Amelioration of Murine Dry Eye Disease by Topical Antagonist to Chemokine Receptor 2

Sunali Goyal, MD; Sunil K. Chauhan, PhD; Qiang Zhang, MD; Reza Dana, MD, MSc, MPH

Objective: To determine the effect of a topical antagonist to the chemokine receptor 2 (CCR2) in a murine model of dry eye disease.

Methods: The effects of a topical CCR2 antagonist and a vehicle control treatment were studied in murine dry eyes. A controlled environment chamber induced dry eye by exposing mice to high-flow desiccated air. Corneal fluorescein staining and enumeration of corneal CD11b^+ cells and conjunctival T cells were performed in the different groups. Real-time polymerase chain reaction was performed to quantify expression of different inflammatory cytokine transcripts in the cornea and conjunctiva.

Results: Eyes receiving the formulation containing CCR2 antagonist showed a significant decrease in corneal fluorescein staining and decreased infiltration of corneal CD11b^+ cells and conjunctival T cells compared with the vehicle-treated and untreated dry eye groups. The CCR2 antagonist also significantly decreased messenger RNA expression levels of interleukins 1α and 1β in the cornea, and tumor necrosis factor α and interleukin 1β in the conjunctiva.

Conclusion: Topical application of CCR2 antagonist is associated with significant improvement in dry eye disease and is reflected by a decrease in inflammation at the clinical, molecular, and cellular levels.

Clinical Relevance: Topical application of CCR2 antagonist may hold promise as a therapeutic modality in dry eye disease.

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Dry eye syndrome or keratoconjunctivitis sicca is one of the most common problems facing patients seeking ophthalmological care. In dry eye disease (DED), the homeostatic balance between the tear film and the ocular surface epithelium is impaired, causing the ocular surface to respond abnormally to environmental challenges. The clinical presentation can be highly variable, ranging from mild ocular discomfort to sight-threatening corneal complications such as persistent epithelial defects or sterile stromal ulceration. Epidemiological studies report that more than 6% of the population older than 40 years and 15% of the population older than 65 years have DED.

Clinically significant DED is associated with ocular surface inflammation, although the exact immunopathogenesis is not known. It is now recognized that the ocular surface (cornea and conjunctiva) and the lacrimal glands function as an integrated unit called the lacrimal functional unit, and inflammation in any component can compromise the function of the others through soluble mediators, generation of autoreactive T cells, and inhibition of neural transmission.

Chemokines are small cytokines with chemotactic properties that coordinate leukocyte migration in immunity and inflammation and hence play a role in the pathogenesis of many human diseases. Inflammatory macrophages, derived from peripheral blood monocytes, are well-characterized cell mediators of tissue destruction in various chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis. Once inside tissues, they orchestrate tissue destruction by secreting various proinflammatory cytokines (tumor necrosis factor α [TNF-α] and interleukin 1 [IL-1]) and chemokines (CXCL8, CCL5, and CCL2) that further cause influx of other inflammatory cells. Our laboratory has previously shown that DED is associated with ingress and activation of corneal CD11b^+ cells. It is known that desiccating stress upregulates chemokines and their receptors in DED, and monocyte chemotactic protein 1 (MCP-1)/CCL2 has been identified as the key molecule for the chemotaxis and influx of mononuclear leukocytes into sites of inflammation. Monocyte chemotactic protein 1 is secreted by monocytes, memory T cells, macrophages, fibroblasts, endothelial cells, and mast cells, and it stimulates the movement of leukocytes along a chemotactic gradient after binding to its cell surface receptor CCR2. The critical role of the MCP-1/CCR2 pair in inflammation has been...
demonstrated using MCP-1 and CCR2 knockout mice, suggesting that the inhibition of migration of CCR2-bearing mononuclear cells may be an effective mechanism to modulate disease progression in chronic inflammation.10 Herein, we hypothesized that a topical antagonist to CCR2 could have therapeutic value for the treatment of dry eyes in humans.

**METHODS**

**ANIMALS**

We used female C3BL/6 mice (Charles River Laboratories, Wilmington, Massachusetts) aged 8 to 12 weeks for this study. The research protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee, and it conformed to the standards in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

**INDUCTION OF EXPERIMENTAL DRY EYE**

We induced dry eye in the mice by placing them in a controlled environment chamber (CEC), which allows continuous regulation of relative humidity below 30%, a constant temperature of 21°C to 23°C, and airflow of 15 L/min, 24 hours a day.11 To achieve maximum ocular surface dryness, the conditions in the CEC were supplemented with topical application of atropine sulfate, 1% (Falcon Pharmaceuticals, Fort Worth, Texas), twice daily for the first 48 hours. In addition, the mice also received subcutaneous 0.1-mL injections of 5-mg/mL scopolamine hydrobromide (Sigma-Aldrich Corp, St Louis, Missouri) 3 times a day (9 AM, 1 PM, and 3 PM) on their dorsal surface for the duration of the experiment.

**TOPICAL CCR2 ANTAGONIST FORMULATION AND TREATMENT REGIMEN**

The topical CCR2 antagonist and the vehicle control treatment were provided by Johnson & Johnson Vision Care, Inc, Jacksonville, Florida. The CCR2 antagonist at a concentration of 5.0 mg/mL (J&J-17166864-AAG) was the tested formulation. Physiologic phosphate-buffered saline (J&J-17166864-AAG) consisting of purified water, sodium phosphate monobasic monohydrate, sodium phosphate dibasic dihydrate, and sodium chloride was used as the vehicle control.

Forty-eight hours after the induction of dry eye, the mice in the CEC were randomly divided into the following 3 different groups (n=5 in each group): an untreated group, a group receiving the CCR2 antagonist formulation. Three microliters of the topical formulation were applied to both the eyes of the unanesthetized mice twice a day (9 AM and 5 PM) from days 3 to 9. The untreated group received no eyedrops. Ocular signs of dry eye were evaluated using fluorescein staining at days 2, 5, and 9. Mice were then euthanized on day 10 for cellular and molecular studies.

**CORNEAL SURFACE STAINING**

Fluorescein staining of the corneal epithelium was used as a diagnostic tool to study the effect of desiccating stress on the ocular surface of the mice. Corneal fluorescein staining was performed at baseline (day 0, before placing the mice in the CEC), day 2 (before instillation of topical therapy), day 5, and finally at day 9. A 0.7-µL quantity of fluorescein, 2.5% (Sigma-Aldrich Corp), was applied to the inferior conjunctival sac of each eye (n=10 eyes per group) by using a micropipette as previously described.11 After 5 minutes, punctate staining on the corneal surface was evaluated in a masked fashion with the help of a slitlamp biomicroscope under a cobalt blue light. The National Eye Institute grading system was used to assess the corneal surface staining.

**RNA ISOLATION AND MOLECULAR ANALYSIS USING REAL-TIME POLYMERASE CHAIN REACTION**

Total RNA was isolated from the cornea and conjunctival tissues using a commercially available kit (RNasey [catalog No. 74004]; Qiagen, Valencia, California). Two corneas and 3 to 4 conjunctivae per group were isolated, pooled, and stored at -80°C in a reagent (TRizol [catalog No. 1596026]; Invitrogen Corporation, Carlsbad, California) until future use.

The first strand of complementary DNA (cDNA) was synthesized with random hexamers using reverse transcription (SuperScript III [catalog No. 18080]; Invitrogen Corporation) according to the manufacturer's recommendations. Real-time polymerase chain reaction was performed using dye-labeled predesigned primers (FAM-MGB; Applied Biosystems, Foster City, California) for TNF-α (assay Mm09999068_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay Mm99999915_g1), IL-1α (assay Mm00439620_m1), and IL-1β (assay Mm00434228_m1). One microliter of cDNA was loaded in each well, and the assays were performed in duplicate. A nontemplate control was included in all of the experiments to evaluate DNA contamination of the reagent used. The GAPDH gene was used as the endogenous reference for each reaction. The results were normalized by the cycle threshold of GAPDH, and the relative messenger RNA (mRNA) level in the untreated group was used as the normalized control for the other 2 groups.

**ANALYSIS OF CELLULAR INFILTRATION BY IMMUNOHISTOCHEMICAL STAINING**

The following primary antibodies were used for immunohistochemical staining: fluorescein isothiocyanate–conjugated rat anti–mouse CD11b (monocyte/macrophage marker [catalog No. 557396; BD Pharmingen, San Diego, California]); isotype fluorescein isothiocyanate–conjugated rat anti–mouse IgG2b [catalog No. 533988BD; Pharmingen] and purified hamster anti–mouse CD3e (T-cell marker [catalog No. 553057; BD Pharmingen]; isotype purified hamster IgG1 [catalog No. 553969BD; Pharmingen]). The secondary antibody used was the Cy3–conjugated goat anti–Armenian hamster antibody (code 127165-160; Jackson Laboratories, Bar Harbor, Maine).

For whole-mount immunofluorescence corneal staining, freshly excised corneas were washed in phosphate-buffered saline and fixed in acetone for 15 minutes. The immunostaining was performed as described previously.1 Three eyeballs from 3 mice per group were taken, and cells were counted in 5 to 6 areas in the periphery (0.5-µm area from the limbus) and 1 to 2 areas in the center (central 2-µm area) of each cornea in a masked fashion by using an epifluorescence microscope (model E800; Nikon, Melville, New York) until magnification ×40. The mean number of cells was obtained by averaging the total number of cells in all the areas studied, and the result was expressed as the number of positive cells per square millimeter.

For cross-sectional staining of the conjunctiva, whole eyeballs were excised, frozen in optimal cutting temperature compound, cut into 7-µm frozen sections, and fixed in acetone for 15 minutes at room temperature.13 Three eyeballs from 3 mice per group (14-16 cross sections per group) were used for T-cell enumeration in the bulbar and tarsal conjunctivae. Serial cross sections per slide were studied under the epifluores-
Chemokines are a group of small-molecular-weight basic proteins (8-10 kDa) that represent the largest family of cytokines encoded in the human genome, constituting a group of more than 50 distinct chemokines and 20 chemokine receptors. They are secreted at sites of inflammation by resident tissue cells, resident and recruited leukocytes, and cytokine-activated endothelial cells, and they exert their functions by binding to specific G-protein–coupled cell surface receptors on target cells. The main stimuli for chemokine production are proinflammatory cytokines such as IL-1 and TNF-α and bacterial products such as lipopolysaccharides.

**Statistical Analysis**

We performed a 2-tailed t test, and P < .05 was deemed statistically significant. Results are presented as the mean (SEM) of at least 3 experiments.

**RESULTS**

**EVALUATION OF CLINICAL SIGNS OF DED USING CORNEAL FLUORESCEIN STAINING**

On day 0, mice were placed in the CEC. As validated previously, 248 hours after placing mice in the CEC, all mice showed an increase in corneal staining corresponding to dry eye induction. Treatment with the CCR2 antagonist and the vehicle control was started on day 3, and corneal fluorescein staining scores were measured at days 5 and 9. Treatment with the topical CCR2 antagonist showed a significant decrease in corneal fluorescein staining compared with the untreated group (P < .001) but was comparable to the vehicle-treated group at day 5 (P = .07). However, by day 9, the CCR2 antagonist–treated eyes showed significantly decreased corneal staining compared with the untreated and vehicle-treated groups (P < .001 for both groups; Figure 1). An overall reduction of 45% was seen in the corneal fluorescein staining score from baseline (day 2) in the CCR2 antagonist–treated eyes compared with only an 8% reduction in the vehicle-treated group.

**ENUMERATION OF CD11b+ MONOCYTES IN CORNEAS**

It has been shown that the normal cornea has a resident population of bone marrow–derived immature CD11b+ antigen-presenting cells, and the induction of dry eye increases the number of CD11b+ cells in the cornea. Therapy with the CCR2 antagonist, compared with the untreated dry eye group, significantly decreased the total number of CD11b+ cells in the periphery (11.6 [0.3] vs 19.3 [0.3] cells/mm2 [P < .001]) and in the center (7.4 [0.3] vs 14.0 [0.3] cells/mm2 [P < .001]) of the cornea. Similarly, the CCR2 antagonist–treated eyes showed a significant decrease in the number of CD11b+ cells in the cornea in the periphery and in the center compared with the vehicle-treated group (16.9 [0.3] cells/mm2 in the periphery [P < .001] and 12.4 [0.3] cells/mm2 in the center [P < .001]) (Figure 2).

**ENUMERATION OF CD3+ T CELLS IN THE CONJUNCTIVA**

Studies in various animal models and humans have made it clear that desiccating stress is often associated with T-cell infiltration of the conjunctiva. The T cells migrate into the peripheral tissues along a chemokine gradient and can mediate chronic ocular surface inflammation in a variety of dry eye states. The effect of topical CCR2 antagonist application on dry eyes was studied, and we found that the treatment significantly decreased the total number of CD3+ T cells in the conjunctiva compared with the untreated and the vehicle-treated groups (P < .001 for both groups) (Figure 3).

**mRNA EXPRESSION LEVELS OF TNF-α, IL-1α, AND IL-1β IN CORNEA AND CONJUNCTIVA**

The levels of mRNA encoding different proinflammatory cytokines, such as TNF-α, IL-1α, and IL-1β, are elevated in the cornea and conjunctiva in DED. Real-time polymerase chain reaction was used to quantify the transcripts encoding TNF-α, IL-1α, and IL-1β in the cornea and the conjunctiva of different groups (4-6 per group). Among-group comparisons showed that treatment with the CCR2 antagonist significantly decreased the mRNA expression levels of IL-1α (P = .04) and IL-1β (P = .01) in the cornea compared with the vehicle-treated group (Figure 4A). Although a similar downward trend was seen in levels of TNF-α, statistical significance could not be reached because of the high variance.

In the conjunctiva, topical therapy with the CCR2 antagonist significantly decreased mRNA levels of TNF-α (P = .02) and IL-1β (P = .04) compared with the vehicle-treated eyes (Figure 4B). The levels of IL-1α, although decreased, were not statistically significant.

**Comment**

Chemokines are a group of small-molecular-weight basic proteins (8-10 kDa) that represent the largest family of cytokines encoded in the human genome, constituting a group of more than 50 distinct chemokines and 20 chemokine receptors. They are secreted at sites of inflammation by resident tissue cells, resident and recruited leukocytes, and cytokine-activated endothelial cells, and they exert their functions by binding to specific G-protein–coupled cell surface receptors on target cells. The main stimuli for chemokine production are proinflammatory cytokines such as IL-1 and TNF-α and bacterial products such as lipopolysaccharides.
The chemokines are divided into the CXC subfamily, which is primarily responsible for recruitment of neutrophils, and the CC subfamily, which preferentially attracts monocytes, eosinophils, basophils, and lymphocytes with variable selectivity. Monocyte chemoattractant protein 1, also known as CCL2, an important member of the CC chemokine subfamily, is a potent chemoattractant for monocytes and binds solely to CCR2, a 7-transmembrane-spanning protein that is linked to the downstream signaling pathways.21 Wide expression of CCR2 occurs on peripheral blood monocytes, activated T cells, natural killer cells, dendritic cells, and basophils,22 and the MCP-1/CCR2 pair has been implicated in a number of chronic inflammatory diseases such as multiple sclerosis, atherosclerosis, and experimental autoimmune uveitis. Studies have shown that, when exposed to appropriate stimulus, human corneal keratocytes and endothelial cells express a high level of MCP-1,23,24 suggesting that, in inflammatory conditions under the regulation of MCP-1, CCR2-expressing cells can migrate into all layers of the cornea. Given these observations, we hypothesized that disruption of this chemokine-chemokine receptor axis with topical CCR2 antagonist could be used as a therapeutic target for controlling pathological inflammation in DED.

Dry eye disease causes ocular surface inflammation evidenced by increased levels of inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) and T lymphocytes at the ocular surface.16-18,25 Despite continuous exposure to desiccating stress and rigorous anticholinergic treatment, CCR2 antagonist–treated eyes showed reversal in corneal epithelial damage as seen by a decrease in corneal fluorescein uptake. This could have been caused by a decrease in the expression of proinflammatory cytokines because studies have shown that elevated cytokine levels in the tear film create an environment in which terminal differentiation of the ocular surface epithelium is impaired, thereby impairing the epithelial surface production of mature surface-protective molecules.26 Topical therapy with the CCR2 antagonist decreased the corneal and conjunctival expression of IL-1 and TNF-α. These cytokines have been implicated in the pathogenesis of corneal ulceration, uveitis, and corneal transplant rejection.27-29 These cytokines also are released by activated macrophages; hence, decreased macrophage infiltration in the CCR2 antagonist–treated eyes may account for decreased expression of these cytokines. Our laboratory has previously shown corneal infiltration by mature major histocompatibility complex class II–expressing antigen-presenting cells in DED.7 Consistent with
our hypothesis, blockade of MCP-1/CCR2 interaction by using CCR2 antagonist resulted in decreased CD11b⁺ monocyte infiltration into the cornea. This is important because the corneal antigen–presenting cell mobilization can significantly affect the pathogenesis and severity in various corneal immune reactions such as graft rejection and herpes keratitis.30

Studies in various animal models and in humans have shown that chronic DED is associated with T-cell infiltration in the conjunctiva.16 Chemokines are not only capable of attracting and activating T cells, but the chemokine-receptor ligation is also involved in T-cell differentiation.31,32 Our study showed significantly reduced T-cell infiltration in the conjunctiva in the eyes treated with the CCR2 antagonist. The blockade of CCR2 receptor-ligand interaction possibly modulates the immunoinflammatory response at the ocular surface by downregulating RNA transcripts for inflammatory cytokines in the conjunctival epithelium and ultimately causing decreased T-cell homing.

The chemokine system has emerged as a critical regulator of dendritic cell and lymphocytic trafficking.33 Chemokines arising under inflammatory conditions act on various chemokine receptors and attract immature dendritic cells to the affected sites. In the inflamed tissue, these immature cells pick up antigen and mature with time. During maturation, chemokine receptor expression changes; this switch results in the dendritic cells leaving the tissues and being drawn into lymphatics and, ultimately, into the T-cell–rich regions of lymph nodes.34 Our results strongly suggest that MCP-1/CCR2 dynamics are critical in the pathogenesis of DED. Beneficial effects of CCR2 blockade in DED may be primarily caused by decreased recruitment of CCR2⁺ mononuclear cells in response to local MCP-1 (CCL2) to the ocular surface, which in turn reduces the trafficking of antigen-presenting dendritic cells to the draining lymph nodes, hence decreasing the T-cell effector responses. In addition, blockade of the MCP-1/CCR2 axis may inhibit recruitment of CCR2⁺ T cells to the eye in response to local MCP-1 and therefore may disrupt the ongoing cycle of T-cell recruitment, reactivation, and effector responses at the ocular surface.

In conclusion, treatment with the topical CCR2 antagonist in our DED mouse model led to significant improvement in the dry eye state at the clinical, molecular, and cellular levels. The reversal of clinical signs and underlying inflammatory changes by using a topical CCR2 antagonist in the present study suggests that CCR2 antagonism could hold promise as a therapeutic modality in DED. However, the chemokine system is characterized by significant redundancy, pleiotropy, and differences among different species, which complicates development of new therapeutics. It is hoped, however, that

Figure 3. Enumeration of conjunctival T cells. A, Representative images of conjunctival cross sections immunostained for CD3 (red) and nucleus (blue) of untreated and vehicle-treated eyes and eyes treated with a topical chemokine receptor 2 (CCR2) antagonist (T cells are marked by arrows). Ep indicates epithelial layer; St, stromal layer. B, Treatment with topical CCR2 antagonist significantly decreased the total number of conjunctival T cells compared with untreated and vehicle-treated eyes. Bars represent mean values; limit lines, SEM.
more effective therapeutics will be found in the near future as the intricacies of the chemokine system are better understood.

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Correspondence: Reza Dana, MD, MSc, MPH, Schepens Eye Research Institute, 20 Stanford St, Boston, MA 02114 (reza.dana@schepens.harvard.edu).

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Figure 4. Real-time polymerase chain reaction analysis showing transcript levels of tumor necrosis factor α (TNF-α), interleukin 1α (IL-1α), and IL-1β in the cornea and conjunctiva. Treatment with a topical chemokine receptor 2 (CCR2) antagonist significantly decreased relative expression of IL-1α and IL-1β in the cornea (A) and TNF-α and IL-1β in the conjunctiva (B) compared with the vehicle-treated group. The dotted line represents results as the intricacies of the chemokine system are better understood.

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Correspondence: Reza Dana, MD, MSc, MPH, Schepens Eye Research Institute, 20 Stanford St, Boston, MA 02114 (reza.dana@schepens.harvard.edu).

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