free RPMI medium alone or in the presence of corneal and conjunctival explants (20 corneas and conjunctivas/1 mL/well) removed from DS10 or NS mice (DC+DS, DC+NS, respectively). After 48 hours, corneal and conjunctival explants were removed, and the remaining non-adherent cells consisting of DCs and cells that migrated out of the

explants were lysed for total RNA extraction and evaluation of mRNA expression. Supernatants were collected for measuring cytokine concentrations by immunobead assay (Luminex; Upstate-Millipore). A separate group of DCs was cultured for 48 hours in serum-free conditioned RPMI media of cornea and conjunctiva explants obtained from NS and DS10 mice.

To stimulate T cells, DCs (1×10^6) were cultured in 24-well plates in complete RPMI-1640 medium supplemented in the presence or absence of corneal and conjunctival explants (20 corneas and conjunctivas/1 mL/well) removed from NS or DS10 mice for 24 hours. Then CD4⁺ T cells (1×10^7 T cells/well per 1×10^6 DCs) were added for an additional 1 to 7 days. Three groups were evaluated: CD4⁺ T cells cocultured with DCs in the absence of corneal and conjunctival explants (T cell+DC); CD4⁺ T cells cocultured with DCs in the presence of corneal and conjunctival explants from dry eye mice (T cell+DC+DS); CD4⁺ T cells cocultured with DCs in the presence of corneal and conjunctival explants from normal control mice (T cell+DC+NS). CD4⁺ cells cocultured with DCs exposed to conditioned media from NS or DS corneal and conjunctiva served as a control group. After 1, 2, and 4 days, the corneal and conjunctival explants were removed from groups 2 and 3, the remaining nonadherent cells consisting of DCs, CD4⁺ T cells, and cells that migrated out of the explants were lysed, and total RNA was extracted for evaluation of mRNA expression. Supernatants were collected after 4 days, and cytokine protein concentrations were measured by immunobead assay (Luminex; Upstate-Millipore). After 7 days, nonadherent cells were harvested, and the number of IL-17- and IFN- γ -producing T cells was evaluated by ELISPOT bioassay.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA from the corneas and conjunctivas collected and pooled from NS, DS5, and DS10 mice (one sample consisted of five pooled mice per group for each experiment, in three independent experiments) from DCs and CD4⁺ T cells (in four independent experiments) was extracted with a purification kit (RNeasy Micro Kit; Qiagen, Valencia, CA) according to the manufacturer's instructions, quantified with a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE), and stored at -80°C . First-strand cDNA was synthesized with random hexamers by M-MuLV reverse transcription (Ready-to-Go You-Prime First-Strand Beads; GE Healthcare, Inc., Arlington Heights, NJ), as previously described.^{44,45}

Real-time PCR was performed with specific MGB probes (*TaqMan*; Applied Biosystems, Inc. [ABI], Foster City, CA) and PCR master mix (*TaqMan* Gene Expression Master Mix; ABI), in a commercial thermocycling system (Mx3005P QPCR System; Stratagene, La Jolla, CA), according to the manufacturer's recommendations. Murine MGB probes were GAPDH, IL-6, IL-23, TGF- β 1, TGF- β 2, IL-1 β , IL-1 α , IL-17A, IL-17F, ROR γ t, IL-22, CCL20 (Th17 inducers and Th17 pathway) IFN- γ , IL-2, IL-12, T-bet (Th1 pathway), IL-4, IL-13, and GATA-3 (Th2 pathway) (assay IDs Mm99999915, Mm00446190, Mm00518984, Mm004417241, Mm00436952, Mm00434228, Mm00439620, Mm00439619, Mm00521423, Mm00441139, Mm00444241, Mm00444228, Mm00801778, Mm00434256, Mm00434165, Mm00450960, Mm00445259, Mm00434204, and Mm00484683, respectively). The GAPDH gene was used as an endogenous reference for each reaction. Results of the relative-quantitative real-time PCR were analyzed by the comparative threshold cycle (C_t) method⁴⁶ and normalized by GAPDH as an internal control.

Multiplex Immunobead Assay

Concentrations of total TGF- β 1 and TGF- β 2 were measured with a bead assay (Luminex; Upstate-Millipore) after HCl activation. Levels of IL-6, IL-1 β , and IL-17 were measured (Multiplex Immunobead Assay; Upstate-Millipore). Total protein concentration in the 2-day supernatants of corneal and conjunctival explants from NS, DS5, and DS10, 2-day supernatants of DCs cocultured with the explants, and 4-day

supernatants of CD4⁺ T cells cocultured with the DCs and explants were measured with a protein assay kit (Micro BCA; Pierce, Rockford, IL). For the TGF- β 1 and TGF- β 2 assay, 1 N HCl was added to lysates for 1 hour, in a shaker, at room temperature, followed by addition of 1 N NaOH. Samples were then added to wells containing 10 μL appropriate cytokine bead mixture that included mouse monoclonal antibodies specific for TGF- β 1, TGF- β 2, IL-17A, IL-6, and IL-1 β (Upstate-Millipore). Serial dilutions of cytokines were added to wells in the same plate as the supernatant samples to generate a standard curve. The plate was incubated overnight at 4°C to capture the cytokines by the antibody-conjugated fluorescent beads. After three washes with assay buffer, 25 μL biotinylated secondary cytokine antibody mixture was applied for 1.5 hours in the dark at room temperature. Reactions were detected with streptavidin-phycoerythrin with an analyzer system (100 IS 2.3; Luminex). Assays were performed on samples from three separate experiments, and the results were averaged.

ELISPOT Assay for IL-17- or IFN- γ -Producing T Cells

Replicate 50- μL cell suspensions containing 3×10^5 CD4⁺ T cells isolated (as described) were added to 96-well polyvinylidene difluoride (PVDF) plates (Millipore, Billerica, MA), precoated with anti-mouse IL-17 or IFN- γ capture antibody (R&D Systems). Wells containing either cells or positive controls (3 ng/well recombinant mouse IL-17A or IFN- γ ; R&D Systems) or media alone (negative control) were incubated at 37°C with CO_2 for 24 hours in RPMI-medium (Invitrogen-Gibco). After washing, the plate was incubated overnight at 4°C , with biotinylated goat anti-mouse IL-17 or IFN- γ detection antibody (R&D Systems), followed by incubation with streptavidin-HRP (R&D Systems) the next day for 2 hours. Red color development was achieved by incubating peroxidase substrate (NovaRed; Vector Laboratories) for 15 minutes. The PVDF membrane was dried, and the individual wells were punched out from the plate. The positive red spots were counted under a dissecting microscope (SMZ 1500; Nikon, Melville, NY). Replicate wells were averaged from three individual experiments. Results are presented as number of spots/ 3×10^5 cells.

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey post hoc testing was used for statistical comparisons of multiple groups. $P \leq 0.05$ was considered statistically significant. These tests were performed using statistical software (Prism 4.0; GraphPad Software, Inc., San Diego, CA).

RESULTS

CD11c⁺ Dendritic Cells Are Present in the Ocular Surface Epithelia

To confirm the presence and location of DCs on the ocular surface, we performed immunohistochemistry for the DC marker CD11c in frozen sections of cornea and conjunctiva from nonstressed mice. CD11c⁺ cells were noted in the conjunctival epithelia, in close contact with the goblet cells (Fig. 1A). Flow cytometry analysis demonstrated that approximately 5% of the cells obtained from the cornea and conjunctival epithelium of nonstressed mice were CD11c⁺ (Fig. 1B). Because of the relatively low density of DCs in the ocular surface epithelia, we elected to use bone marrow-derived CD11c⁺ DCs for the coculture experiments.

Desiccating Stress Increases Levels of IL-17 Family Cytokines on the Ocular Surface

In a previously reported survey of Th1, Th2, and Th17 family cytokines in the ocular surface after DS, we found a significant increase in Th17-related cytokines.³⁷ To confirm that cytokines characterizing the Th17 phenotype increase in the cornea and

FIGURE 1. (A, arrows) Representative image of conjunctival section of nonstressed mice stained for CD11c⁺. Inset, dotted square: high magnification of CD11c⁺. Note dendritic cell appearance of stained cells. (B) Flow cytometry analysis of freshly isolated cells from the corneal (CN) and conjunctival (CJ) epithelia stained with CD11c-FITC conjugated antibody. Lymphocytes were gated based on characteristic light-scatter properties, and single lymphocytes were gated based on forward scatter height versus forward scatter area (FSC-A). Mean $\pm$ SD percentage of positive cells in three independent experiments is noted on the graph.

conjunctiva in response to experimental DS, we reevaluated the effects of DS on levels of Th17 family cytokines in these ocular surface tissues.

Evaluation by reverse transcription and real-time PCR (Fig. 2A) revealed that levels of IL-17A transcripts significantly increased in the corneal and conjunctival epithelia of eyes exposed to DS for 5 (DS5) and 10 (DS10) days compared with the

NS control group. These results were consistent with our previous study³⁸ that found an increased number of IL-17-secreting cells in the cornea and conjunctiva after DS.

As shown in Figure 2B, DS significantly increased IL-6 (at DS5 and DS10), TGF- β 1, TGF- β 2, IL-23, and IL-1 β (at DS5) transcripts in the corneal epithelium compared with the NS group. Significantly higher levels of IL-6, IL-1 β (both at DS5 and DS10), TGF- β 1, and IL-23 (at DS5) transcripts were observed in the conjunctiva of DS mice than the NS group. The greatest increase at DS5 was observed for IL-6, both in the cornea (7.6 ± 1.5 -fold) and in the conjunctiva (6.9 ± 1.5 -fold).

To confirm the gene expression studies, release of Th17-inducing cytokines into the supernatants of 2-day cultured corneal and conjunctival explants from NS, DS5, and DS10 groups were evaluated by immunobead assay (Luminex; Fig. 2C). Significantly higher release of IL-6 and TGF- β 1 at DS5 and DS10 and of IL-1 β at DS10 were observed in the DS supernatants compared with the NS group. The level of TGF- β 1 was greater in the DS10 than in the DS5 group ($P < 0.05$). Because a higher concentration of cytokines was detected in DS10 than DS5 explants, we selected DS10 for subsequent coculture study.

Stimulated Production of Th17-Inducing Cytokines in the DCs Cocultured with Ocular Surface Tissues from DS Mice

To investigate the role of desiccated ocular surface tissues on stimulating DCs to produce Th17-inducing cytokines as the

FIGURE 2. Stimulated production IL-17A- and Th17-inducing cytokines in the corneal and conjunctival epithelia of dry eye mice. (A) Real-time PCR data showing the relative expression (x-fold) of IL-17A in cornea (CN) and conjunctiva (CJ) in nonstressed controls mice (NS) and mice subjected to DS for 5 or 10 days (DS5, DS10, respectively; $n = 5$ per group). (B) Real-time PCR data showing the relative expression (x-fold) of Th17-inducing cytokines (IL-6, TGF- β 1, TGF- β 2, IL-23, IL-1 β) in cornea (CN) and conjunctiva (CJ) in NS, DS5, and DS10 groups. (C) Concentrations of IL-6, TGF- β 1, and IL-1 β in the supernatants of 2-day cultured corneal and conjunctival explants obtained from NS, DS5, and DS10 groups measured by immunobead assay. Data are expressed as the mean $\pm$ SD of three separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; DS5 or DS10 versus NS group.

FIGURE 3. Stimulated Th17-inducing cytokines in the DCs cocultured with ocular surface tissue explants from non-stressed (NS) mice and mice subjected to DS (DS) for 10 days. **(A)** Real-time PCR data showing the relative expression (α -fold) of Th17-inducing cytokines (TGF- β 1, TGF- β 2, IL-6, IL-23, IL-1 β , IL-1 α) in DCs cultured for 2 days alone without corneal and conjunctival explants, or with corneal and conjunctival explants from a nonstressed control group (DC+NS) or eyes subjected to DS for 10 days (DC+DS). **(B)** Concentrations of IL-6, TGF- β 1, and IL-1 β in 2-day supernatants of DCs alone or DCs cocultured with corneal and conjunctival explants removed from NS eyes or eyes subjected to DS for 10 days, measured by immunobead assay. Data are expressed as mean $\pm$ SD of three separate experiments. * P < 0.05; ** P < 0.01; *** P < 0.001.

initial step in the ocular surface Th17 response to DS, we evaluated the expression of Th17-inducing cytokines by DCs cultured with or without explanted ocular surface tissues using real-time PCR. The data are summarized in Figure 3A. Overall, the DC+DS group showed the greatest increase in Th17-inducing cytokines, including TGF- β 1, IL-6, IL-23, and IL-1 β , all significantly different from the DC+NS and DC alone groups. The DC+DS group also had significantly greater levels of IL-1 α than the DC alone group. In support of the PCR findings, the relative concentrations of Th17-inducing cytokines measured by Luminex immunobead assay in 2-day supernatants of DCs cocultured with or without corneal and conjunctival explants were similar to the PCR findings (Fig. 3B). Concentrations of IL-1 β and IL-6 in the supernatants of cultured DS explants were not significantly different from those measured in the DC alone group (data not shown).

Th17 Differentiation of CD4⁺ T Cells Was Induced after Coculture with DCs and DS Ocular Surface Tissues

Based on our findings that Th17-inducing cytokines were stimulated in ocular surface tissues exposed to DS and in DCs cultured with DS cornea and conjunctival explants and our previously reported finding that DS increased the number of IL-17-producing cells on the ocular surface,³⁷ we hypothesized that the production of Th17-associated cytokines would be promoted in CD4⁺ T cells cocultured with DCs for 1, 2, and 4 days in the presence of corneal and conjunctival explants from eyes subjected to DS. In our preliminary experiments, we evaluated whether CD4⁺ T cells isolated from cervical lymph nodes and spleens cocultured with cornea and conjunctival explants alone induced IL-17 production; however, levels of IL-17 transcripts were low and no higher than levels of media alone (data not shown). Consequently, subsequent coculture experiments were performed in the presence of DCs.

IL-17A and IL-17F levels in the T cell+DC+DS group were significantly higher than the T cell+DC level (24 hours, 48 hours, and 4 days) and were higher than the T cell+DC+NS levels at 24 hours and 4 days for IL-17A and at 48 hours for IL-17F. IL-22 was significantly higher in T cell+DC+DS than T cell+DC+NS at 24 hours and 48 hours and higher than T cell+DC at 4 days. CCL-20, a Th17-attracting chemokine, was higher in the T cell+DC+DS group than in the other groups at 24 hours (Fig. 4A). Of note, the T cell+DC+NS group was

higher than the T cell+DC control group for IL-17A at 24 hours and 4 days, IL-17F at 48 hours, IL-22 at 24 hours and 48 hours, and CCL-20 at 24 hours. Furthermore, the level of IL-17A in the cells that migrated out of DS explant-only cultures was >100-fold lower than the T cell+DC+DS group, indicating that explant-derived cells were not responsible for the observed increase of this cytokine (data not shown).

This experiment was repeated using DCs cultured for 2 days in conditioned media of cornea and conjunctiva explants from normal and DS eyes to determine whether DCs prepared in this fashion could stimulate Th17 differentiation in the absence of the ocular surface tissue explants. We found that DCs prepared in this manner stimulated the production of Th17-associated factors in CD4⁺ T cells less efficiently (IL-17A, 1.8-fold; IL-17F, 2.1-fold; ROR γ t, 2.4-fold vs. DCs cultured in conditioned media from NS explants).

To confirm the PCR results, the production of IL-17 and IFN- γ by CD4⁺ T cells was evaluated by ELISPOT bioassay and Luminex immunobead assay. Our results, presented in Figures 4B–E, showed a significant increase in the number of IL-17-producing cells in the T cell+DC+DS group compared with the T cell+DC+NS and T cell+DC groups (P < 0.05, respectively). The T cell+DC+NS group also had a significantly greater number of IL-17-producing cells than the control group. There was a slight, but nonsignificant, decrease in the number of IFN- γ -producing T cells in the T cell+DC+DS group compared with the T cell+DC+NS and T cell+DC groups. We also observed a significant increase in the concentration of IL-17 in the supernatant of the T cell+DC+DS group (Fig. 4F) compared with either the T cell+DC+NS or the T cell+DC group (P < 0.05 and P < 0.001, respectively). The IL-17 concentration was also significantly increased in the T cell+DC+NS group compared with control. The increased concentration of IL-17 in the coculture was not caused by cells in the explants because the IL-17 concentration in the DS explant-only cultures was >60-fold lower than in the T cell+DC+DS group. These findings indicate that DS creates an environment on the ocular surface capable of inducing Th17 differentiation by CD4⁺ T cells.

As further evidence in support of this phenomenon, we evaluated the expression of the Th17 cell transcription factor ROR γ t in the three groups (n = 4) by real-time PCR (Fig. 4G). The ROR γ t level was significantly increased in the T cell+DC+DS group compared with the T cell+DC+NS and T

FIGURE 4. Th17-induced Th17 differentiation of CD4⁺ T cells cocultured with DCs in the presence of cornea and conjunctival tissues subjected to DS. (A) Real-time PCR data showing the relative expression (x-fold) of Th17-expressed cytokines (IL-17A, IL-17F, IL-22, CCL-20) in CD4⁺ T cells cocultured with DCs for 1 day, 2 days, and 4 days in the T cell+DC group (absence of corneal and conjunctival explants) or DCs and corneal and conjunctival explants from a nonstressed control group (T cell+DC+NS) or from a 10-day DS group (T cell+DC+DS) (*n* = 4). (B, C) ELISPOT bioassay showing the numbers of IL-17 or IFN- γ -producing cells in T cell+DC group, T cell+DC+NS explant group, and T cell+DC+DS explant group. Cells (3×10^5) were seeded per well. (D, E) Mean $\pm$ SD of three independent IL-17 and IFN- γ ELISPOTS showing IL-17 or IFN- γ -producing cells derived from CD4⁺ T cells cocultured with DCs for 7 days in the absence or presence of corneal and conjunctival explants removed from NS and 10-day DS mice, respectively. (F) IL-17 concentration in the supernatant of CD4⁺ T cells cocultured with DCs for 4 days in the absence or presence of corneal and conjunctival explants removed from NS and 10-day DS mice, respectively. (G) Real-time PCR data showing relative expression (x-fold) of the Th17 cell transcription factor-ROR γ t in CD4⁺ T cells cocultured with DCs for 1 day, 2 days, and 4 days in the T cell+DC group, T cell+DC+NS group, and T cell+DC+DS group (*n* = 4). Data are presented as mean $\pm$ SD of three or four independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

cell+DC groups at all three time points. ROR γ t expression was also greater in the T cell+DC+DS group than in the T cell+DC control at 24 hours and 4 days.

To determine whether the effect of desiccated ocular surface epithelia on T-cell differentiation was specific for the Th17 pathway, we investigated the effects of coculture on the levels of Th1-associated factors (IFN- γ , IL-12, IL-2, and T-bet) and Th2-associated factors (IL-4, IL-13, and GATA-3) in the CD4⁺ T cells cocultured with DCs for 4 days in the presence or absence of corneal and conjunctival explants by real-time PCR (Fig. 5). Levels of IL-2, T-bet, IL-4, IL-13, and GATA-3 were decreased in the T cell+DC+DS and T cell+DC+NS groups compared with the T cell+DC control. No difference was observed between the T cell+DC+DS group and the T cell+DC+NS group. There was a nonsignificant trend toward decreased levels of IFN- γ and IL-12 transcripts in the T cell+DC+DS group.

DISCUSSION

We have previously reported that DS increases the number of CD4⁺ T cells in the ocular surface epithelium of mice.^{29,38} We

have also found significantly increased levels of IL-17-producing cells and Th17-associated cytokine mRNAs in the cornea and conjunctiva after DS. The present study was performed to determine whether DS creates an environment on the ocular surface that fully promotes Th17 differentiation. Using a mouse model we found that desiccating environmental stress increased levels of factors involved in the differentiation and survival of IL-17-producing T cells in the cornea and conjunctiva. With this knowledge, we attempted to further study the initiating steps in the immune cycle of dry eye that has been proposed by our group and others^{47,48} using a coculture system of desiccated ocular surface tissues and DCs and found exposure to the stressed ocular surface tissues increased the production of Th17-inducing factors by DCs. Finally, we found that coculture of the desiccated ocular surface tissues with DCs and CD4⁺ T cells increased the production and release of IL-17A- and Th17-associated factors. These findings provide a mechanism for development of the Th17-biased immune response on the ocular surface to DS.

FIGURE 5. Decreased levels of Th1- and Th2-associated factors in $CD4^+$ T cells cocultured with DCs in the presence of corneal and conjunctival tissue subjected to DS. Real-time PCR data showing the relative expression ($\times$ -fold) of Th1-associated factors (IFN- γ , IL-12, IL-2, T-bet) and Th2-associated factors (IL-4, IL-13, GATA-3) in $CD4^+$ T cells cocultured with DCs for 4 days in the T cell+DC group, T cell+DC+NS group, and T cell+DC+DS group ($n = 4$). Data are expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. T cell+DC+DS or T cell+DC+NS group versus T cell+DC group.

Several key cytokines have been identified that promote Th17 differentiation. Specifically, TGF- β and IL-6 have been found to initiate Th17-cell differentiation of naive $CD4^+$ cells,^{15–17} whereas IL-23 and IL-1 finalize the differentiation program and maintain differentiated Th17 cells in the presence of IL-6 and TGF- β .^{18,19,49} Similar to our findings on the mouse ocular surface,³⁸ these cytokines have been found to increase in inflamed nonocular mucosal tissues.^{50,51} The predilection of Th17 for mucosal tissues suggests the cytokine microenvironment at these sites may promote the Th17 differentiation pathway.

Consistent with the findings in our experimental mouse dry eye model, evidence for Th17 inflammation has been found in non-Sjögren and Sjögren syndrome-associated dry eye in humans.^{30,31} Increased levels of IL-23 and IL-6 were found in tears, saliva, and salivary glands biopsy specimens obtained from patients with Sjögren syndrome, an autoimmune disease that causes severe dry eye.^{36,37} Furthermore, we found increased production of these factors in the conjunctival epithelium of patients with dry eye from a variety of causes.³⁸

Our experiments suggest that factors produced by the ocular surface epithelia exposed to DS stimulate the production of Th17-inducing cytokines by DCs and augment their ability to promote Th17 differentiation. DCs are antigen-presenting cells specialized to activate T cells and to initiate primary immune responses. DCs reside in the skin and mucosal tissues, including the airways and intestines, sites that are frequently exposed to allergens, pathogens, and chemical toxins. We detected $CD11c^+$ DCs in the ocular surface epithelium, particularly the conjunctiva. DCs prime $CD4^+$ T cells by presenting antigenic peptides cradled in major histocompatibility complex class II molecules on their cell surfaces. TCR ligation with costimulation drives T cells to develop into effector cells. Recent studies have found Th17 differentiation could be induced through peptidoglycan (PGN)-stimulated DCs.⁵² In addition to the surface epithelial cells, DCs appear to be an important source of Th17-inducing cytokines.^{17,26–28} We found that exposure of DCs to ocular surface tissues from mice subjected to DS significantly increased the production of TGF- β 1, IL-6, IL-1 β , and IL-23. The production of certain cytokines by DCs (i.e., IL-6, IL-23, and IL-1 β) was also noted to increase after coculture with nonstressed ocular surface tissues, suggesting there is a slight bias toward promoting a Th17 phenotype by the normal ocular surface, which is the only exposed mucosal surface in the body. These DC-produced factors, combined with those

produced by the ocular surface tissues, were found to fully promote Th17 differentiation in cocultured $CD4^+$ T cells.

Consistent with the findings of our previous study,³⁸ the present study showed that DS induces a Th17 skewed response on the murine ocular surface. Increased expression of the Th17 cytokine IL-17A was observed in corneal and conjunctival epithelia of the mice with dry eye. Given that IL-17A is produced by T cells but not by epithelial cells, the Th17 reaction on the ocular surface was likely caused by $CD4^+$ T cells, which have previously been found by our group to infiltrate ocular surface tissues after experimental DS.^{29,34,44} $CD4^+$ T cells that were cocultured with epithelial explants and DCs were found to express increased levels of Th17 cytokines (IL-17A, IL-17F, IL-22) and CCL-20 transcripts and to produce and release IL-17A. Levels of IL-17 transcripts and protein measured in the 5-day DS explant-only control group were found to be >100 - and >60 -fold lower, respectively, than those measured in the DS explant+DC+T cell coculture group, indicating that IL-17-producing lymphocytes residing in or infiltrating the desiccated ocular surface tissues were not responsible for the marked increase in IL-17 observed in the coculture group. These findings provide clear evidence that changes in the ocular surface environment after DS are capable of inducing Th17 differentiation.

We found that efficient differentiation of $CD4^+$ T cells to IL-17 producers required the combination of ocular surface explants from DS mice and DCs. Exposure of DCs to conditioned media from DS ocular surface explants were not as effective in promoting Th17 differentiation as those cocultured with ocular surface explants. Possible explanations for these findings include the need for direct contact among these cells, more efficient activation of cytokines such as TGF- β 1 produced by the ocular surface epithelial cells, and higher concentrations of Th17-inducing factors in the explant+DC coculture group than the explant-only or DC-only groups. These findings are consistent with the proposed mechanism of Th17 differentiation as a multistep process that starts with partial differentiation of $CD4^+$ T cells after exposure to factors (e.g., IL-6 and TGF- β) produced by activated DCs in the regional nodes, followed by full differentiation by epithelial- and DC-derived factors such as IL-1 and IL-23 after the cells traffic to the ocular surface.⁴⁹

The transcription factor ROR γ t was identified as a candidate master regulator that drives Th17-cell lineage differentiation.²⁵ Expression of ROR γ t is induced by TGF- β , IL-6, and IL-23. Overexpression of ROR γ t was found to promote Th17-cell differentiation when both Th1- and Th2-cell differentiation were blocked. In a model of experimental autoimmune encephalomyelitis, mice with ROR γ t-deficient T cells were found to have attenuated autoimmune disease and lacked tissue-infiltrating Th17 cells.²⁵ We found robust up-regulation (up to 100-fold) of the level of the Th17-cell transcription factor ROR γ t in T cells cocultured with desiccated ocular surface tissues and DCs, providing further evidence of the potent Th17-prone environment induced by desiccation.

IL-17-producing T cells are distinct from Th1 cells.¹⁷ In vitro stimulation of YFP-negative sorted naive $CD4^+$ T cells from Yeti mice indicated that differentiation to IL-17 progresses without opening of the IFN- γ locus. Analysis of the expression of transcription factors showed clearly that IL-17-producing T cells express neither GATA-3 nor T-bet, Th1- and Th2-associated transcription factors, respectively.¹⁷ Indeed, Th17 differentiation has been found to be suppressed by Th1 (IFN- γ)- and Th2 (IL-4)-associated cytokines.⁴⁹ In the T cell+DC+DS group, we found lower expression of Th1 (IL-2, T-bet)- and Th2 (IL-4, IL-13, GATA-3)-associated factors. There was no change in the production of IFN- γ and IL-12 transcripts in the T cell+DC+DS group and no increase in the number of

IFN- γ -producing CD4⁺ T cells in the cells cocultured with DS explants and DCs. Taken together, these findings support the mechanism proposed by Chauhan and Dana⁴⁷ that the migration of DCs activated by stressed ocular surface epithelia migrate to the regional lymph nodes, where they prime CD4⁺ T cells and promote early-stage Th17 differentiation. These partially differentiated CD4⁺ T cells migrate to the ocular surface where Th17 differentiation is maximally promoted by epithelial- and DC-derived factors.

In summary, this study demonstrates that DS stimulates the expression and production of Th17-inducing cytokines by corneal and conjunctival epithelia and that desiccation creates an environment promoting Th17 differentiation through a dendritic cell-mediated pathway.

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