

PDE4 Inhibition Suppresses IL-17–Associated Immunity in Dry Eye Disease

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PURPOSE. To determine the effect of phosphodiesterase type-4 (PDE4) inhibition on IL-17–associated immunity in experimental dry eye disease (DED).

METHODS. Murine DED was induced, after which a PDE4 inhibitor (cilomilast), dexamethasone, cyclosporine, or a relevant vehicle was administered topically. Real-time PCR, immunohistochemical staining, and flow cytometry were employed to evaluate the immuno-inflammatory parameters of DED with a focus on IL-17–associated immunity. Corneal fluorescein staining (CFS) was performed to evaluate clinical disease progression.

RESULTS. DED induction increased proinflammatory cytokine expression, pathogenic immune cell infiltration, and CFS scores. Cilomilast significantly decreased the expression of TNF- α in the cornea ($P \leq 0.05$) and IL-1 α , IL-1 β , and TNF- α in the conjunctiva ($P \leq 0.05$) as compared with vehicle control. Cilomilast treatment markedly decreased the presence of CD11b⁺ antigen-presenting cells in the central and peripheral cornea ($P \leq 0.05$), and led to decreased conjunctival expression of cytokines IL-6, IL-23, and IL-17 ($P \leq 0.05$). Moreover, cilomilast decreased the expression of IL-17 and IL-23 in the draining lymph nodes ($P \leq 0.05$). Topical cilomilast was significantly more effective than vehicle at reducing CFS scores ($P \leq 0.05$). The therapeutic efficacy of cilomilast was comparable or superior to that of dexamethasone and cyclosporine in all tested measures.

CONCLUSIONS. Topical cilomilast suppresses the generation of IL-17–associated immunity in experimental DED. (*Invest Ophthalmol Vis Sci.* 2012;53:3584–3591) DOI:10.1167/iov.11-9110

The pathogenesis of dry eye disease (DED) has not been fully elucidated; however, there is a growing body of evidence indicating that DED is an immune-mediated disorder. Inflammation of the ocular surface and lacrimal glands, collectively known as the “lacrimal functional unit” (LFU), is an intrinsic characteristic of both Sjögren’s syndrome– and non-Sjögren’s syndrome–associated DED.¹ Elevated tear film

osmolarity, a feature common to all forms of DED, is thought to precipitate inflammation of the LFU by activating intracellular stress-associated mitogen-activated protein (MAP) kinase pathways that induce the production of proinflammatory cytokines such as IL-1 and TNF- α .² These cytokines promote the activation and maturation of antigen-presenting cells (APCs) that subsequently migrate to draining lymphoid tissues and prime autoreactive effector T cells.^{3,4} Adoptive transfer of CD4⁺ T helper (Th) cells from DED-induced donor mice to athymic (nude) recipient mice produces inflammation of the LFU similar to that observed in conventional DED, suggesting that DED is a T-cell mediated autoimmune disorder.⁵ Th17 cells, a recently discovered class of Th cells, have been implicated in the pathogenesis of numerous autoimmune diseases, including DED.^{3,6–8} Th17 cells that are resistant to T-regulatory cell–mediated suppression have been described in the regional lymph nodes of DED-induced mice.⁶ DED involves increased expression of the Th17-associated cytokines IL-6, IL-23, and IL-17.^{6,7} Th17-secreted IL-17 promotes disruption of the corneal epithelial barrier, and administration of anti-IL-17 antibody results in a marked attenuation of DED severity.^{6,7}

Anti-inflammatory and immunomodulatory medications, such as corticosteroids and cyclosporine, are utilized clinically in the treatment of DED.⁹ Corticosteroids (e.g., dexamethasone) are potent immunosuppressants that downregulate the activity of proinflammatory molecules and lymphocytes.¹⁰ Corticosteroids are capable of ameliorating many cases of severe DED; unfortunately, the side effects of prolonged corticosteroid use (e.g., cataract, glaucoma) generally make this an untenable choice.¹¹ Topical cyclosporine reduces DED severity by inhibiting the activity of T cells and promoting tear fluid secretion.^{12,13} Cyclosporine’s efficacy in the treatment in DED is well established; however, many patients fail to respond favorably or adequately to cyclosporine therapy.¹⁴ Medications that modulate various proinflammatory molecules have shown promise in the treatment of experimental DED, but these have not yet materialized in the clinical setting.¹⁵ Most ophthalmologists agree that the currently available treatment modalities for moderate to severe DED are limited in both number and efficacy.¹⁶

Cyclic nucleotide phosphodiesterases (PDEs) are involved in the regulation of numerous intracellular signal transduction pathways.¹⁷ The PDE4 family predominates in inflammatory cells, and PDE4 inhibition is a promising method of potentially abrogating pathogenic inflammation.¹⁸ The present study evaluated the therapeutic potential of topically applied PDE4 inhibitor (cilomilast) in a murine model of DED. Cilomilast was compared with the anti-inflammatory medications dexamethasone and cyclosporine. We determined the effect of topical cilomilast and dexamethasone on several well-described measures of ocular surface inflammation. Subsequently, we investigated the effects of topical cilomilast and cyclosporine on measures of IL-17–associated immunity.

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METHODS

Animals

Six- to 8-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used for this study. Mice were housed in a secure, pathogen-free environment at the Schepens Eye Research Institute Animal Care Facility. All procedures and protocols were approved by the Schepens Eye Research Institute Animal Care and Use Committee. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

DED Induction

DED was induced by housing mice in a controlled environment chamber that allows for the continuous regulation of airflow (15 L/min), relative humidity (15% to 20%), and temperature (21 to 23°C) as previously described.^{4,15,19,20} Clinical DED severity was increased by administering topical and systemic anticholinergic medications. Atropine sulfate 1.0% (Bausch & Lomb, Rochester, NY) was administered via topical ocular instillation twice per day for the first 2 days of DED induction. Subcutaneous injections of 0.1 mL scopolamine hydrobromide, 5.0 mg/mL (Sigma-Aldrich Corporation, St Louis, MO), were performed three times per day for the duration of the experiment.^{4,15,20}

Medication Formulations

Cilomilast 0.05% (Alcon Laboratories, Inc., Fort Worth, TX), cyclosporine ophthalmic emulsion 0.05% (Allergan, Inc., Irvine, CA), dexamethasone 0.1% (Alcon Laboratories, Inc.), and relevant vehicles were utilized in a blinded fashion by two examiners.

Treatment Regimen

To evaluate the cilomilast effect on innate immunity, dry eye induction was limited to 2 days. Following DED induction, 16 mice were randomized to four groups (four mice per group): (1) cilomilast, (2) cilomilast vehicle, (3) dexamethasone, (4) DED-untreated. Each treatment group received 3 μ L of its respective medication via ocular surface instillation three times per day over a period of 7 days.

To evaluate the effect of cilomilast on IL-17-associated immunity, the induction of DED was extended to 6 days. Twenty-four mice were randomized to four groups (six mice per group): (1) cilomilast, (2) cilomilast vehicle, (3) cyclosporine, (4) DED-untreated. Each treatment group received 3 μ L of its respective medication via ocular surface instillation three times per day over a period of 7 days.

Corneal Fluorescein Staining

Corneal fluorescein staining (CFS) was performed on day 0 (before DED induction), day 2 (before initiating topical therapy), and 7 days after treatment (7-day Tx). A 1.0- μ L quantity of fluorescein, 1.0% (Sigma-Aldrich), was applied to the inferior conjunctival sac of each eye as previously described.¹⁹ CFS was examined 3 minutes later using slit-lamp biomicroscopy under cobalt blue light. The National Eye Institute's standardized grading system was used to score punctuate staining.²¹ To evaluate the effect of cilomilast on IL-17-associated immunity, the DED induction phase was extended to 6 days. CFS was performed on day 0 (before DED induction), day 6 (before initiating topical therapy), and 7-day Tx.

Real-Time PCR

Real-time PCR was performed on tissues from the same mice that underwent CFS examination. Corneas, conjunctiva, and lymph nodes from naïve, DED-untreated, and DED-treated mice were harvested. In each group, total RNA was isolated using a commercially available kit

(RNeasy; Qiagen, Valencia, CA). The first strand of cDNA was synthesized by reverse transcriptase using random hexamers (SuperScript III; Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Real-time PCR was performed using Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA) and dye-labeled, predesigned primers for IL-1 α (Mm00439620_m1), IL-1 β (Mm00434228_m1), TNF- α (Mm99999068_m1), IL-6 (Mm00446190_m1), IL-17A (Mm00439619_m1), IL-23 (Mm00518984_m1), FoxP3 (Mm00475156_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1) and all assays were performed in duplicate. The GAPDH gene was used as the endogenous reference for each reaction. The results of quantitative PCR were analyzed by the C_T method, in which the target change = $2^{-\Delta\Delta C_T}$. The results were normalized by the C_T value of GAPDH, and the mean C_T of relative mRNA level in the normal, DED-untreated group or treated DED group was used as the calibrator.

Immunohistochemical Staining

The following primary antibodies were used for immunohistochemical staining: FITC-conjugated rat anti-mouse CD11b (monocyte/macrophage marker; BD Biosciences, San Diego, CA) and FITC-conjugated rat IgG2bk (isotype control; BD Biosciences). For whole-mount corneal staining, freshly excised corneas were washed in PBS and fixed in acetone for 15 minutes. Nonspecific staining was blocked with anti-FcR CD16/CD32 antibody (BD Biosciences). Specimens were immunostained with primary or isotype antibody for 2 hours, washed with PBS, incubated with secondary antibodies, and mounted with Vector Shield mounting medium (Vector Laboratories; Burlingame, CA). Corneas were analyzed using confocal microscopy at $\times 40$ magnification. CD11b⁺ cells were enumerated in four areas in the periphery (0.5- μ m area from the limbus) and two areas in the center (central 2- μ m area) of each cornea. The mean number of cells was obtained by averaging the cell number in each area examined.

Flow Cytometry

Draining lymph nodes and conjunctiva were harvested separately and aseptically from naïve and DED mice (three mice per group) at the end of the treatment period. Briefly, conjunctivae were removed and cut into pieces followed by digestion with 2 mg/mL collagenase type IV (Sigma-Aldrich) and 0.05 mg/mL DNase I (Roche, Basel, Switzerland) for 1 hour at 37°C with agitation. The suspension and remaining tissue were then homogenized and filtered through a 70- μ m cell strainer (BD Biosciences). Blocked with anti-FcR mAb, cells were then double-stained with phycoerythrin (PE)-Cy5-conjugated anti-CD45 and PE-conjugated anti-IL-17 at 4°C for 45 minutes. Single-cell suspensions were also prepared from lymph node (LN). Briefly, LN cells in 1000 μ L media were stimulated in six-well flat-bottom plates with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin (Sigma-Aldrich) for 18 hours at 37°C; GolgiStop (BD Biosciences) was added to the cultured cells for the last 6 hours (4 μ L/6 mL cell culture; BD Biosciences) to inhibit cytokine secretion.⁶ The cells were double-stained with FITC-conjugated anti-CD4, and PE-conjugated anti-IL-17. Control samples were stained with appropriate isotype-matched control antibodies. Stained cells were analyzed using a flow cytometer (Epics XL; Beckman Coulter, Fullerton, CA). The percentage of stained cells in the conjunctivae was calculated with respect to isotype control staining. All antibodies, matched isotype controls, and the fix-perm buffer were purchased from eBioscience (San Diego, CA).

Statistical Analysis

Using SPSS software (SPSS Inc., Chicago, IL), the Shapiro-Wilk test was employed to test the data for normality. All variables were normally distributed. Then using Microsoft Excel, the two-tailed *t*-test was used and $P \leq 0.05$ was considered statistically significant. Results are presented as the mean \pm SEM of two or more trials.

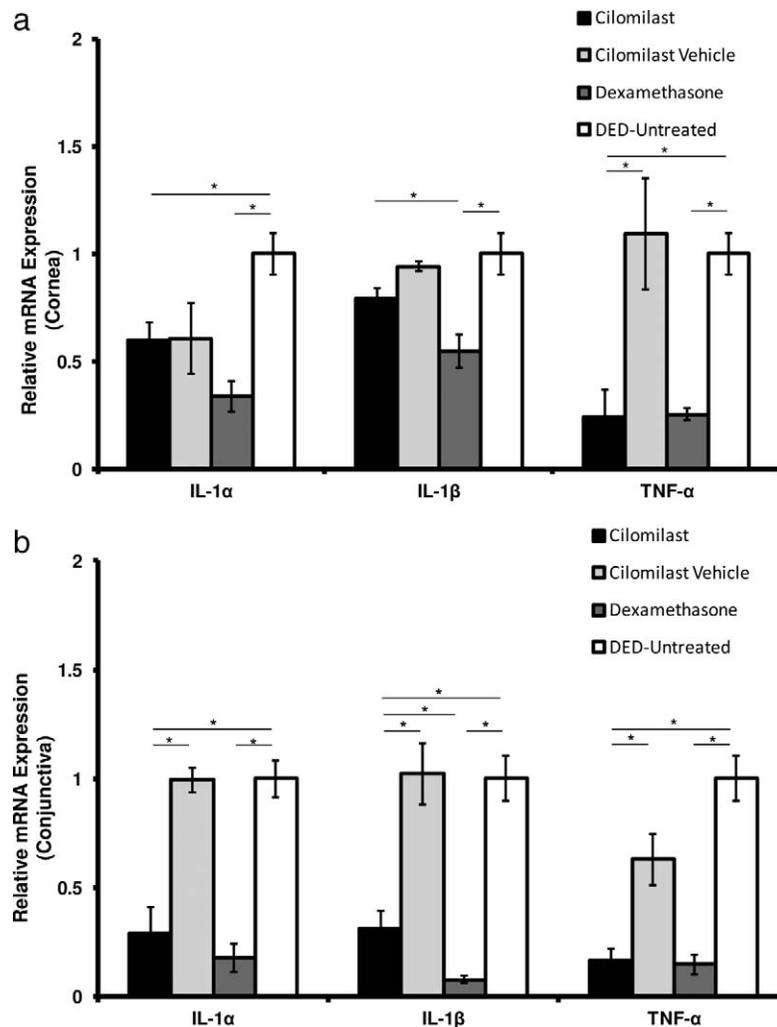


FIGURE 1. At 7-day Tx, treatment with either cilomilast or dexamethasone significantly reduced the corneal expression of IL-1 α and TNF- α as compared with the DED-untreated corneas. Compared with the cilomilast-treated group, dexamethasone significantly reduced the corneal expression of IL-1 β ($n = 4$ corneas/group/experiment) (a). Cilomilast significantly reduced the conjunctival expression of IL-1 α , IL-1 β , and TNF- α as compared with the vehicle-treated and DED-untreated eyes. Dexamethasone significantly reduced the conjunctival expression of IL-1 β as compared with the cilomilast-treated group. Compared with the DED-untreated group, dexamethasone significantly reduced conjunctival expression of IL-1 α , IL-1 β , and TNF- α ($n =$ eight eyes/group/experiment) (b). Results are normalized to mRNA expression of the DED-untreated dry eye group. Data are represented as mean of three experiments (SEM displayed).

RESULTS

Cilomilast Decreased the Expression of Proinflammatory Cytokines in the Cornea and Conjunctiva

Real-time PCR was used to quantify the expression of transcripts encoding IL-1 α , IL-1 β , and TNF- α in the corneas and conjunctivae of DED-induced mice ($n =$ six corneas/eight conjunctivae per group). Treatment with topical cilomilast significantly decreased the corneal expression of TNF- α ($P = 0.04$) as compared with the vehicle-treated group. Compared with the DED-untreated corneas, treatment with cilomilast significantly reduced IL-1 α and TNF- α expression ($P = 0.04$ and $P = 0.02$, respectively). Topical dexamethasone significantly reduced the expression of IL-1 α ($P = 0.01$), IL-1 β ($P = 0.03$), and TNF- α ($P = 0.002$) as compared with the DED-untreated corneas. Topical dexamethasone significantly reduced the expression of IL-1 β compared with the cilomilast-treated group ($P = 0.05$). Corneal expression of IL-1 α in the dexamethasone-

treated group was lower than the cilomilast-treated eyes; however, differences did not reach statistical significance (Fig. 1a). In the conjunctiva, topical cilomilast significantly decreased the expression of IL-1 α ($P = 0.006$), IL-1 β ($P = 0.01$), and TNF- α ($P = 0.02$) as compared with the vehicle-treated group. Topical cilomilast significantly decreased the expression of IL-1 α ($P = 0.02$), IL-1 β ($P = 0.01$), and TNF- α ($P = 0.004$) as compared with the DED-untreated groups. As compared with the DED-untreated group, dexamethasone significantly reduced the expression of IL-1 α ($P = 0.005$), IL-1 β ($P = 0.001$), and TNF- α ($P = 0.002$). The expression levels of IL-1 β ($P = 0.04$) in the dexamethasone-treated group was comparable to that of seen in the cilomilast-treated group (Fig. 1b).

Cilomilast Decreased the Corneal Infiltration of CD11b⁺ APCs

Corneal CD11b⁺ cells have been implicated in the induction of corneal immunity and in the immunopathogenesis of DED.¹⁵

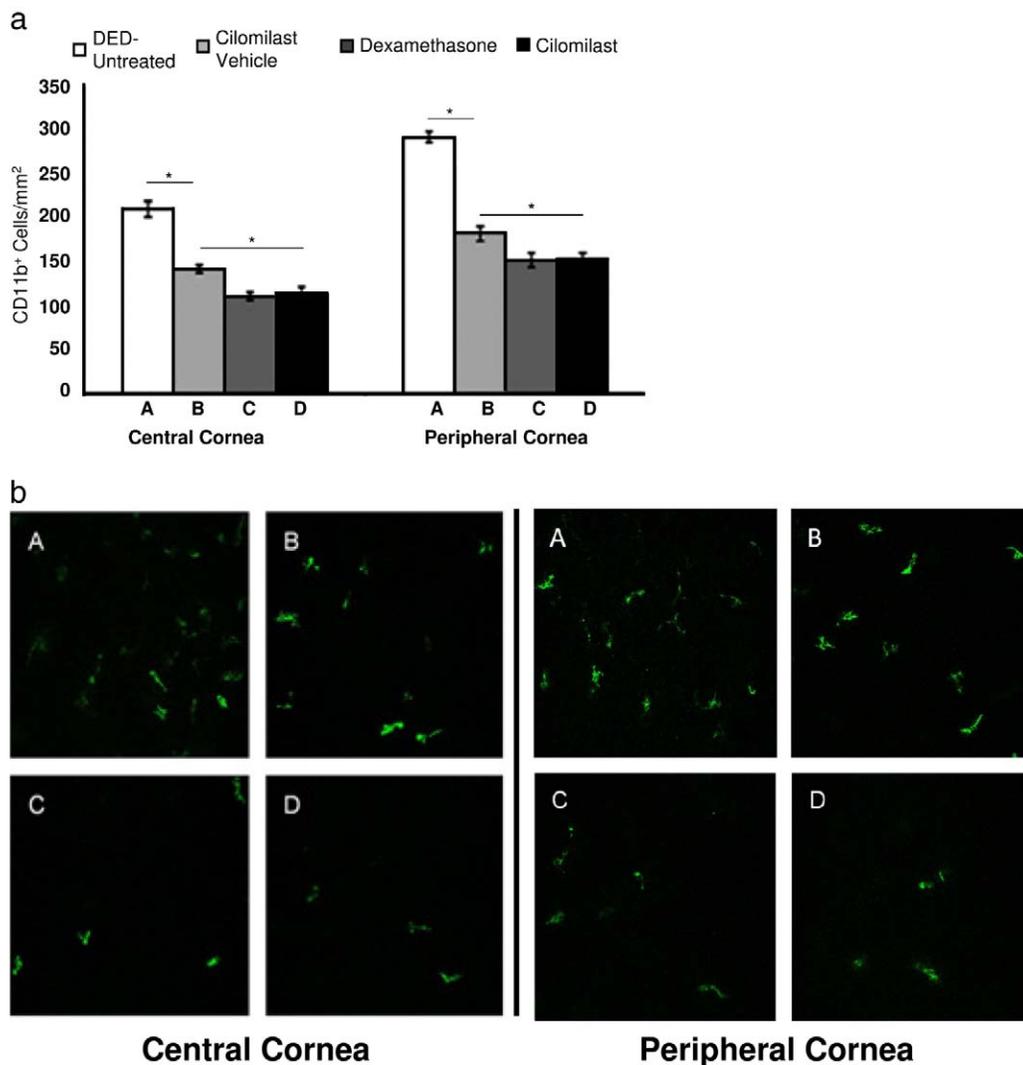


FIGURE 2. (a) Compared with the DED-untreated, topical cilomilast and dexamethasone significantly decreased the infiltration of CD11b⁺ cells into the central and peripheral cornea at 7-day Tx ($P < 0.02$). Cilomilast significantly reduced CD11b⁺ cell infiltration as compared with the vehicle ($P < 0.05$). (b) Representative confocal images of CD11b⁺ staining cells in the central and peripheral cornea: (A) DED-untreated, (B) cilomilast vehicle, (C) dexamethasone, (D) cilomilast ($n = 4$ corneas/group).

To assess the effect of PDE4 antagonism on these cells, immunohistochemical staining was used to quantify the presence of CD11b⁺ cells in the cornea ($n =$ four corneas per group). Treatment with either topical cilomilast or dexamethasone significantly reduced the presence of CD11b⁺ cells in both the central and peripheral cornea as compared with the DED-untreated group ($P < 0.001$). Similarly, topical cilomilast significantly reduced the presence of CD11b⁺ cells in the central and peripheral cornea as compared with the vehicle-treated group ($P < 0.02$). Compared with the DED-untreated cornea, cilomilast vehicle significantly reduced CD11b⁺ cell infiltration in the central and peripheral cornea ($P < 0.001$) (Figs. 2a, 2b).

Cilomilast Reduced Th17-Associated Cytokine Expression in the Conjunctiva

Th17 cells are involved in the development of immune-mediated disease such as DED. Real-time PCR was used to measure the expression of Th17-associated cytokines in the cornea and conjunctiva. No statistically significant differences

were noted in the corneal expression of IL-6, IL-23, or IL-17 in the cilomilast-treated corneas compared with the vehicle-treated group (data not shown). Treatment with topical cilomilast resulted in a significant decrease in the conjunctival expression of IL-6 ($P = 0.05$), IL-23 ($P = 0.004$), and IL-17 ($P = 0.05$) as compared with the vehicle-treated groups (Fig. 3a). Topical cilomilast significantly decreased the expression of IL-6, IL-23, and IL-17 as compared with the DED-untreated group ($P = 0.0001$, $P = 0.02$, $P = 0.04$, respectively). Topical cyclosporine also significantly decreased the conjunctival expression of IL-6 when compared with the DED-untreated group ($P = 0.002$). There was no significant difference between cyclosporine- and cilomilast-treated eyes with respect to IL-6, IL-23, and IL-17 expression (Fig. 3a).

Cilomilast Reduced IL-17⁺ Cell Infiltration in the Conjunctiva

Conjunctival cells were isolated and stained with anti-CD45 PE-CY5 and anti-IL-17 PE. Flow cytometry was performed to measure the infiltration of IL-17⁺CD45⁺ cells. Topical cilomilast

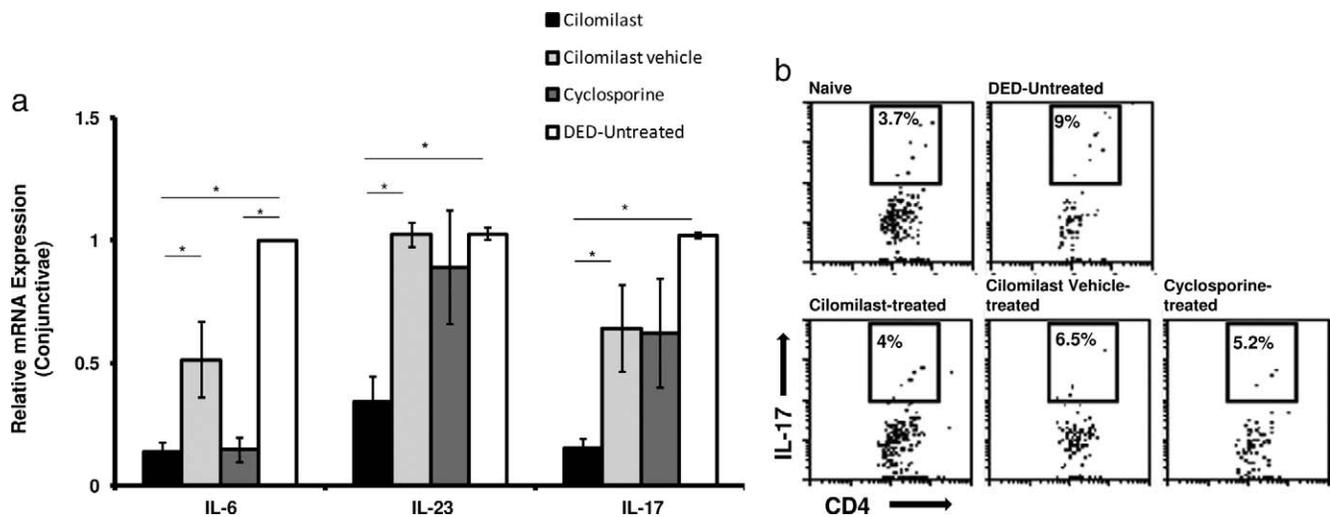


FIGURE 3. Topical cilomilast significantly decreased the conjunctival expression of IL-6, IL-23, and IL-17 as compared with both the DED-untreated and relevant vehicle-treated groups ($P \leq 0.05$). Cyclosporine significantly reduced IL-6 expression as compared with the DED-untreated group. Results are normalized to mRNA expression of the DED-untreated dry eye group. Data are represented as mean of three experiments (SEM displayed) ($n =$ six eyes/group/experiment) (a). Cilomilast also decreased the frequency of IL-17⁺CD45⁺ cell infiltration of the conjunctiva. Conjunctivae from six eyes per group were pooled and experiment was performed once (b).

decreased the frequency of IL-17⁺CD45⁺ cells (4.0%) as compared with the DED-untreated (9.0%), cyclosporine (5.2%), and cilomilast (6.5%) vehicle-treated groups. Topical cyclosporine treatment also led to a decrease in the frequency of IL-17 expressing cells as compared with the DED-untreated group (Fig. 3b).

Cilomilast Decreased the Presence of Th17-Associated Cytokines in the Regional Lymph Nodes

Treatment with topical cilomilast significantly decreased the expression of IL-23 ($P = 0.03$) and IL-17 ($P = 0.01$) as compared with the vehicle-treated group. Compared with the DED-untreated group, cilomilast significantly decreased the expres-

sion of IL-17 ($P = 0.0003$) and IL-23 ($P = 0.001$). There was no statistically significant difference in IL-6 expression between the various groups. IL-23 expression was significantly decreased in the lymph nodes of vehicle-treated and cyclosporine-treated mice as compared with the DED-untreated group ($P = 0.02$, $P = 0.01$). Cyclosporine significantly decreased IL-17 expression as compared with the DED-untreated group ($P = 0.002$) (Fig. 4a).

Cilomilast Reduced IL-17⁺ Cell Infiltration in the Draining Lymph Nodes

Topical cilomilast decreased the frequency of IL-17⁺CD4⁺ cells (5.3%) as compared with the DED-untreated (8.0%), and vehicle-treated (7.0%) groups (Fig. 4b). Treatment with topical

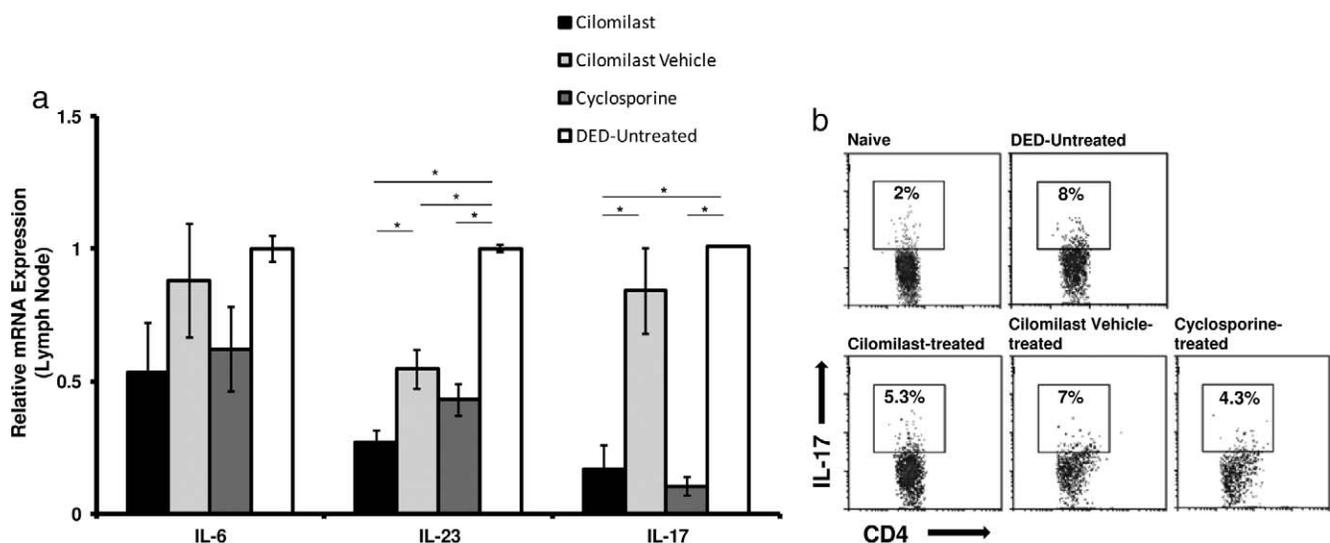


FIGURE 4. As compared with the vehicle, treatment with topical cilomilast significantly decreased the expression of IL-23 and IL-17 in the regional lymph nodes. Cyclosporine significantly reduced IL-17 expression as compared with the DED-untreated lymph nodes. Results are normalized to mRNA expression of the DED-untreated dry eye group. Data are represented as mean of three experiments (SEM displayed) ($n =$ three mice/group/experiment) (a). Compared with the vehicle-treated group, cilomilast decreased the frequency of IL-17⁺CD4⁺ T-cell infiltration in the draining lymph nodes. Draining lymph nodes from three mice per group were pooled and experiment was performed once (b).

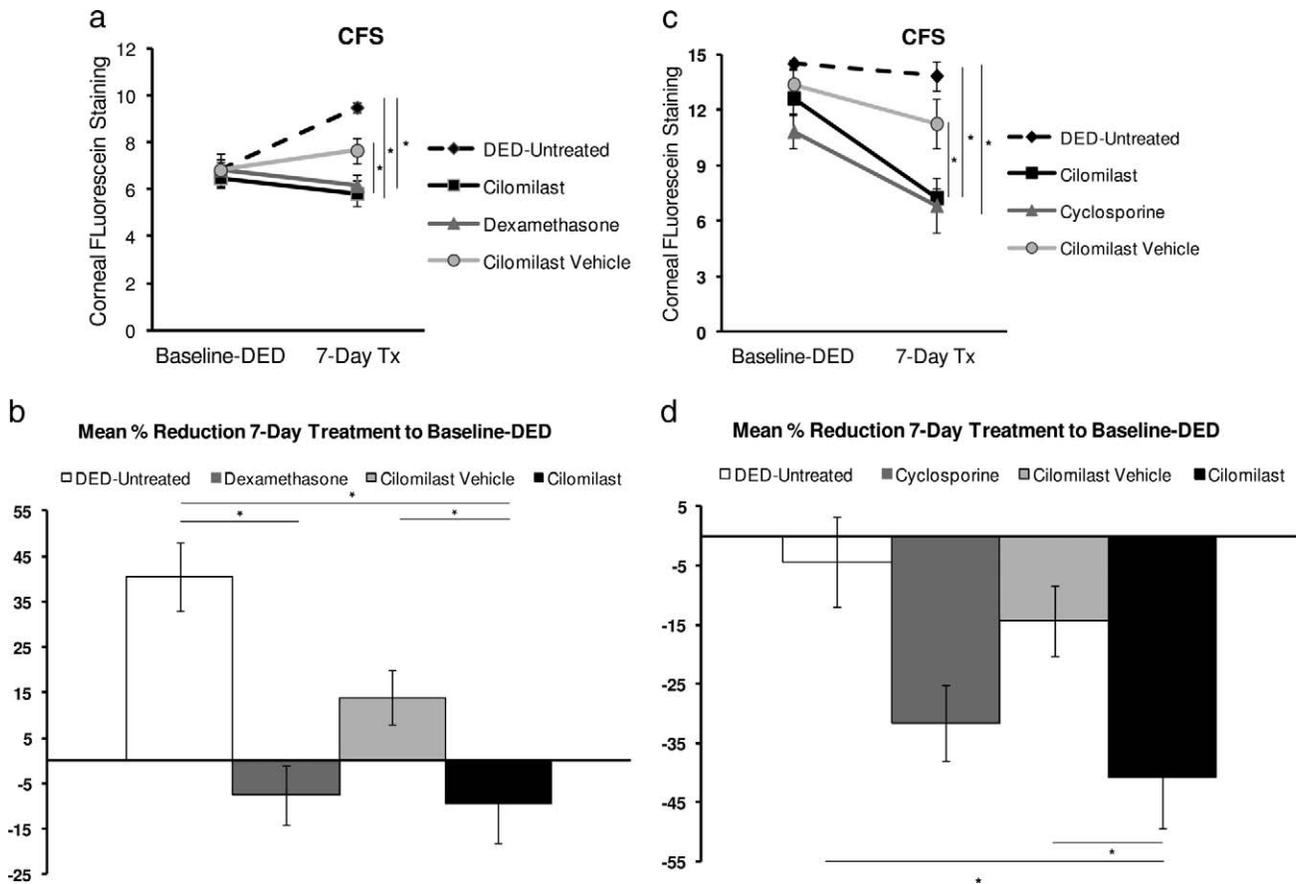


FIGURE 5. At 7-day Tx, topical cilomilast produced a significant decrease in the CFS as compared with the vehicle-treated and DED-untreated corneas. As compared with the DED-untreated group, dexamethasone significantly reduced mean CFS (a). Compared with the DED-untreated group, treatment with either topical cilomilast or dexamethasone resulted in a statistically significant corneal fluorescein percent reduction between baseline DED and 7-day Tx ($P < 0.05$). Each experiment consisted of four mice (eight eyes) per group (b). Only topical cilomilast significantly reduced the severity of CFS as compared with both the DED-untreated and relevant vehicle-treated groups (c). Compared with the vehicle-treated and DED-untreated corneas, treatment with topical cilomilast resulted in a significant corneal fluorescein percent reduction ($P < 0.05$) (d). Each experiment consisted of six mice (12 eyes) per group.

cyclosporine (4.3%) also led to a decrease in the frequency of IL-17 expressing cells as compared with the DED-untreated group.

Cilomilast Decreased the Clinical Severity of DED

To determine the potential therapeutic effect of cilomilast on clinical disease severity, we performed CFS on DED-induced mice. Normal mice demonstrated only minimal corneal punctate staining at baseline. Mice were placed in the controlled environment chamber and anticholinergic treatment was initiated on day 0. CFS was performed on day 2, revealing a significant increase in CFS scores. Therapeutic treatment with cilomilast, cilomilast vehicle, and dexamethasone was initiated on day 3 and continued for 7 days. All treatment groups demonstrated a reduction in average CFS scores after 7-day Tx, and there was a statistically significant difference observed when comparing all treatment groups with the DED-untreated group ($P < 0.001$). Treatment with either topical cilomilast or dexamethasone produced a statistically significant decrease in CFS as compared with the DED-untreated group by 7-day Tx ($P = 0.003$ and $P = 0.0006$, respectively). Cilomilast-treated eyes showed a significant reduction in CFS by 7-day Tx compared with the cilomilast vehicle-treated eyes ($P = 0.05$). There was no statistically significant difference between cilomilast- and dexamethasone-treated eyes in CFS reduction (Fig. 5a). There was a reduction

of 10% in the CFS score in the cilomilast-treated eyes from baseline, whereas in the vehicle-treated group there was a 13% increase in CFS score (Fig. 5b). To evaluate the effect of cilomilast on IL-17-induced immunity, treatment with cilomilast, cilomilast vehicle, and cyclosporine was initiated on day 6 and continued for 7 days. CFS was performed on days 6, 11, and 14 to evaluate clinical disease progression. All treatment groups demonstrated a reduction in average CFS scores between days 6 and 14 (Fig. 5b); however, only the cilomilast-treated group experienced a statistically significant reduction in CFS scores when compared with the DED-untreated and relevant vehicle-treated groups ($P = 0.001$ and 0.05 , respectively) (Fig. 5c). Topical cilomilast treatment resulted in a 40% reduction in CFS scores between baseline and 7-day Tx, whereas vehicle-treated eyes showed a 14% reduction (Fig. 5d), suggesting that topical PDE4 blockade reduces DED severity. Topical cyclosporine produced a comparable average CFS percent reduction, but this was not statistically significant due to considerable variability.

DISCUSSION

The cyclic nucleotide PDE enzyme “superfamily” comprises numerous PDE families necessary for the degradation of cyclic nucleotides, such as cAMP and cGMP.²² Each PDE family is

characterized by a distinct combination of genetic, enzymatic, and pharmacologic profiles. The prototypic secondary messenger cAMP is involved in a diverse array of biologically essential intracellular signal transduction pathways.¹⁷ The cAMP-specific PDE4 family predominates in bone marrow-derived inflammatory cells, including T cells and APCs.²³ The potent anti-inflammatory effects of PDE4 inhibition have been demonstrated in experimental models of asthma, atopic dermatitis, chronic obstructive pulmonary disease (COPD), acute lung injury, rheumatoid arthritis, and multiple sclerosis.²⁴ PDE4 inhibitors show promise in the clinical treatment of asthma, and a specific PDE4 inhibitor was recently approved by the European Medicines Agency for the clinical treatment of severe COPD.²⁵⁻²⁷ The present study investigated the therapeutic efficacy of the specific PDE4 inhibitor cilomilast in the treatment of DED.

We investigated the effect of cilomilast on several well-described measures of ocular surface inflammation: proinflammatory cytokine expression and APC infiltration. Dexamethasone was chosen as the comparative treatment because its therapeutic efficacy has been demonstrated in numerous DED experiments.⁹ The proinflammatory cytokines IL-1 α , IL-1 β , and TNF- α are elevated in the tears and ocular surface of DED patients.^{20,28} Topical cilomilast significantly reduced the corneal expression of TNF- α and the conjunctival expression of IL-1 α , IL-1 β , and TNF- α . These cytokines are produced in large quantities by leukocytes, including APCs such as dendritic cells.²⁹ These cytokines are also produced by corneal and conjunctival epithelium.^{30,31} Infiltration of the cornea by APCs has been described in both experimental and clinical DED.^{20,32} APC enumeration was performed, revealing that cilomilast significantly decreased the presence of CD11b⁺ APCs in the peripheral and central cornea. These findings are consistent with previous reports indicating that PDE4 inhibition reduces the ability of APCs to produce proinflammatory cytokines (e.g., TNF- α) and promote immune activity.²⁹

We also investigated the effect of cilomilast treatment on IL-17-associated immune responses in DED. For this experiment, the DED induction phase was extended to 6 days to ensure that a robust adaptive immune response was generated. Infiltration of the conjunctiva by pathogenic T cells is a universal feature of DED.³³ Th17 cells are a recently discovered population of T cells that have been implicated in the immunopathogenesis of DED.^{3,6-8} IL-6 and IL-23 promote the differentiation and expansion of Th17 cells.^{34,35} IL-17, the signature cytokine of Th17 cells, promotes DED by disrupting the corneal epithelial barrier following desiccating stress.⁷ Cyclosporine was chosen as the comparative treatment because cyclosporine's method of action specifically targets T cells, including Th17 cells.³⁶ Treatment with topical cilomilast significantly reduced the conjunctival expression of IL-6, IL-23, and IL-17, and the draining lymph node expression of IL-23 and IL-17. These findings indicate that treatment with topical cilomilast reduces the IL-17-associated immunity.

There was a reduction of 10% to 20% in CFS score in the vehicle-treated compared with the DED-untreated eyes. The vehicle effect was also seen on the infiltration of CD11b⁺ cells in the cornea and CD45⁺IL17⁺ cells in the conjunctiva. This is perhaps due to the lubricating effect of the vehicle on the ocular surface. Lower expression of IL-23 in the draining lymph nodes might be due to the reduced migration of CD11b⁺ cells to the draining lymph nodes.

Importantly, treatment with topical cilomilast consistently reduced corneal epitheliopathy scores by approximately 40% from baseline. Moreover, topical cilomilast was significantly more effective than its associated vehicle at reversing ocular surface damage, suggesting that the beneficial effects of cilomilast treatment are not solely attributable to lubrication of

the ocular surface. Topical application of the anti-inflammatory medications dexamethasone and cyclosporine produced CFS reductions that were comparable to those produced by the topical application of cilomilast. In summary, our findings indicate that topical cilomilast ameliorates DED as determined by clinical and inflammatory measures. The therapeutic efficacy of cilomilast was comparable or superior to that of dexamethasone and cyclosporine. These findings suggest that topical cilomilast may be an effective therapeutic modality for clinical DED. Additional investigations will be required to determine the optimal dosage and duration of cilomilast treatment.

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