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Characterization of Effector T Cells in Dry Eye Disease

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PURPOSE. Dry eye disease (DED) is associated with ocular surface inflammation that is thought to be mediated primarily by CD4⁺ T cells. The purpose of this study was to investigate whether this T cell-mediated immune response is generated in the lymphoid compartment and to characterize the functional phenotype of the T cells activated in DED.

METHODS. DED was induced in female C57BL/6 mice by exposure to a desiccating environment in the controlled environment chamber and to systemic scopolamine. T cells from regional draining lymph nodes (LNs) of DED mice and normally sighted mice were analyzed for surface activation markers (CD69 and CD154), chemokine and cytokine receptors, and proliferation potential.

RESULTS. Draining LNs of DED mice showed increased frequencies of CD69- and CD154-expressing T cells with higher proliferative capacity. In addition, these LN T cells primarily showed a helper T-cell (Th)1 phenotype, expressing significantly higher levels of IFN- γ and IL-12R β 2 but not IL-4R. Similarly, the LNs of DED mice showed significantly increased frequencies of T cells expressing CXCR3 and CCR5, but not CCR4, suggesting a bias toward a Th1 phenotype.

CONCLUSIONS. These data demonstrate that a Th1-type immune response is induced in the regional LNs of DED mice. The identification of specific cytokine/chemokine receptors overexpressed by these T cells may signify potential novel targets/strategies for the treatment of DED. (*Invest Ophthalmol Vis Sci.* 2009;50:3802–3807) DOI:10.1167/iovs.08-2417

Dry eye disease (DED) is one of the most common causes for patients to seek ophthalmic care for symptoms of ocular irritation and blurred vision.¹ In more severe cases, DED can lead to blindness caused by corneal ulceration or infection.^{2,3}

To date, the pathogenesis of DED has not been fully understood, although it is widely recognized that DED is associated with ocular surface inflammation.^{4–8} This inflammation is thought to be mediated primarily by CD4⁺ T cells.^{9,10} The significance of T cell-mediated inflammation in DED is supported by several distinct lines of observation. First, corticosteroids are effective at treating most patients with DED. Cyclosporine A, an immunomodulatory agent that suppresses T-cell lymphokines (IL-2), is also effective at improving the signs and symptoms of severe DED.⁹ Second, T-cell infiltration

of the conjunctiva is observed in clinical and experimental DED,^{9,10} concomitant with expression of the CCR5 chemokine receptor, which is critical for the recruitment of CD4⁺ T cells.⁶ Third, adoptive transfer of CD4⁺ T cells from DED mice into T cell-deficient nude mice leads to the development of DED.¹¹ Although the immune system of T cell-deficient nude mice is different from that of wild-type mice, this observation strongly suggests a pathogenic role for T cells in DED. Despite the strong evidence for T-cell involvement in the pathogenesis of DED, the site and mechanism of T-cell activation in DED are unknown.

Regional lymph nodes (LNs) have been shown to be critical sites for the induction of immunity to tissue antigens because they serve as large reservoirs of lymphoid cells. In corneal transplantation, for example, the critical role of draining LNs in the induction of alloimmunity has been established.^{12–14} The purpose of the present study was to investigate whether T cells in the draining LNs are activated in DED by detecting the expression of surface activation markers and their proliferative capacity. We also investigated whether T cells differentiate into a primarily helper T cell (Th)1 or Th2 response through detection of their cytokine and chemokine expression profiles.

MATERIALS AND METHODS

Mouse Model of Dry Eye

Eight-week-old female C57BL/6 mice (Taconic Farms, Germantown, NY; Charles River Laboratories, Wilmington, MA) were used in these experiments. The protocol was approved by the Institutional Animal Care and Use Committee, and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Dry eye was induced by placement of mice in a controlled-environment chamber (CEC)¹⁵ for 10 days modified with subcutaneous administration of scopolamine to maximize ocular dryness.¹⁶ Age-matched mice not placed in the CEC were used as normally sighted controls.

Flow Cytometry

Draining cervical LNs were harvested aseptically and pooled ($n = 3$ –5 mice) separately from DED and normally sighted mice. Single-cell suspensions obtained from these nodes were blocked with anti-FcR mAb (BD PharMingen, San Diego, CA) for 30 minutes at 4°C in 1% BSA/0.02% NaN₃/PBS. Cells were then stained with the following antibodies for 45 minutes at 4°C: anti-CD4 FITC or anti-CD8 FITC, anti-CD69 phycoerythrin (PE), anti-CD154 PE, anti-CCR5 PE, anti-IL-12R β 2, anti-IL-4R PE (BD PharMingen), anti-CXCR3 PE (R&D), anti-CCR4 (Capralogics Inc., Hardwick, MA), and their isotype-matched controls. Goat anti-hamster PE and donkey anti-goat PE (Jackson ImmunoResearch, West Grove, PA) were the secondary antibodies applied for 45 minutes at 4°C against anti-IL-12R β 2 and anti-CCR4, respectively. Finally, cells were washed and analyzed on a flow cytometer (Beckman Coulter, Fullerton, CA).

Proliferation Assay

Draining LNs were harvested as described. CD4⁺ effector T cells from draining LNs and T cell-depleted syngeneic splenocytes were sorted by magnetically activated cell sorting (MACS) isolation kit (Miltenyi Biotec, Auburn, CA). CD4⁺ effector T cells from draining LNs of

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normally sighted mice served as controls. In proliferation assays, CD4⁺ effector T cells (1×10^5) were cocultured with T cell-depleted syngeneic splenocytes (1×10^5) and 1 μ g/mL anti-CD3 antibody for 3 days. Proliferation was measured using BrdU incorporation assay (Millipore, Billerica, MA).

ELISPOT

The ELISPOT assay used to detect cytokine secretion by T cells is a modification of the previously described procedure.^{17,18} Briefly, 96-well ELISPOT plates (Polyflectronics, Rockland, MA) were coated with 4 mg/mL primary anti-IFN- γ mAb or IL-4 mAb (BD Pharmingen) in sterile PBS overnight. The plates were then washed three times with PBS and blocked for 1.5 hours with PBS containing 1% BSA. Next, cells were harvested aseptically from draining LNs ($n =$

3–5 mice) of DED and normally sighted mice. T cells were sorted by MACS isolation kit (Miltenyi Biotec) using anti-CD90 magnetic beads and were added to wells previously loaded with CD3e mAb (BD Pharmingen) to a final volume of 200 μ L medium (AIM-V; Invitrogen, Carlsbad, CA). Cells were incubated for 48 hours. The plates were washed three times with PBS and then four times with PBS containing 0.025% Tween 20. Biotinylated anti-IFN- γ and anti-IL-4 detection mAbs were added at 2 μ g/mL (BD Pharmingen) and incubated for 2 hours at room temperature. The washing steps were repeated, and after 1 hour of incubation with avidin-HRP, the plates were washed again three times with PBS/0.025% Tween 20 and three times with PBS alone. The spots were developed by the addition of the aminoethylcarbazole staining solution (Sigma-Aldrich). The resultant spots were counted and analyzed on a com-

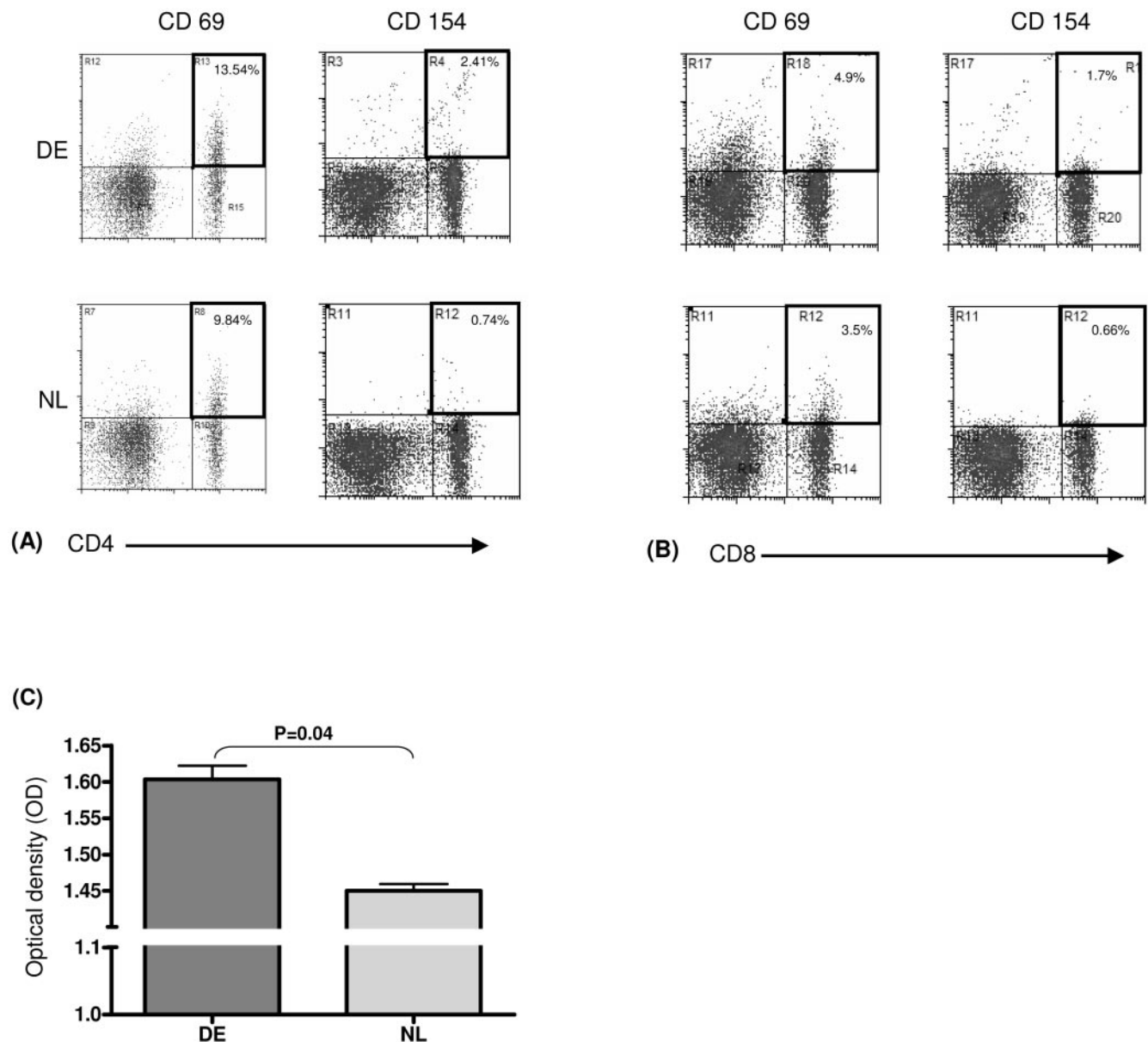


FIGURE 1. Analysis of T-cell surface activation marker expression and its proliferative capacity. Draining LNs were harvested from DED mice and normally sighted (NL) mice; single-cell suspensions were prepared and dual stained with anti-CD4 FITC (A) or anti-CD8 FITC (B) and activation marker antibody anti-CD69 PE or anti-CD154 PE. Data shown are representative of five independent experiments. (C) CD4⁺ T cells were sorted from the draining LNs of DED mice compared with NL mice using the MACS cell isolation kit. CD4⁺ T effector cells were cocultured with T cell-depleted syngeneic splenocytes and anti-CD3 antibody for 3 days. Proliferative response was assessed by BrdU incorporation assay. Results are presented as optical density (OD) reading \pm SEM. Data shown are representative of three independent experiments.

puter-assisted image analyzer (ELISPOT; CTL Laboratories, Shaker Heights, OH).

Statistical Analysis

Data are expressed as the mean \pm SEM of the results for at least three separate experiments. Two-tailed Student's *t*-test was performed (Excel; Microsoft, Redmond, WA). $P < 0.05$ was taken as indicative of statistical significance.

RESULTS

Increased Frequency and Proliferative Capacity of Activated T Cells in DED

To investigate whether T cells are activated in the regional lymphoid compartment (draining LNs) of DED mice, we measured the expression of the surface activation markers CD69 and CD154 (CD40L) on CD4⁺ and CD8⁺ T cells by two-color flow cytometry (Figs. 1A, 1B). We observed a significant increase in the percentages of CD4⁺ and CD8⁺ T cells expressing CD69 (40%; $P = 0.01$ and $P = 0.002$, respectively) and CD154 (200%; $P = 0.004$ and $P = 0.02$, respectively) in the draining LNs of DED mice compared with those from the normally sighted controls.

Antigen-stimulated T cells in the draining LNs undergo proliferation to provide a larger population of antigen-specific effector T cells that mediate immune disease. To determine whether T cells from DED mice undergo expansion, we compared the proliferative potential of T cells from DED mice and normally sighted mice. CD4⁺ T cells were magnetically sorted from harvested LN suspensions and stimulated with anti-CD3 antibody for 3 days in culture. Proliferation was measured with the BrdU incorporation assay (Fig. 1C). We observed a 12% increase in the proliferative capacity of CD4⁺ T cells isolated from the draining LNs of DED mice ($P = 0.04$) compared with those from the normally sighted controls.

Th1-Mediated Immunity in Draining LN T Cells

To determine whether the T-cell response in the lymphoid compartment in DED is biased toward a Th1 rather than a Th2 phenotype, sorted T cells were stimulated with anti-CD3 antibody, and ELISPOT assay was performed to quantify the frequencies of IFN- γ - versus IL-4-positive T cells (Fig. 2). We found a significant 117% increase in IFN- γ secretion ($P = 0.005$) and a significant 79% decrease in IL-4 secretion ($P = 0.03$) by T cells of DED mice compared with those of normally sighted controls. In another set of experiments, fluorescence-activated cell sorter analyses for intracellular IFN- γ and BrdU incorporation showed that proliferating BrdU+CD4⁺ T cells of DED mice also display enhanced IFN- γ expression compared with those of normally sighted mice (data not shown).

To further confirm the T-cell immune response differentiation in the draining LNs, we investigated the expression of the cytokine receptors IL-12R β 2 and IL-4R on draining LN T cells by two-color flow cytometry (Fig. 3). IL-12R β 2 is expressed predominantly on Th1 cells, whereas IL-4R is expressed on Th2 cells.^{19,20} We observed a significant 90% increase in the percentage of CD4⁺ T cells ($P = 0.03$) but not CD8⁺ T cells ($P = 0.40$) expressing IL-12R β 2 in DED mice compared with those in normally sighted controls. We also found that there was no change in the percentage of CD4⁺ T cells ($P = 0.60$) or CD8⁺ T cells ($P = 0.49$) expressing IL-4R in DED mice compared with those in normally sighted controls.

Increased Frequency of CCR5- and CXCR3-Expressing T Cells

Effector T cells acquire chemokine receptors to migrate out of lymphoid compartments along chemokine gradients toward

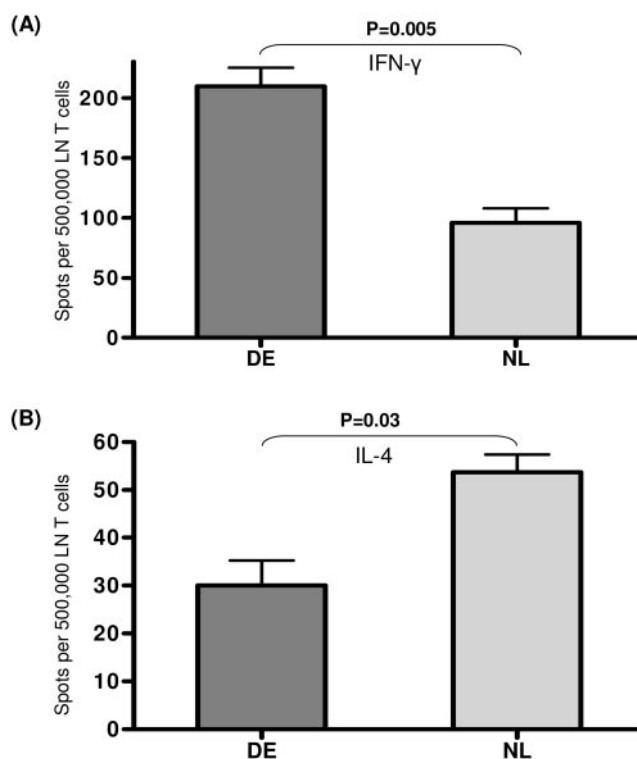


FIGURE 2. Analysis of T-cell cytokine secretion. Frequencies of reactive T cells on CD3 stimulation from draining LNs of DED and normally sighted (NL) mice were evaluated with the ELISPOT assay for (A) IFN- γ and (B) IL-4. Results are depicted as the mean \pm SEM number of spots per 0.5 million responder T cells loaded. Data shown are representative of three independent experiments.

sites of inflammation in the periphery. Th1 and Th2 cells express distinct patterns of chemokine receptors. CCR5 and CXCR3 are predominantly expressed by Th1 cells, whereas CCR4 is expressed by Th2 cells.²¹ To determine the differential expression of these receptors by the draining LN T cells in DED, we performed two-color flow cytometry of CCR5, CXCR3, and CCR4 on CD4⁺ and CD8⁺ T cells (Fig. 4). We observed a significant increase in the percentage of CD4⁺ and CD8⁺ T cells expressing CCR5 (190% [$P = 0.02$] and 100% [$P = 0.005$], respectively) and CXCR3 (70% [$P = 0.002$] and 16% [$P = 0.01$], respectively). This increase was 45% greater in CD4⁺ T cells than in CD8⁺ T cells. We have also found that 80% to 85% of CXCR3-expressing CD4⁺ T cells coexpress CCR5 in DED mice and in normally sighted mice (data not shown). However, there was no significant change in the percentage of CD4⁺ ($P = 0.96$) or CD8⁺ ($P = 0.76$) T cells expressing CCR4 in the draining LNs of DED mice compared with those of normally sighted controls.

DISCUSSION

The data presented herein demonstrate that T cells are activated in the regional LNs of DED mice. These T cells have a Th1 phenotype, as demonstrated by their ability to secrete IFN- γ and to acquire Th1 type chemokine (CCR5 and CXCR3) and cytokine (IL-12R β 2) receptors.

We found a significantly increased percentage of CD4⁺ and CD8⁺ T cells expressing CD69 and CD154 in DED mice compared with normally sighted controls. This increase was most notable among CD4⁺ T cells. CD69 is an early T-cell activation marker and is not detected in resting lymphocytes. CD154, also known as CD40 ligand (CD40L), is predominantly expressed

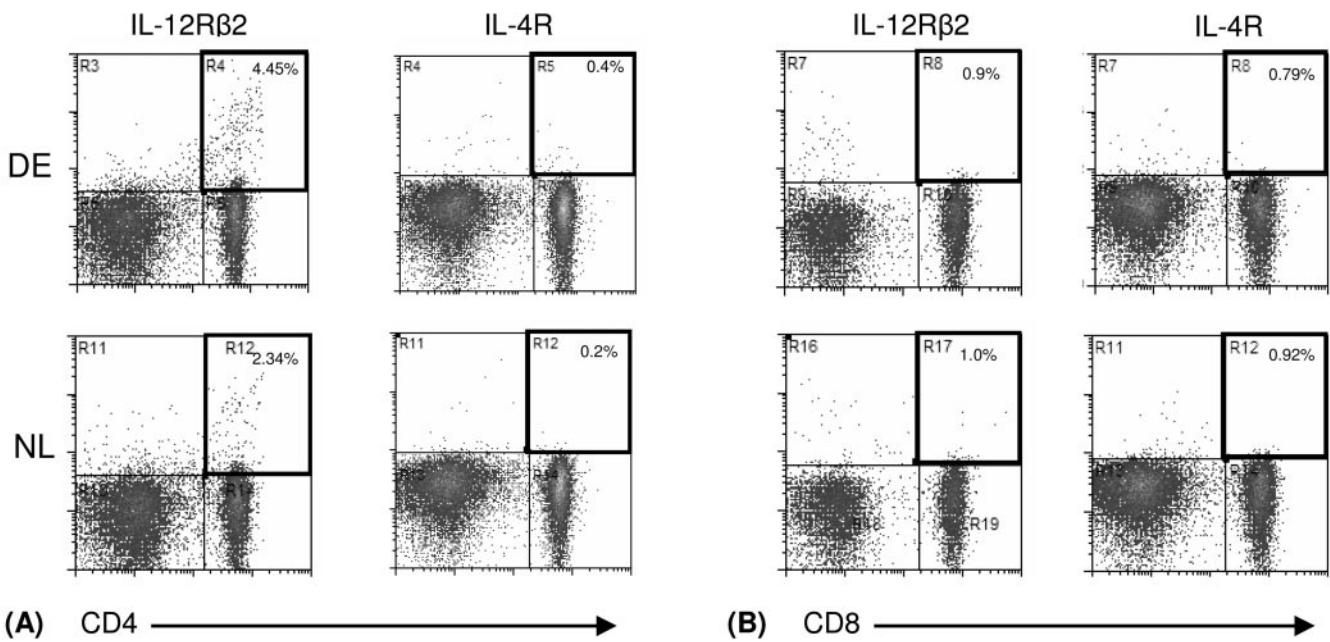


FIGURE 3. Flow cytometric analysis of cytokine receptors by T cells. Draining LNs were harvested from DED and normally sighted (NL) mice. Single-cell suspensions were prepared and dual stained with anti-CD4 FITC (A) or anti-CD8 FITC (B) and with cytokine receptor antibody anti-IL-12Rβ2 PE or anti-IL-4R PE. Data shown are representative of four independent experiments.

on activated CD4⁺ T cells, and its expression enhances the activation and differentiation of these cells.^{22,23} The expression of these cell surface markers is increased by activated T

cells in humans with various autoimmune diseases.^{24–26} To confirm that the activation of the T cells is associated with their enhanced capacity to expand, we performed BrdU incorpora-

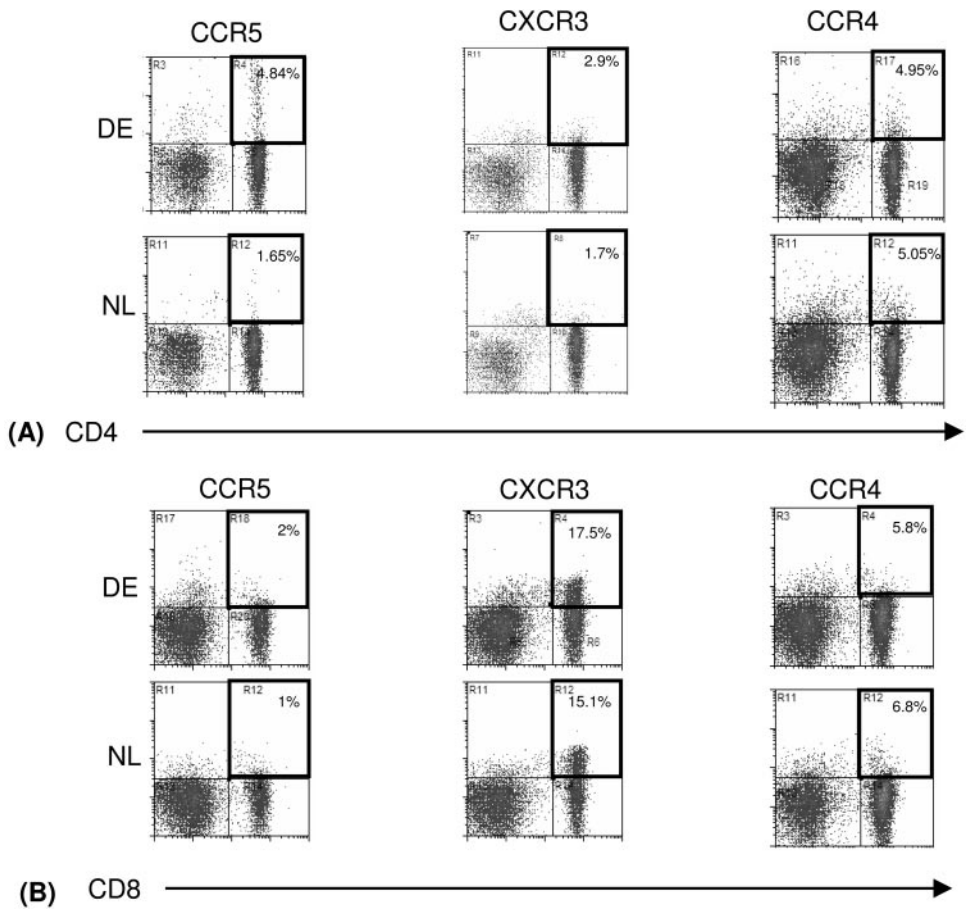


FIGURE 4. Flow cytometric analysis of chemokine receptors by T cells. Draining LNs were harvested from DED and normally sighted (NL) mice. Single-cell suspensions were prepared and dual stained with anti-CD4 FITC (A) or anti-CD8 FITC (B) and chemokine receptor antibody anti-CCR5 PE, anti-CXCR3 PE, or anti-CCR4 PE. Data shown are representative of four independent experiments.

tion assay to detect the proliferative potential of these cells. We observed a higher proliferation of CD4⁺ T cells of DED mice compared with those isolated from draining LNs of normally sighted controls. In the aggregate, these data point toward the generation of a T cell-mediated immune response in the lymphoid compartment of DED mice. Although it is not formal proof, the increased expression of CD40L suggests an immune process driven by specific antigens, potentially autoantigens.²³ It has been shown that autoantigens are exposed in cells undergoing apoptosis; an example is the α -fodrin protein exposed in apoptotic lacrimal gland cells.²⁷ Further studies are clearly needed to characterize the potential autoantigens exposed by the ocular surface epithelium in DED.

Our data also demonstrate that T cells in DED mice experience significantly increased secretion of IFN- γ but not IL-4 and an increased percentage of CD4⁺ T cells expressing IL-12R β 2 but not IL-4R compared with those in normally sighted controls. The differentiation of T cells into a Th1 or a Th2 response is determined by several factors; one principal factor is the cytokine milieu during the interaction of antigen-presenting cells (APCs) with naive T cells (Th0). The ligation of CD154 with CD40 on the surfaces of APCs increases the production of IL-12 by these cells.²³ IL-12 is a key cytokine in Th1-mediated autoimmune diseases,²⁸ and it promotes the secretion of IFN- γ by Th1 cells.^{29–31} The primary signal transduction of IL-12 is through IL-12R β 2,³² whose expression is believed to be confined to Th1 cells.¹⁹ Thus, these findings confirm a more notable involvement of CD4⁺ T cells in the initiation of a regional Th1 immune response in DED.

Chemokine receptors are needed for the trafficking of activated T cells in vivo. Th1 and Th2 cells express distinct patterns of chemokine receptors. CCR5 and CXCR3 are predominantly expressed by Th1 cells, whereas CCR4 is expressed by Th2 cells.²¹ We found a significantly increased proportion of primarily CD4⁺ T cells expressing CCR5 and CXCR3 in the draining LNs of DED mice compared with normally sighted controls. We have previously shown that CCR5 expression is increased significantly by the conjunctivae of humans with different forms of DED,⁶ suggesting an important role for CCR5 in ocular surface inflammation. In addition, it has been shown that the expression of CCR5 and CXCR3 and their corresponding chemokine ligands is increased by the conjunctiva and cornea of DED mice.³³ Taken together, these findings suggest that the draining LN T cells acquire the chemokine receptors required for their migration and homing to the ocular surface. Recently, it has been shown that Th17 cells play a role in autoimmune diseases.³⁴ In addition, Th17 cells express CCR5 and CXCR3; therefore, their role in DED requires further investigation.³⁵

The present study demonstrates the induction of a Th1 immune response in the regional LNs of DED mice, coincident with their acquisition of specific chemokine receptors that assist their homing to the inflamed ocular surface in DED. It is unknown whether the blockade of specific chemokine ligands/receptors may hold promise in the treatment of DED. However, several CXC and CC chemokines, and their respective receptors, have emerged as promising targets in transplantation and autoimmune disease.^{36–38} Future studies evaluating specific chemokine blockade, singly or in combination with cytokine systems involved in Th1 activation (e.g., CD40L, IL-12), may provide novel strategies for the treatment of DED.

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