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The CCR6/CCL20 Axis Mediates Th17 Cell Migration to the Ocular Surface in Dry Eye Disease

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PURPOSE. Th17 cells are believed to be the primary effector cells in the pathogenesis of dry eye disease (DED). However, the mechanisms by which Th17 cells migrate from the lymphoid tissues to the ocular surface are unknown. The purpose of this study was to investigate the role of the C-C chemokine receptor 6/C-C chemokine ligand 20 (CCR6/CCL20) chemokine axis in mediating Th17 cell migration in DED.

METHODS. DED was induced by housing C57BL/6 mice in a low-humidity environment supplemented with scopolamine treatment. Th17 cell expression of CCR6 was evaluated using flow cytometry and ocular surface expression of CCL20 was measured using PCR and ELISA assays. CCL20 neutralizing antibody was administered subconjunctivally to DED mice and disease severity, including the frequency of conjunctival Th17 cells, was evaluated.

RESULTS. CCR6 is preferentially expressed by Th17 cells in both normal and DED mice and DED significantly upregulates ocular surface expression of CCL20. Disruption of CCR6/CCL20 binding with CCL20 neutralizing antibody decreases T-cell migration in vitro and reduces Th17 cell infiltration of the conjunctiva when administered in vivo, significantly improving clinical signs of DED. These changes were accompanied by a decrease in ocular surface inflammatory cytokine levels and corneal CD11b+ cell frequencies. Treatment also significantly reduced the generation of Th17 cells.

CONCLUSIONS. Local neutralization of CCL20 decreases Th17 cell infiltration of the ocular surface in DED, leading to improvement in clinical signs of disease. This suggests that CCR6/CCL20 interactions direct Th17 cell migration in DED and that disruption of this axis may be a novel therapeutic approach to this condition.

Keywords: dry eye, Th17 cell, cell migration, CCL20, CCR6

Dry eye disease (DED) is an immunoinflammatory disorder of the ocular surface that affects millions of patients in the United States alone.^{1,2} Inflammation of the ocular surface, tear film instability, and tear film hyperosmolarity are hallmarks of this disease and may lead to symptoms ranging from chronic ocular discomfort to corneal perforation.³

A current hypothesis of DED pathogenesis postulates that insult to any component of the lacrimal functional unit (consisting of the cornea, conjunctiva, lacrimal glands, and associated neural pathways) may result in ocular surface inflammation and the induction of an adaptive immune response.⁴ Antigen-presenting cells from the inflamed ocular surface home to the draining lymph nodes, where they prime naïve T cells to differentiate into pathogenic T effectors, including interleukin-17 secreting CD4+ T cells (Th17). These Th17 cells then migrate from the lymph nodes⁵ to the conjunctiva,⁶ where they propagate inflammation and cause tissue damage by secreting potent inflammatory cytokines.⁷ Although several studies now implicate Th17 cells as the dominant pathogenic effectors in DED,^{5,7,8} the specific mechanisms by which they migrate to the ocular surface are unknown.

Chemokines are a family of “chemotactic cytokines” that direct the movements of immune cells by binding to specific chemokine receptors that are expressed at the cell surface. At

the vascular endothelium, chemokines bind their corresponding receptors to induce changes in integrin conformation, promoting leukocyte extravasation.⁹ Once out of the vasculature, chemokine binding induces the directed migration of leukocytes, which follow a chemotactic gradient of chemokines to arrive at their intended destination.¹⁰ Chemokine receptors are expressed in a leukocyte-subset-specific manner, and the receptor C-C chemokine receptor 6 (CCR6) has been reported to be preferentially expressed by Th17 cells.^{11,12} Although chemokines and their receptors generally display some promiscuity in binding specificity, CCR6 is unique in that it binds only one chemokine, C-C chemokine ligand 20 (CCL20, also known as MIP-3 α , LARC, and exodus-1).¹³ CCL20 is constitutively expressed by several types of epithelial^{14–16} and immune cells^{17,18} in normal, homeostatic conditions and is upregulated in various inflammatory settings.¹⁹

In the present study, we hypothesized that CCR6/CCL20 interactions direct Th17 cell migration to the ocular surface in DED. To validate our hypothesis, we systematically explored a well-characterized murine model of dry eye.^{6,20} We evaluated the generation of Th17 cells and their expression of the chemokine receptor CCR6, as well as the presence of CCL20 at the ocular surface. Furthermore, we used local, in vivo neutralization of CCL20 to demonstrate the functional rele-

vance of the CCR6/CCL20 axis in mediating Th17 cell migration and DED pathogenesis.

METHODS

Mice

Female C57Bl/6 mice (8–10 weeks old; Charles River Laboratories, Wilmington, MA) were used in all experiments. The experimental protocol was approved by the Schepens Eye Research Institute Institutional Animal Care and Use Committee. The study was designed and animals were treated in accordance with the guidelines established in the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and visual research.

Dry Eye Model

Disease was induced according to an established model of the disease.^{6,20} Mice were housed in a low-humidity (relative humidity: <20%) controlled environment chamber (CEC) with constant air flow (15 L/min) for 12 days and received 5 mg/mL subcutaneous scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) injections three times per day. Topical atropine sulfate (1%; Bausch & Lomb, Rochester, NY) was administered twice per day on days 1 and 2 to enhance anticholinergic effects. Age- and sex-matched mice housed in room-air conditions served as naïve controls. Clinical signs of DED were assessed by corneal fluorescein staining (CFS) scoring using 1% fluorescein (Sigma-Aldrich) in accordance with the standard National Eye Institute scoring system.²¹

Flow Cytometry

Draining lymph nodes (submandibular and cervical) were harvested using jewelers forceps (Katena Products, Inc., Denville, NJ) and small straight scissors. Conjunctiva was harvested by lifting at the junction of bulbar and palpebral conjunctiva and dissecting along both bulbar and palpebral insertion points (Vannas Scissors; Storz, Bausch & Lomb). Single-cell suspensions of the draining lymph nodes were made and cells stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL; Sigma-Aldrich) and Ionomycin (500 ng/mL; Sigma-Aldrich) for 5 hours in the presence of a commercial protein transport inhibitor (0.7 μ L/100 μ L media, Golgistop; BD Biosciences, San Jose, CA). Conjunctiva samples were stimulated in RPMI (Thermo Fisher Scientific, Waltham, MA) +10% fetal bovine serum (FBS) for 24 hours at 37°C and 5% CO₂ with PMA (50 ng/mL; Sigma-Aldrich) and Ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of a protein transport inhibitor (0.7 μ L/100 μ L media, Golgistop; BD Biosciences), and then single-cell suspensions were made. Cells were stained with PE/Cy5-conjugated anti-CD4 (eBioscience, San Diego, CA) and PE-conjugated anti-CCR6 (BioLegend, San Diego, CA). Intracellular cytokine staining with FITC-conjugated anti-interleukin 17A or IFN- γ (eBioscience) was performed after fixation and permeabilization. At least 100,000 events per sample were collected on a flow cytometer (LSR II; BD Biosciences) and data were analyzed using commercial software (Summit v4.3; Dako Colorado, Inc., Fort Collins, CO). For each experiment, $n = 9$ to 10 mice per group (3–4 mice/flow sample) or $n = 5$ mice per group (neutralization studies, 1–2 mice/flow sample) were used. Each flow cytometry experiment was performed at least twice.

Real-Time PCR

Full-thickness cornea (including epithelium and stroma) and bulbar and palpebral conjunctiva were excised. Samples were stored in ready-to-use reagent (TRIzol; Invitrogen, Carlsbad, CA) at -80°C until RNA was isolated and reverse-transcribed (RNeasy micro kit; Qiagen, Valencia, CA and SuperScript III kit; Invitrogen, Carlsbad, CA, respectively). Real-time PCR was performed using a commercial PCR master mix (TaqMan Universal PCR Master Mix; Applied Biosystems, Carlsbad, CA) and predesigned primers (Applied Biosystems): IL-6 (Mm00446190), IL-23 (Mm01160011_g1), TNF- α (Mm00443260), MMP-3 (Mm00440295), IFN- γ (Mm01168134_m1), and GAPDH (Mm99999915_g1). Samples were analyzed using a real-time PCR system (LightCycler 480 II System; Roche Applied Science, Indianapolis, IN) and comparative threshold (C_T) values were collected. Target C_T values were normalized to GAPDH C_T values from the same sample, and fold change for each group relative to naïve, room-air mice was calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

Two full-thickness corneas (including epithelium and stroma) or bulbar and palpebral conjunctiva from two eyes from normal or DED mice were collected per well and stimulated in RPMI + 10% FBS for 24 hours at 37°C and 5% CO₂ with PMA (50 ng/mL; Sigma-Aldrich) and Ionomycin (500 ng/mL; Sigma-Aldrich) to measure production of secreted CCL20. Supernatants were collected and stored with protease inhibitor (Sigma-Aldrich) at -80°C until analyzed with a murine CCL20 ELISA kit (R&D Systems, Minneapolis, MN).

In Vitro Migration Assay

Transwell migration assays were performed using 8- μ m pore size cell culture inserts (BD Falcon, Franklin Lakes, NJ) in 24-well culture plates (BD Falcon). Cornea and conjunctiva from two eyes of normal or DED mice were placed in the lower well and stimulated with PMA (50 ng/mL; Sigma-Aldrich) and Ionomycin (500 ng/mL; Sigma-Aldrich) for 3 hours. Draining lymph nodes from five DED mice were collected and single-cell suspensions made. CD4⁺ cells were isolated using a magnetic cell-sorting kit (Miltenyi Biotec, Cologne, Germany); 5×10^5 DED CD4⁺ cells were placed in the upper well of each transwell and the plate was incubated for 2 hours at 37°C and 5% CO₂. Anti-CCL20 neutralizing antibody (R&D Systems), and Rat IgG isotype control antibody (R&D Systems) groups received 30 μ g of respective antibody at the time of stimulation and 30 μ g of antibody at the time of upper well cell placement. After 2 hours migrated cells were collected and quantified using a hemocytometer, minus the number of migrated cells in a gravity control group.

Immunohistochemistry

Whole-mount corneas from normal and isotype control and anti-CCL20-treated DED mice were harvested and epithelium was removed. Following fixation in acetone, corneas were stained with FITC-conjugated anti-CD11b⁺ antibody (eBioscience) and 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), then visualized using a confocal laser scanning microscope (Leica TCS-SP2; Leica Microsystems, Wetzlar, Germany). Z-stack photographs of the full corneal stroma thickness were compiled and double-positive CD11b⁺DAPI⁺ cells were counted using ImageJ software (developed by Wayne Rasband, National Institutes of Health,

Bethesda, MD; available at <http://rsbweb.nih.gov/ij/index.html>).

CCL20 Neutralization

DED was induced for 3 days and mice were randomly assigned to receive either anti-CCL20 neutralizing antibody (1% concentration) or Rat IgG isotype control (1% concentration). On days 4, 6, 8, and 10 mice were anesthetized with 2% isoflurane in 100% oxygen and 10 μ L of anti-CCL20 neutralizing antibody ($n = 10$ eyes) or Rat IgG ($n = 10$ eyes) was administered via subconjunctival injection. To confirm disease induction and follow disease severity, CFS was performed on days 3, 7, and 10. Mice were euthanized and tissues analyzed on day 11. Tissue from each group was processed as follows: six corneas: PCR, three corneas: immunohistochemistry, conjunctiva from four eyes: PCR, conjunctiva from five eyes: flow cytometry, lymph nodes from five mice: flow cytometry, one whole eye (cornea and conjunctiva): frozen cross-sections (not used). The CCL20 neutralization experiment was performed twice.

Statistical Analysis

Groups were compared using the Student's *t*-test or ANOVA where appropriate. Data are reported as the average \pm SEM. Values of $P < 0.05$ were considered statistically significant and error bars represent SEM. Data normality was verified using "Normal Quantile Plots," developed by Scott Gurth (Mt. San Antonio College), as performed previously.²²

RESULTS

Dry Eye Disease Generates CCR6-Expressing Th17 Cells

To examine the expression of CCR6 by Th17 cells in our model of dry eye, we first induced DED by housing mice in the controlled environment chamber (CEC) for 12 days. Disease induction was confirmed by evaluating CFS, a commonly used indicator of corneal epitheliopathy (Fig. 1a). Flow cytometry was used to compare populations of effector T cells from normal (room-air) mice and DED mice. Draining lymph nodes were harvested and single-cell suspensions created. Cells were evaluated for surface staining of CD4 and CCR6, as well as intracellular staining for the signature Th17 secreted cytokine, interleukin-17A, and the signature Th1 secreted cytokine, interferon- γ . We found that although CD4+IL-17A+ (Th17) and CD4+IFN- γ + (Th1) cells are present in both normal and DED conditions (Fig. 1b), DED induces a significant increase in Th17 cell frequency (DED: $0.61 \pm 0.05\%$ vs. Nor: $0.38 \pm 0.03\%$, $P = 0.01$) but not Th1 cell frequency (DED: $0.44 \pm 0.03\%$ vs. Nor: $0.37 \pm 0.03\%$, $P = 0.15$) (Fig. 1c).

To evaluate the expression of CCR6 by these effector T cells, gates were placed on CD4+IL-17A+ and CD4+IFN- γ + cells and CCR6 mean fluorescence intensity (MFI) was measured (Fig. 2a). Th17 cells express a significantly higher level of CCR6 compared with that of Th1 cells in both the normal (MFI: 103.46 ± 5.4 vs. MFI: 56.39 ± 4.4 , $P = 0.002$) and DED state (MFI: 131.65 ± 9.1 vs. MFI: 74.19 ± 4.3 , $P = 0.005$) (Fig. 2b). To investigate whether CCR6 continues to be expressed by Th17 cells that have migrated to the ocular surface, conjunctiva from normal and DED mice was collected and analyzed by flow cytometry. This analysis showed that Th17 cells that have migrated to the conjunctiva in normal and DED mice do express CCR6 (Fig. 2c), confirming receptor

expression both at the time of immune induction in the draining lymph nodes, and after Th17 cell peripheralization to the ocular surface. The CCR6 MFI of Th17 cells isolated from the draining LN increases in DED compared with normal, but does not reach statistical significance (Normal MFI: 103.46 ± 5.4 vs. DED MFI: 131.65 ± 9.1 , $P = 0.06$), whereas the CCR6 MFI of Th17 cells in the conjunctiva remains constant (Normal MFI: 139.75 ± 10.85 vs. DED MFI: 139.06 ± 10.69 , $P = 0.97$) (Fig. 2d).

CCL20 Expression at the Ocular Surface Is Upregulated in Dry Eye Disease

Immune cells migrate via the process of chemotaxis, in which cells expressing chemokine receptors are attracted to chemokine ligands in the environment.¹⁰ Accordingly, after demonstrating the expression of the chemokine receptor CCR6 by Th17 cells at the draining cervical lymph nodes and ocular surface, we next investigated the expression of the corresponding CCR6 ligand, CCL20, in the conjunctiva and cornea of normal and DED mice. Using real-time PCR, we found that messenger RNA (mRNA) levels encoding CCL20 in the cornea (Fig. 3a, $P = 0.03$) and conjunctiva (Fig. 3b, $P < 0.001$) of DED mice are upregulated approximately 12-fold compared with that of normal mice. In addition, to further investigate the expression of CCL20 at the protein level, ELISA was performed on supernatants from cornea and conjunctiva samples. We found that CCL20 protein is expressed at a basal level in normal mice and production by both cornea (Fig. 3c, $P = 0.02$) and conjunctiva (Fig. 3d, $P = 0.01$) is significantly upregulated in DED.

CCL20 Blockade Reduces T-Cell Migration In Vitro

After confirming the presence of CCR6-expressing T cells and the chemokine ligand CCL20 at the ocular surface in DED, we sought to determine the functional significance of the CCR6/CCL20 axis in T-cell migration by performing in vitro migration studies. Using an 8- μ m pore size transwell system, we evaluated the ability of DED-derived CD4+ T cells to migrate toward normal and DED ocular surface tissue (Fig. 4). Tissue from DED mice caused the migration of significantly more CD4+ T cells (17×10^4 cells $\pm 0.5 \times 10^4$) than normal tissue (13.6×10^4 cells $\pm 1 \times 10^4$, $P = 0.04$), and administration of CCL20 neutralizing antibody significantly decreased T-cell migration (9×10^4 cells $\pm 0.9 \times 10^4$) compared with both no treatment (17.1×10^4 , $P = 0.001$) and isotype control treatment (14.8×10^4 cells $\pm 0.1 \times 10^4$, $P = 0.003$).

CCL20 Blockade Improves Clinical Signs of DED

Given the effectiveness of CCL20 neutralization in reducing CD4+ T-cell migration in vitro, we next wanted to determine whether disruption of the CCR6/CCL20 axis could prevent the migration of Th17 cells in DED in vivo. Following the induction of DED for 3 days, mice received either 1% anti-CCL20 neutralizing antibody or 1% isotype control antibody in sterile PBS delivered via subconjunctival injection every other day for a total of four treatments (Fig. 5a), and were evaluated by CFS scoring on days 3, 7, and 10 to assess disease severity. Mice were blindly scored according to the National Eye Institute's standardized (0-15) grading criteria. At the end of the treatment period (day 10), mice receiving anti-CCL20 antibody showed a significant 54% decrease in average CFS score ($P < 0.001$) compared with control antibody recipients (Fig. 5b), indicating that CCL20 blockade reduced corneal epitheliopathy in DED mice.

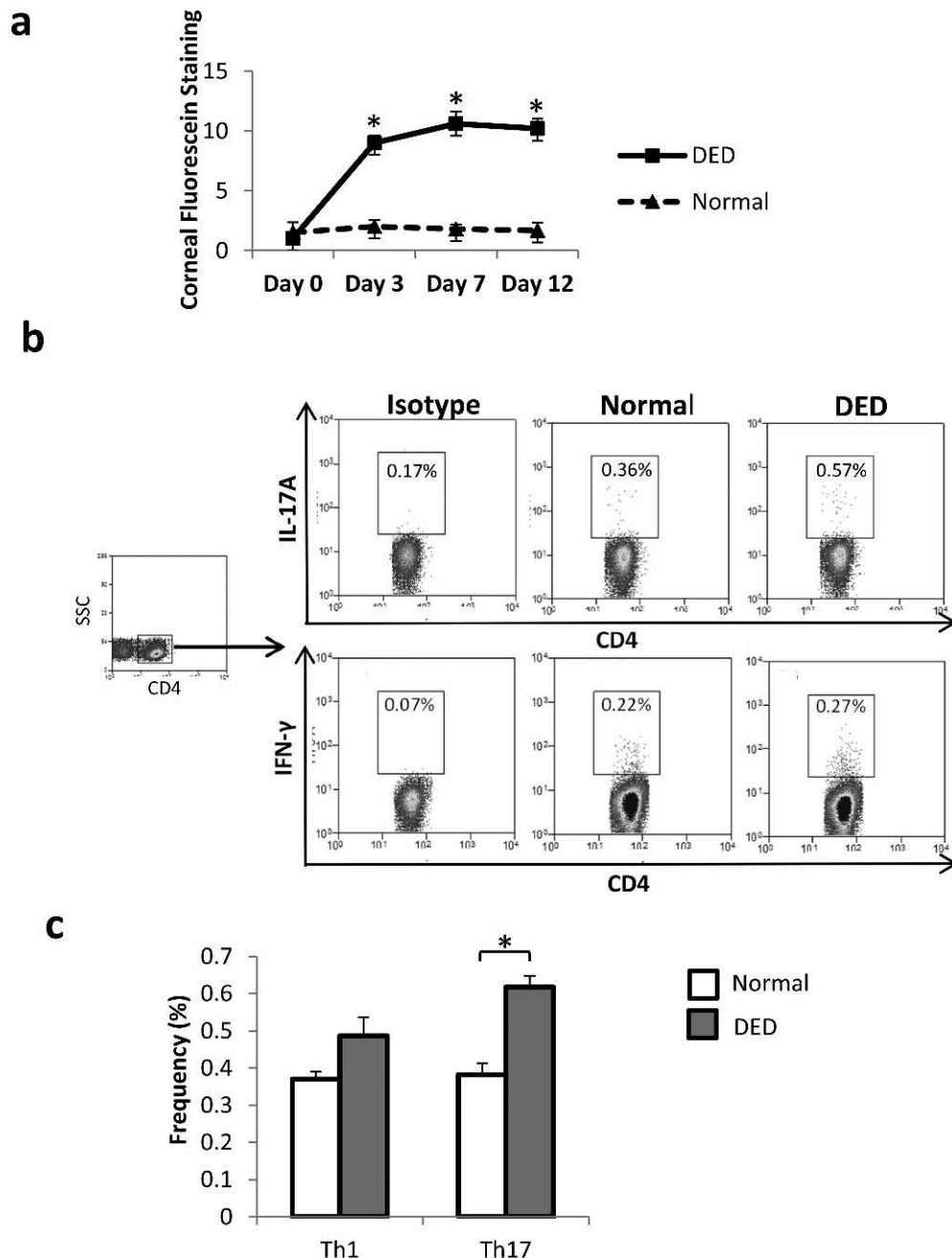


FIGURE 1. (a) DED was induced by housing mice in a low-humidity-controlled environment and administering scopolamine hydrobromide. CFS, the principal clinical measure of corneal epitheliopathy, was used to confirm disease induction. At days 3, 7, and 12, DED mice (solid line) exhibit a significant increase in CFS scores compared with normal, room-air mice (dashed line) ($n = 10$ mice per group, $*P < 0.001$; representative data from one of three experiments are shown). (b) Representative flow cytometry histograms showing the generation of Th17 cells (top) and Th1 cells (bottom) in the draining LNs of DED mice. Frequencies are of the representative plot. (c) The frequency of Th1 and Th17 cells in the LNs of normal (white bars) and DED mice (gray bars) at day 12 are compared. There is a statistically significant increase in Th17 cell frequency but not Th1 cell frequency following induction of DED ($n = 9-10$ mice per group, $*P = 0.01$; averages are of three experiments).

CCL20 Blockade Suppresses Immune Cell Infiltration of the Ocular Surface in Dry Eye Disease

The administration of anti-CCL20 neutralizing antibody was successful in improving clinical signs of DED. To evaluate the efficacy of this intervention in specifically preventing Th17 cell migration to the conjunctiva in DED, we quantified the frequency of conjunctiva-infiltrating Th17 cells. At day 11, normal, untreated DED, and DED mice treated with anti-CCL20

and isotype control antibodies were euthanized and conjunctiva was collected for flow cytometric analysis. As expected, there was a significant increase in conjunctival Th17 cells in the untreated DED group compared with the normal group ($5.64 \pm 0.44\%$ vs. $3.13 \pm 0.07\%$, $P = 0.005$). However, treatment with anti-CCL20 neutralizing antibody led to a significant decrease in Th17 cell frequency ($2.93 \pm 0.22\%$, compared with both the isotype control treated ($4.45 \pm 0.06\%$, $P = 0.005$) and untreated DED groups ($P = 0.002$) (Fig. 6a).

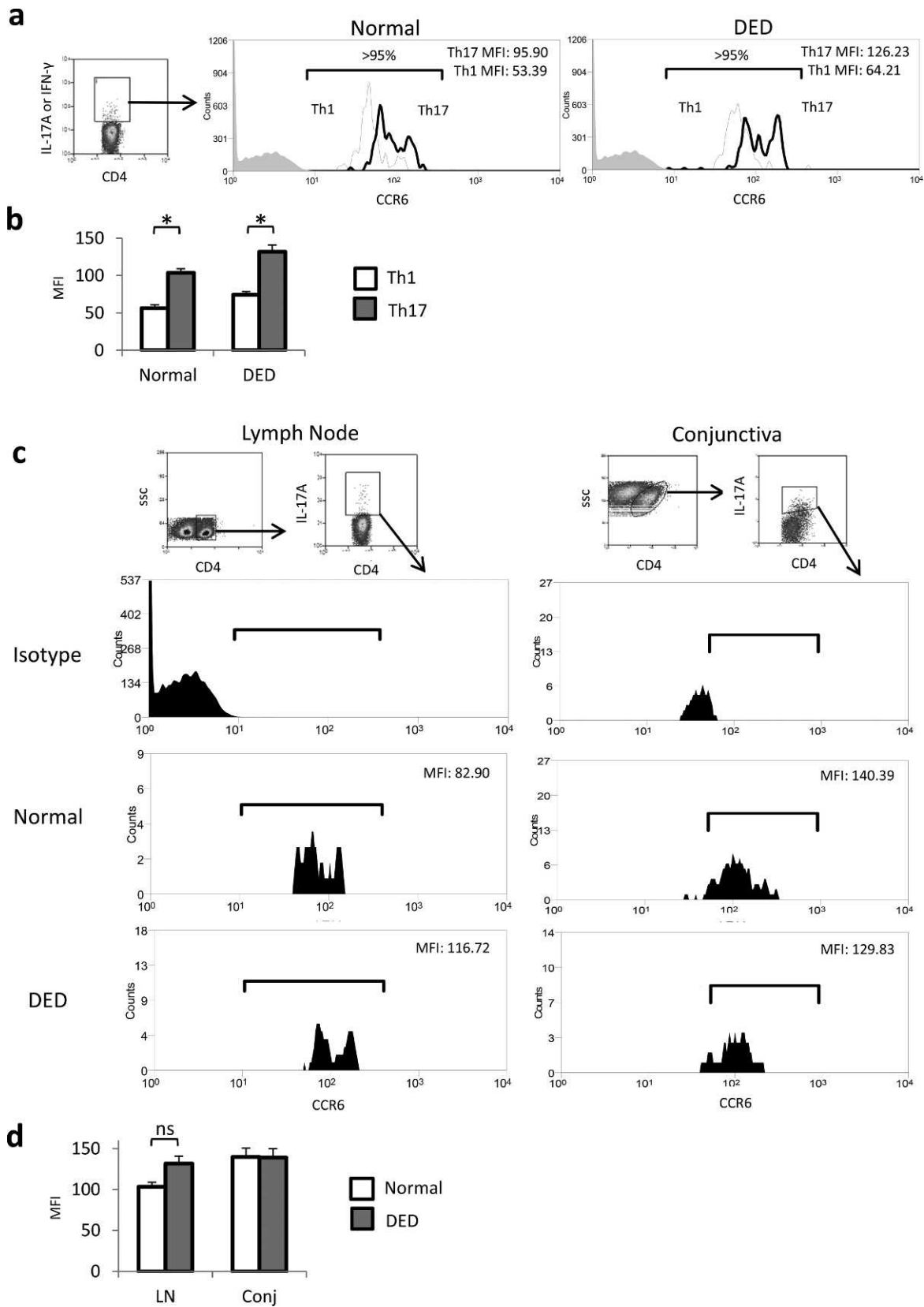


FIGURE 2. (a) IL-17A+ (Th17) and IFN- γ + (Th1) CD4+ cells were evaluated for CCR6 expression by comparing the MFI of each subset to isotype control (gray). Th17 cells (dark line) isolated from the draining LNs of both normal and DED mice express approximately twice as much CCR6 as their Th1 cell counterparts (light line). Representative histograms shown. (b) In both normal (left) and DED (right) mice, Th17 cells (gray bar) express a significantly higher level of CCR6 than that of Th1 cells (white bar) (Normal: $P = 0.002$, DED: $P = 0.005$, $n = 9-10$ mice per group; averages are from one experiment of three). (c) Representative histograms showing the expression of CCR6 by Th17 cells in the LNs and conjunctiva in both normal and DED mice. (MFIs are of the representative histogram.) (d) CCR6 MFI on Th17 cells remains constant between normal and DED conditions ($n = 9-10$ mice per group; representative data from one of three experiments are shown).

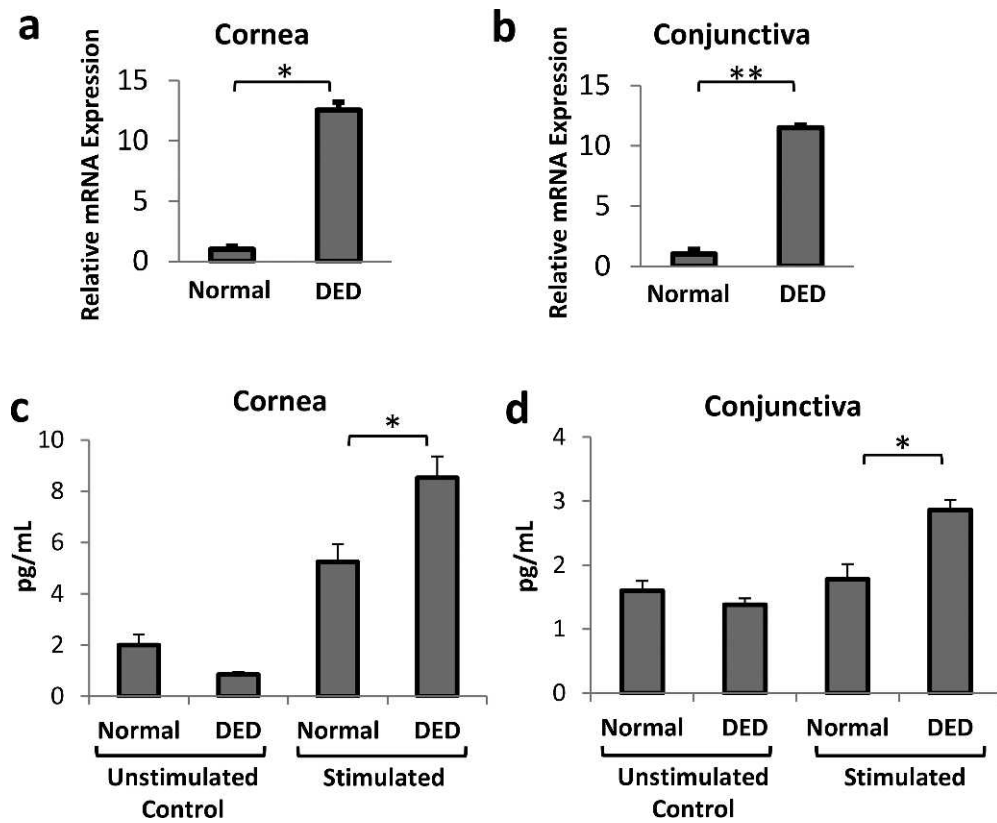


FIGURE 3. CCL20 messenger RNA (mRNA) transcript levels are upregulated in DED in both cornea (a) and conjunctiva (b). CCL20 protein production increases in DED in both cornea (c) and conjunctiva (d), as measured by ELISA ($n = 4-6$ mice per group, $*P < 0.05$, $**P < 0.005$; data from one experiment of two are shown).

CD11b+ cells are bone marrow-derived antigen-presenting cells that are both constitutively present and actively recruited to the cornea in the setting of inflammatory conditions such as DED.^{23,24} To assess CD11b+ cell infiltration of the cornea in

our treatment groups, corneas from anti-CCL20 and isotype control treated mice were stained for CD11b+ cells (green) with DAPI counterstain (blue) and visualized using confocal microscopy (Fig. 6b). To quantify CD11b+ cells, Z-stack images of the peripheral corneal stroma were compiled and CD11b+DAPI+ cells were counted using ImageJ software (National Institutes of Health). DED mice receiving anti-CCL20 treatment showed a significant decrease in CD11b+ infiltration of the peripheral cornea (73 ± 9 cells/mm², $P < 0.01$) compared with those DED mice receiving isotype control antibody (118 ± 17 cells/mm²) (Fig. 6c). There was no statistically significant difference in CD11b+ cell infiltration of the central cornea (data not shown).

Blocking Th17 Cell Migration Downregulates Ocular Surface Inflammation

As an inflammatory disease characterized by the involvement of Th17 cells, DED is known to upregulate ocular surface mRNA expression of proinflammatory cytokines, including the Th17 associated cytokines IL-6 and IL-23, matrix metalloproteinase 3 (MMP-3), TNF- α , and IFN- γ . To evaluate the effect of anti-CCL20 neutralizing antibody on ocular surface inflammation in DED, corneal and conjunctival samples from anti-CCL20-treated DED mice were analyzed for expression of these inflammatory cytokines (Figs. 6d, 6e). Compared with the isotype control group, the conjunctiva of anti-CCL20-treated mice displayed significantly decreased expression of IL-6 ($P = 0.001$), MMP-3 ($P < 0.001$), and IFN- γ ($P < 0.05$) mRNA. We also found a significant decrease in IL-6 expression in the cornea ($P < 0.05$).

Migrated CD4+ Cells

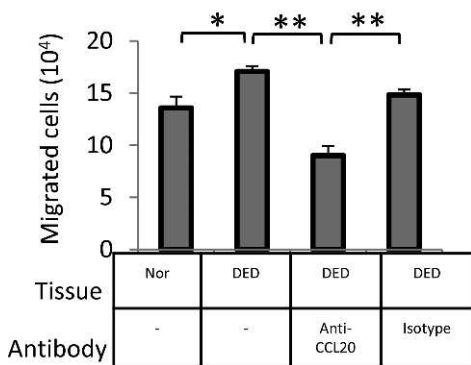


FIGURE 4. CD4+ T cells isolated from the draining lymph nodes of DED mice were placed in the upper well of an 8- μ m transwell filter and cultured with cornea and conjunctiva (lower well) from normal or DED mice. After 2 hours, migrated cells in the lower well were counted. Significantly more CD4+ cells migrated when DED tissue was included in culture compared with normal tissue ($P = 0.04$). Treatment with CCL20 neutralizing antibody decreased the number of migrated CD4+ cells compared with both isotype control antibody treated ($P = 0.003$) and untreated DED groups ($P = 0.001$) ($n = 5$ mice per group, $*P < 0.05$, $**P < 0.005$; data from one of two experiments are shown).

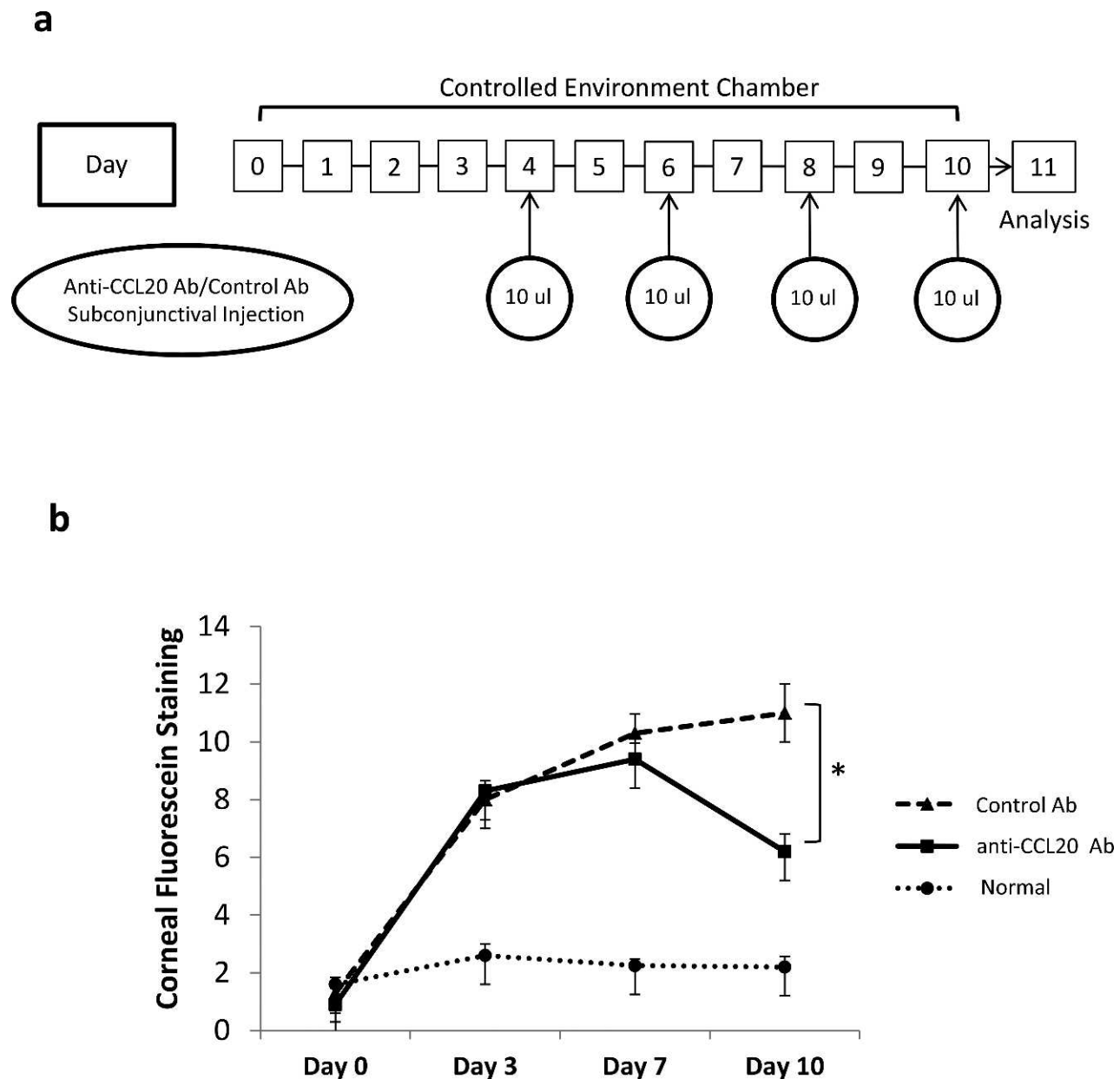


FIGURE 5. (a) Experimental design of CCL20 neutralization experiment. Following the induction of DED, mice were randomly assigned to receive anti-CCL20 neutralizing antibody or control antibody every other day for 4 days via subconjunctival injection. (b) Corneal epitheliopathy was evaluated by scoring CFS at days 0, 3, 7, and 10. At day 10, mice receiving subconjunctival anti-CCL20 neutralizing antibody (*solid line*) showed a statistically significant reduction in CFS score compared with isotype control treated mice (*dashed line*). Mice were blindly scored according to the National Eye Institute CFS grading system ($n = 10$ eyes per group, $*P < 0.001$; data from one of two experiments are shown).

Anti-CCL20 Treatment Inhibits Propagation of Th17 Immunity in DED

As shown previously⁵ and confirmed in the present study (Fig. 1b), DED leads to the expansion of Th17 cells in the regional draining lymph nodes. To evaluate whether treatment with anti-CCL20 antibody had any effect on the generation of Th17 cells, the draining lymph nodes of anti-CCL20 and isotype control treated mice were harvested and prepared for flow cytometric analysis. We evaluated the frequency of Th17 cells and found that in DED mice treated with anti-CCL20 antibody, the frequency of Th17 cells was approximately half the frequency of that seen in DED mice treated with isotype control antibody ($0.36 \pm 0.04\%$ vs. $0.58 \pm 0.02\%$, $P = 0.01$) (Fig. 7).

DISCUSSION

Our data demonstrate that in DED, Th17 cells preferentially express the chemokine receptor CCR6 and expression of the corresponding chemokine ligand CCL20 is upregulated at the ocular surface. The importance of this receptor/ligand pair in Th17 cell migration is illustrated by the functional blockade of CCR6/CCL20 interactions both in vitro and in vivo. Subconjunctival administration of an anti-CCL20 neutralizing antibody prevents the recruitment of Th17 cells to the conjunctiva, leading to an improvement in cellular, molecular, and clinical signs of DED.

Th17 cells are recognized as one of the major players in DED pathogenesis,^{5,7} making Th17 cell-specific therapies attractive strategies for treating this condition. As demonstrat-

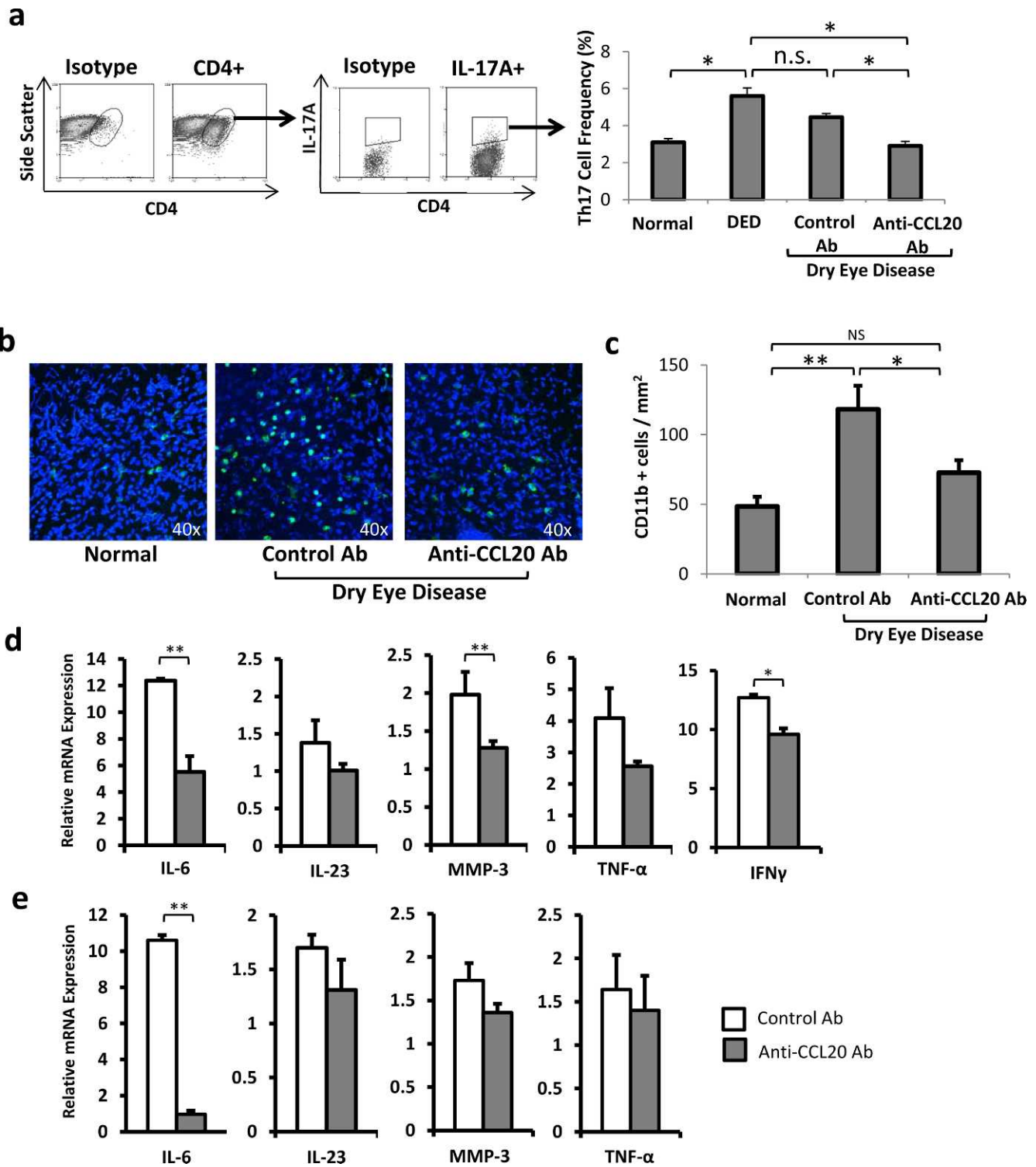


FIGURE 6. (a) Conjunctival flow cytometry samples were gated on CD4 then IL-17A to quantify the frequency of Th17 cells reaching the ocular surface. Conjunctiva from DED mice receiving anti-CCL20 antibody demonstrates a decreased frequency of Th17 cells compared with mice receiving isotype control antibody ($P=0.002$) and untreated DED mice ($P=0.005$) ($*P < 0.005$, $n=5$ mice per group; representative data from one of two experiments are shown). (b) Representative micrographs of whole-mount cornea immunostaining ($\times 40$). Corneas from normal, DED treated with anti-CCL20 antibody, and DED treated with isotype control antibody mice were stained with anti-CD11b antibody (green) and DAPI (blue). (c) Treatment with anti-CCL20 antibody significantly decreased the number of CD11b+ cells in peripheral cornea compared with isotype control antibody (three corneas per group were each evaluated in four distinct peripheral areas; $*P < 0.05$, $**P \leq 0.001$, NS, not statistically significant). (d) Real-time PCR analysis of conjunctiva showing expression levels of IL-6, IL-23, MMP-3, TNF- α , and IFN- γ . (e) Real-time PCR analysis of cornea showing expression levels of IL-6, IL-23, MMP-3, and TNF- α ($n=4-6$ eyes per group, $*P < 0.05$, $**P \leq 0.001$). White bars: DED mice treated with isotype control antibody. Gray bars: DED mice treated with anti-CCL20 antibody. Results are normalized to mRNA levels in normal mice.

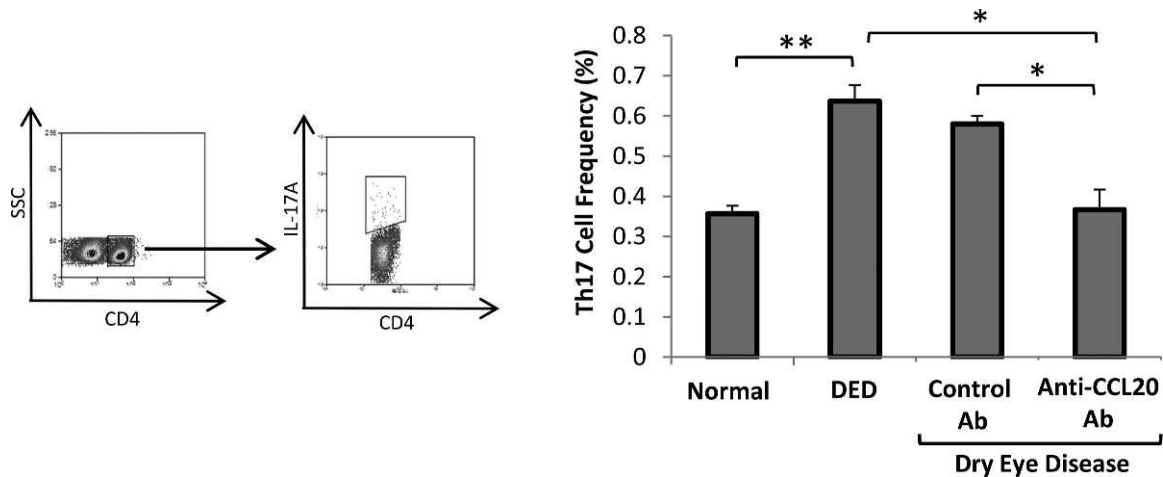


FIGURE 7. Th17 cells in the draining lymph nodes of DED mice were quantified by flow cytometry following treatment with CCL20 neutralizing antibody or control antibody. DED mice generate fewer Th17 cells when treated with anti-CCL20 antibody as compared with both isotype control antibody treatment ($P = 0.01$) and no treatment ($P = 0.01$) ($n = 5$ mice per group, $*P < 0.05$, $**P < 0.005$; representative data from one of two experiments are shown).

ed here, one such approach may be the targeting of chemokine-mediated mechanisms of Th17 migration. Past attempts to modulate cellular migration in DED by VLA-4 and CCR2 blockade have nondiscriminantly affected several subsets of immune cells.^{25,26} In contrast to these studies, we herein demonstrate specific blockade of cells of the Th17 subset through neutralization of CCL20.

To evaluate the role of CCR6/CCL20 dynamics in mediating Th17 cell migration in DED, we first examined the generation of effector T cells in the regional draining lymph nodes of DED mice, and their expression of CCR6. Our data demonstrate a greater increase in Th17 cell frequency than Th1 frequency following the induction of DED, supporting the dominant role of Th17 cells in DED pathogenesis.⁸ Further, we found that although both Th17 and Th1 cells express CCR6, the receptor is preferentially expressed at a higher level by cells of the Th17 subset and continues to be expressed by Th17 cells that have reached the conjunctiva. We did not find a statistically significant increase in CCR6 expression by Th17 cells in DED, suggesting that once a cell has differentiated into the Th17 phenotype, whether in normal or DED mice, the level of CCR6 expression is relatively constant. That is, Th17 cell expression of CCR6 appears to be a reflection of cell lineage/differentiation rather than disease state.¹¹ Indeed, this is consistent with a previous study in which CCR6 MFI was found to be similar in both central (lymph node homing) and effector (periphery homing) memory T cells.²⁷

Knowing that the final destination of effector Th17 cells in DED is the ocular surface, we next investigated the expression of the corresponding CCR6 chemokine, CCL20, by the cornea and conjunctiva. We found a significant upregulation of CCL20 in both the cornea and conjunctiva of DED mice as compared with normal mice. This is consistent with previous work demonstrating increased conjunctival CCL20 mRNA in DED.^{7,28} CCL20 is chemotactic for CCR6-expressing cells, and induces arrest and integrin-dependent adhesion of CD4+ T cells, facilitating CD4+ cell peripheralization.^{29,30} CCL20 is known to be secreted by several cell types, including vascular endothelial cells,³¹ Th17 cells,¹⁷ and monocytes.¹⁸ Corneal epithelial cells are known to express CCL20 in various disease/injury models, including herpetic stromal keratitis,¹⁵ infection with *Staphylococcus aureus*,³² and following corneal abrasion.¹⁶ Like these models, our model of DED is characterized by immune cell infiltration of the ocular surface and the

accompanying elaboration of inflammatory cytokines.⁷ Therefore, the increased levels of CCL20 seen in the present study may be due to direct secretion by infiltrating Th17 cells and monocytes, the stimulation of resident epithelial and vascular endothelial cells by inflammatory cytokines, or both.

To evaluate the functional relevance of the CCR6/CCL20 axis in DED pathogenesis, we sought to disrupt CCR6 and CCL20 interactions using a CCL20 neutralizing antibody. In an in vitro migration assay, we observed decreased CD4+ cell migration toward DED cornea and conjunctiva after treatment with this antibody. We also sought to demonstrate the functional importance of the CCR6/CCL20 axis in vivo in our DED model by administering CCL20 neutralizing antibody via subconjunctival injection. Following this intervention, we observed a significant improvement in corneal epitheliopathy. Because our data demonstrated the production of CCL20 by corneal and conjunctival tissue (Fig. 3), we sought to administer neutralizing antibody at the ocular surface, to achieve specific local blockade of Th17 cell chemotaxis. When antibody was administered topically, we observed a small, statistically insignificant improvement in CFS (data not shown), whereas subconjunctival injection of neutralizing antibody did yield a significant improvement in CFS score (Fig. 5). Compared with topical instillation, subconjunctival administration allowed us to maximize antibody binding³³ while avoiding the more widespread changes frequently seen with less specific interventions such as the systemic administration of agents or use of knockout mice.³⁴ Previous immunohistochemical studies in both experimental and clinical DED have demonstrated CD4+ T-cell infiltration of the subepithelial layer of the conjunctiva.^{25,35,36} Our data suggest that the clinical improvement in disease severity following treatment with CCL20 neutralizing antibody is largely due to a decreased number of CD4+HL-17A+ Th17 cells infiltrating the conjunctiva (Fig. 6a). Thus, the ocular surface is not exposed to Th17-secreted cytokines, which would ordinarily recruit other immune cells and stimulate IL-17 receptor expressing resident corneal and conjunctival cells to secrete additional proinflammatory cytokines.^{5,37}

Decreased ocular surface inflammation following blockade of Th17 cell migration is evident in our analysis of cytokine gene expression. IL-6 and IL-23 are two cytokines required for the induction and proper effector functioning of Th17 cells,^{17,38} and their levels at the ocular surface were noticeably

decreased after CCL20 neutralization. Without these supporting cytokines, not only are Th17 cells that do reach the ocular surface rendered less potent as effectors, but there is also a resultant decrease in overall ocular surface inflammation. This is supported by the significant decrease observed in MMP-3 and IFN- γ expression, two cytokines known to contribute to inflammation and ocular surface damage in DED.^{7,39}

Our data indicate that the therapeutic effect of CCL20 neutralization is not limited to the local benefits of decreased Th17 cell frequencies in the conjunctiva. Treatment also leads to a decrease in CD11b+ cell infiltration of the cornea, which is likely secondary to decreased local inflammation.⁴⁰ CCL20 blockade may also be acting by directly preventing CD11b+ cell migration, because these cells can also express CCR6.⁴¹ Disruption of DED pathogenesis is further illustrated by the decreased Th17 cell frequency in the draining lymph nodes of anti-CCL20-treated mice. This may be due to decreased inflammation limiting the mobilization of antigen-presenting cells, such as CD11b+ cells, from the ocular surface to the lymphoid tissue. This, in turn, leads to decreased priming of naïve T cells and the generation of fewer Th17 effectors. These observations suggest that in addition to preventing Th17 cell migration, CCL20 blockade also disrupts the amplification and progression of the Th17 immune response in DED.

In conclusion, the present study highlights the crucial role of the CCR6/CCL20 axis in mediating Th17 cell migration in DED. DED induces an adaptive immune response that includes the generation of CCR6-expressing Th17 cells in the regional draining lymph nodes and upregulation of CCL20 at the ocular surface, two complementary findings that represent a mechanism for Th17 cell migration from the lymph nodes to the conjunctiva. Following the functional blockade of CCL20, we demonstrate not only a reduction in the number of Th17 cells that have migrated to the conjunctiva, but also a reduction in the DED immune response and ocular surface inflammation. These cellular and molecular changes yield a significant improvement in the clinical presentation of disease. Taken as a whole, these findings provide new understanding of DED pathogenesis and the role of chemokines in mediating Th17 cell migration to the ocular surface in this disease, and suggest that targeting the CCR6/CCL20 axis in DED may be a novel therapeutic strategy in the treatment of this common immunoinflammatory disorder.

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