Chemokine Receptor CCR5 Expression in Conjunctival Epithelium of Patients With Dry Eye Syndrome

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Objective: To characterize chemokine receptor CCR5 expression on the conjunctival epithelium in dry eye syndromes.

Methods: Conjunctival impression cytology samples were obtained from normal subjects (n=15) and patients with dry eye syndrome (n=45). Cells were harvested from impression cytology samples, and flow cytometry was performed to quantitatively analyze the cell surface expression of chemokine receptor CCR5. Characterization of CCR5-positive cells was done by 2-color flow cytometry using fluorescein-conjugated anti-CCR5 and phycoerythrin-conjugated anti-CD45 antibodies (where CD45 is a marker for bone marrow–derived cells). To study CCR5 messenger RNA transcripts, real-time polymerase chain reaction was done on RNA isolated from the impression cytology samples of normal subjects (n=5) and patients with dry eye syndrome (n=14).

Results: We found significant up-regulation in cell surface expression of CCR5 in patients with both aqueous tear–deficient and evaporative forms of dry eye syndrome (*P*<.001). The real-time polymerase chain reaction results (for messenger RNA) corroborated the flow cytometry data (for protein). The majority of the cells expressing CCR5 were non–bone marrow–derived resident epithelial cells of the conjunctiva.

Conclusion: Our findings suggest that CCR5 up-regulation is significantly associated with dry eye syndrome–associated ocular surface disease.

Clinical Relevance: Chemokine receptor CCR5 or its ligands may serve as useful targets for modulation of tissue immunoinflammatory responses in dry eye syndromes.

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THE OCULAR SURFACE IS CONstantly confronted with a large number of pathogens and immunogenic antigens; however, the presence of a healthy tear film shields the eye from these environmental challenges and acts as a deterrent against frequent attacks of inflammation. Dry eye syndrome, or keratoconjunctivitis sicca, is a heterogeneous group of ocular surface disorders characterized by an impairment in the homeostatic balance between the tear film and the ocular surface epithelium, resulting in a diminished ability to respond normally to environmental challenges leading to ocular surface damage. Millions of people, primarily women, in the United States alone have this disorder.[1] Patients with dry eye experience symptoms of chronic ocular dryness and discomfort that can be debilitating.[2]

Dry eye disorders have been classified into 2 major categories: aqueous tear–deficient (ATD) dry eye and evaporative dry eye.[3] Sjogren syndrome, an autoimmune disorder characterized by altered lacrimal and salivary gland function, is one of the leading causes of the ATD form of dry eye.[4] The ATD form is also seen in patients with chronic graft-vs-host disease, which is a major complication of hematopoietic stem cell and bone marrow transplantation.[5] Meibomian gland dysfunction (MGD), which is associated with disturbances in the lipid layer of the tear film resulting in excessive tear evaporation, causes evaporative forms of dry eye.[3,6] Recent studies[7-9] have shown that significant dry eye syndrome of all types is associated with a variable degree of inflammation.

Chemokines (*chemo*tactic cyto*kines*) are a group of relatively small–molecular weight (7-12–kd) structurally related proteins that are produced by a variety of cell types and are divided according to structure into 4 different subfamilies: CXC (α), CC (β), C (γ), and CX3C (δ) chemokines.[10] The human chemokine system comprises more than 50 distinct chemokines and 20 chemokine re-

ceptors.[11] Chemokines are capable of attracting and activating T cells and coordinating dendritic cell function.[12] Additionally, chemokine receptor ligation is involved in T-cell differentiation into helper T-cell 1 (T_H1) and T_H2 cells.[13,14] Accordingly, chemokine receptors CCR5 and CXCR3 are expressed primarily on T_H1 cells[15,16] whereas CCR3 and CCR4 are expressed primarily by T_H2 cells.[17,18] Although there is considerable evidence for the role of chemokines and chemokine receptors in the pathogenesis of many acute and chronic inflammatory diseases (such as asthma and rheumatoid arthritis),[19] very little is known about chemokine receptor-ligand interactions in ocular surface inflammation observed in dry eye syndrome.

Experimental evidence suggests that dry eye syndrome is associated with the T_H1 type of immunoinflammatory response.[20,21] Since CCR5 is the principal chemokine receptor preferentially expressed by T_H1 cells, we conducted the current study to investigate the expression of CCR5 in the conjunctival epithelium of patients with ATD and evaporative forms of dry eye syndrome. Using flow cytometric and real-time polymerase chain reaction (PCR) analyses, we found the following: (1) chemokine receptor CCR5 is constitutively expressed at low levels on the conjunctival epithelium of normal subjects; (2) CCR5 expression is significantly up-regulated in patients with ATD forms as well as evaporative forms of dry eye syndrome; and (3) the majority of cells expressing the CCR5 receptor on the conjunctival epithelium are resident epithelial cells, suggesting a role of ocular surface epithelial cells in modulating immunoinflammatory responses in dry eye syndromes.

METHODS

The study was conducted in compliance with good clinical practice guidelines, institutional review board regulations, informed consent regulations, and the tenets of the Declaration of Helsinki. All of the subjects enrolled in the study were adults older than 18 years who were able to give informed consent.

SUBJECT SELECTION

Patients with dry eye syndrome (n=45) were enrolled at the Cornea Clinic of Massachusetts Eye and Ear Infirmary, Boston, and Campus Bio-medico, University of Rome, Rome, Italy. Normal subjects (n=15) were enrolled at these centers and at the Schepens Eye Research Institute, Boston. All of the prospective subjects completed an institutional review board–approved questionnaire regarding the presence and frequency of symptoms of dry eye, use of dry eye therapy, other current medications, and history of ocular allergies. Detailed ophthalmologic examination was performed, including the Schirmer test (with and without anesthesia), visual acuity, biomicroscopy, tear break-up time, corneal staining with sodium fluorescein, and meibomian gland assessment.[6]

Patients with dry eye who were enrolled in the study included patients with the ATD form of dry eye as well as those with MGD, which is a prototype of evaporative dry eye. The inclusion criteria for the ATD group were the following: (1) Schirmer reading of less than 10 mm without anesthesia; (2) diagnosis of Sjogren syndrome according to the modified criteria of Vitali et al[22]; or (3) dry eye associated with chronic graft-vs-host disease. Inclusion criteria for the MGD group were the following: (1) tear break-up time of less than 5 seconds; (2)

presence of meibomian gland disease; and (3) Schirmer reading greater than 10 mm without anesthesia. The control group (normal subjects) had no evidence of symptomatic or clinical dry eye disease. Exclusion criteria were a history of recent contact lens wear (within 1 month), recent ocular or eyelid surgery (within the previous 6 months), and ocular allergies.

CONJUNCTIVAL IMPRESSION CYTOLOGY

To obtain conjunctival cells for flow cytometry, impression cytology specimens were collected as previously described[23] from all of the subjects enrolled in the study. Briefly, eyes were anesthetized with topical 0.5% proparacaine hydrochloride drops. A sterile piece of polyethersulfone filter (13.0×6.5 mm) was applied to the inferior bulbar conjunctiva without exerting pressure. The filter membrane was removed after a few seconds, and membranes from both eyes were dipped into a tube containing 1.5 mL of cold phosphate-buffered saline with 0.05% paraformaldehyde. Tubes were stored at 4°C and processed within 1 week for flow cytometry. Impression cytology specimens for RNA isolation were collected from subjects enrolled at Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute only. These samples could not be obtained from investigators at the University of Rome owing to international shipping restrictions. Sterile nitrocellulose filter paper (10 mm in diameter) was used to collect impression cytology samples from superotemporal bulbar conjunctiva. Filters from both eyes were stored in tubes containing RNA extraction reagent (TRIzol; Invitrogen Corp, Carlsbad, Calif) at –80°C until further analysis.

ANALYSIS OF CELL SURFACE EXPRESSION OF CCR5 PROTEIN

Harvesting Cells for Flow Cytometry

Conjunctival cells were harvested from the impression cytology filters, which were dipped in 0.05% paraformaldehyde, by gently shaking the tubes for 20 to 30 minutes to extract as many cells as possible without trauma. The cells were then centrifuged at 1600 rpm for 5 minutes and resuspended in phosphate-buffered saline with 1% bovine serum albumin (Sigma-Aldrich Corp, St Louis, Mo). The cells were counted and immunostained for flow cytometric analysis. Cell numbers obtained from impression cytology samples ranged from 10 000 to 120 000 cells. Human peripheral blood mononuclear cells (PBMCs) were used as staining controls. Peripheral blood was obtained from 2 healthy volunteers, and PBMCs were isolated by the gradient centrifugation technique.[24] Using PBMCs, we determined that prefixation with 0.05% paraformaldehyde did not influence the level of CCR5 on the cell surface.

Monoclonal Antibodies

Fluorescein-conjugated monoclonal antibody against CCR5 as well as isotype IgG control monoclonal antibody were obtained from R&D Systems, Minneapolis, Minn. Phycoerythrin-conjugated anti-CD45 and the corresponding isotype control monoclonal antibodies were obtained from BD Pharmingen, San Diego, Calif.

Immunostaining Procedures and Flow Cytometric Analysis

Cells were incubated with fluorescein-conjugated anti-CCR5 antibody (clone number 45549.111) in a final dilution of 1:50 for 30 minutes at 4°C. Cell suspensions were washed in phos-

phate-buffered saline with 1% bovine serum albumin and then resuspended in phosphate-buffered saline for flow cytometric analysis. Flow cytometry was performed using a Coulter Epics-XL flow cytometer with System Work II software (Beckman Coulter, Inc, Fullerton, Calif) for data analysis. Cells were gated using forward-scatter and side-scatter characteristics. Logarithmic fluorescence histograms were obtained, and results were expressed as percentages of positive cells over and above the negative isotype control–stained cells.

To characterize the CCR5-positive cells, we performed 2-color flow cytometric analysis using fluorescein-conjugated anti-CCR5 and phycoerythrin-conjugated anti-CD45 antibodies. Since CD45 is a panleukocyte marker and is expressed on all bone marrow–derived cells, the double-positive population (CCR5$^+$CD45$^+$) is indicative of CCR5-positive cells that are bone marrow–derived. The resident epithelial cells of the conjunctiva are CD45 negative.

Analysis of CCR5 Transcripts by Real-time PCR

Total RNA was isolated from impression cytology samples using the extraction reagent TRIzol as previously described.[25] One microgram of total RNA was reverse transcribed to complementary DNA using a first-strand synthesis system for reverse transcription PCR (Superscript; Invitrogen Corp) according to the manufacturer's instructions. Real-time PCR amplification of complementary DNA was performed using an ABI Prism 7900 HT sequence detection system instrument (Applied Biosystems, Foster, Calif). Primers and probes used for human CCR5 (TaqMan Gene Expression Assays; Applied Biosystems) and glyceraldehyde-3-phosphate dehydrogenase (human *GAPD* endogenous control, VIC-MGB–labeled probe, primer limited; Applied Biosystems) gene amplification were obtained. The comparative threshold cycle method was used for analysis of CCR5 mRNA expression using glyceraldehyde-3-phosphate dehydrogenase as the endogenous control. The threshold cycle value is the fractional cycle number at which the amount of amplified target reaches a fixed threshold of detectable fluorescence, and it is a reliable measurement of starting copy numbers of mRNA in a real-time PCR amplification process. Samples were assayed in triplicate in a total volume of 50 µL using thermal cycling conditions of 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A nontemplate control was included in all of the experiments to confirm lack of DNA contamination of reagents used for amplification.

STATISTICAL ANALYSES

Based on the normality of the data distribution, the *t* test or Mann-Whitney *U* test was used for statistical comparison of assay results between 2 groups. An analysis of variance test was applied to calculate differences among 3 or more groups. All of the results are expressed as the mean±SEM; *P*<.05 was considered statistically significant.

RESULTS

ANALYSIS OF CCR5 PROTEIN EXPRESSION BY FLOW CYTOMETRY

Impression cytology samples containing low cell numbers (<10 000) (n=7), a nonhomogeneous population of cells (n=2), and cell aggregates (n=3) were discarded. Flow cytometric analysis results described here are from conjunctival impression cytology samples obtained from 25 patients with the ATD form of dry eye (22 women, 3 men), 13 patients with MGD (9 women, 4 men), and 10 normal subjects (6 women, 4 men). The ATD group included 9 patients with Sjogren syndrome, 6 patients with chronic graft-vs-host disease, and 10 patients with tear deficiency due to other unidentified causes (idiopathic "primary" dry eye) who were grouped in a non-Sjogren non–graft-vs-host disease dry eye group. The age range of normal subjects (n=10) was 25 to 67 years (mean±SEM age, 38.40±4.25 years), and that of patients with dry eye (n=38) was 26 to 80 years (mean±SEM age, 52.42±2.14 years).

Flow cytometry was performed to quantitatively analyze the cell surface expression of CCR5 on conjunctival cells in patients with dry eye and in normal subjects. In control experiments, we found positive expression of CCR5 on PBMCs. The mean±SEM percentage of cells positive for CCR5 in PBMCs from 2 separate experiments was 15.50%±1.50%. Using flow cytometry on impression cytology specimens, we found that the mean±SEM percentage of cells positive for CCR5 in the patients with dry eye (66.92%±2.38%) was significantly higher compared with that in the normal subjects (35.30%±5.54%) (*P*<.001, *t* test). Histograms of percentages of cells expressing CCR5 from a representative patient with dry eye and a normal subject are shown in **Figure 1**. We further analyzed CCR5 expression separately in patients with ATD and evaporative forms of dry eye syndrome. We found significantly higher percentages of cells expressing CCR5 in both ATD (mean±SEM, 65.16%±2.99%) and MGD (mean±SEM, 70.31%±3.91%) groups of patients with dry eye compared with normal subjects (35.30%±5.54%) (*P*<.001, analysis of variance). However, there was no significant difference in the mean percentage of CCR5 expression between the patients with ATD and MGD forms (*P*>.50). **Figure 2** shows the groupwise distribution of mean percentages of CCR5 expression in the various subgroups of ATD forms of dry eye syndrome, in the MGD group, and in the normal subjects. Significant up-regulation of CCR5 is seen in all of the subgroups of patients with dry eye compared with the normal subjects (analysis of variance).

CHARACTERIZATION OF CCR5-POSITIVE CELLS BY 2-COLOR FLOW CYTOMETRY

To determine the bone marrow derivation of the cells that express CCR5, we performed 2-color flow cytometry using anti-CCR5 and anti-CD45 antibodies since CD45 is a marker for bone marrow–derived cells. The percentage of CCR5$^+$CD45$^+$ cells (CCR5-positive bone marrow–derived cells) ranged from 3% to 10%. The mean±SEM percentage of CCR5$^+$CD45$^+$ cells was 4.50%±1.50% in normal subjects and 6.50%±3.50% in the patients with dry eye. Indeed, the significant majority of cells were CCR5$^+$CD45$^-$ (CCR5-positive non–bone marrow–derived) resident cells of the conjunctival epithelium (mean±SEM percentage of CCR5$^+$CD45$^-$ cells, 60.75%±7.69%; range of percentages of CCR5$^+$CD45$^-$ cells, 47%-76%). **Figure 3** shows 2-color flow cytometric analysis of CCR5 on conjunctival cells and PBMCs.
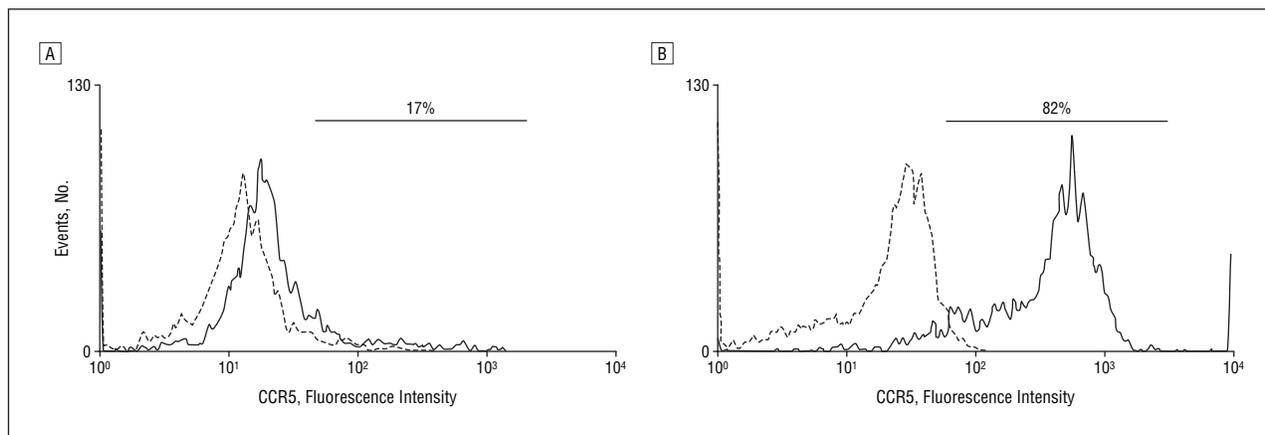
Figure 1. Flow cytometric analysis of CCR5 expression on conjunctival cells (solid lines) obtained from impression cytology samples from a normal subject (A) and a patient with dry eye (B). The percentage of cells positive for CCR5 is indicated above the marker lines. Dashed lines indicate isotype-negative control.

RELATIVE EXPRESSION OF CCR5 TRANSCRIPT BY REAL-TIME PCR

Real-time PCR was done as a second quantitative method to confirm CCR5 up-regulation in dry eye syndrome. Two populations of subjects were compared: patients with dry eye (n=14) and normal subjects (n=5). Owing to the limited number of impression cytology samples available for RNA analysis, the samples used for real-time PCR assay and those used for flow cytometric analysis were not from same subjects. **Figure 4**A shows typical plots generated by real-time PCR amplification of the *CCR5* gene showing threshold cycle values from a representative patient with dry eye and a normal subject. The amount of *CCR5* gene in each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase by subtracting the threshold cycle of glyceraldehyde-3-phosphate dehydrogenase from that of *CCR5* (data not shown). The results are shown as the fold difference in CCR5 mRNA expression in patients with dry eye relative to the amount that is in normal subjects. The mean±SEM relative expression of CCR5 mRNA in the dry eye group (2.02±0.38) was significantly higher compared with that in normal subjects (1.03±0.13) ($P<.05$) (Figure 4B).

Figure 2. Graph showing mean percentages of CCR5 expression in normal subjects (n=10) and in different groups of patients with dry eye. SS indicates Sjogren syndrome (n=9); GVHD, graft-vs-host disease (n=6); NSNG, non–Sjogren syndrome non-GVHD (n=10); MGD, meibomian gland dysfunction (n=13); and error bars, SEM. *$P<.001$. †$P<.05$.

COMMENT

In this study, we investigated the ocular surface of patients with ATD and evaporative forms of dry eye for expression of the chemokine receptor CCR5 and compared these data with those of normal subjects who did not show any clinically evident manifestations of dry eye. We used the technique of conjunctival impression cytology,[26] which is a noninvasive method of collecting cells of the conjunctival epithelium. For quantitative analysis of CCR5 expression, we did flow cytometry (protein) and real-time PCR (mRNA) assays on impression cytology specimens. We found that the conjunctival epithelium expresses CCR5 at low levels in normal subjects, and this expression is significantly up-regulated in both of the principal (ATD and evaporative) forms of dry eye syndrome. The majority of cells expressing the CCR5 receptor on the conjunctival epi-
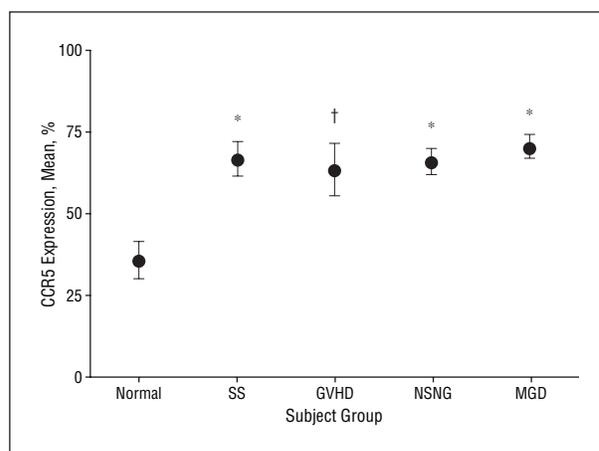
thelium were non–bone marrow–derived resident epithelial cells. Our findings suggest that CCR5 up-regulation is significantly associated with dry eye syndrome–associated ocular surface disease.

Chemokine receptor CCR5 was first identified as a receptor for chemokine ligands CCL3, CCL4, and CCL5, which are also known as macrophage inflammatory protein 1α, macrophage inflammatory protein 1β, and RANTES (regulated on activation, normal T cell expressed and secreted), respectively.[27] Chemokine receptor CCR5 is expressed on the surface of bone marrow–derived cells such as monocytes, immature dendritic cells (antigen-presenting cells) and certain subsets of T cells,[16,27,28] and resident cells in various tissues.[29] It is known that CCR5 plays a critical role in T_H1-mediated responses.[15,16] Thus, our findings are consistent with previous observations that dry eye syndrome is associated with T_H1-mediated inflammation.[20,21] Studies[30] performed on conjunctival biopsies (which include deeper subepithelial layers) have shown increased expression of CD11a and CD23 on T cells, indicating an activated phe-
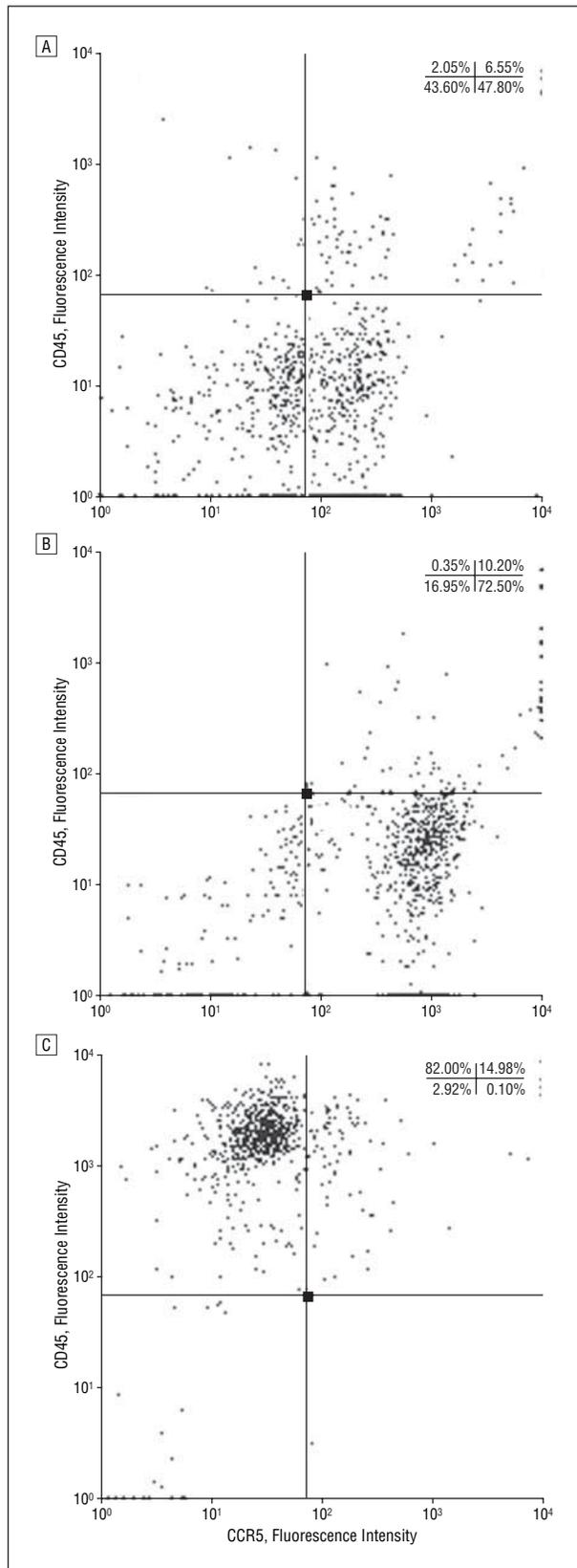
Figure 3. Two-color flow cytometric analysis of CCR5 expression on conjunctival cells from a normal subject (A) and a patient with dry eye (B), where the majority of CCR5⁺ cells are CD45⁻ resident epithelial cells. C, Control data from CD45⁺ peripheral blood mononuclear cells. The numbers appearing in the upper right quadrants indicate percentages in the respective quadrants.

notype, in patients with both Sjogren and non-Sjogren keratoconjunctivitis sicca. Increased expression of CCR5 in the patients with dry eye points to a likely role for CCR5 in the process of mononuclear cell mobilization and, possibly, T-cell activation in the ocular surface inflammation in dry eye syndrome.

Our findings of significant and comparable up-regulation of CCR5 in both ATD and evaporative dry eye indicate that CCR5 plays a role in ocular surface inflammation in dry eye syndrome irrespective of the causality. Chemokines are known to be produced by a variety of cell types in response to inflammatory cytokines, and they operate through regulated expression of specific chemokine receptors.[31] Studies have shown increased RNA transcripts of inflammatory cytokines in the conjunctival epithelium of patients with Sjogren syndrome.[7] Additionally, in vitro stimulation of a human conjunctival epithelial cell line with inflammatory mediators has been shown to produce RANTES.[32] To our knowledge, no studies have been performed so far to describe CCR5 receptor-ligand interactions in human dry eye disease. However, increasing levels of chemokine ligand RANTES with concomitant increase in expression of CCR5 receptor has been described in the lacrimal glands in an animal model of Sjogren syndrome.[33] We speculate that the up-regulated expression of chemokine receptor CCR5 in dry eye syndrome may be secondary to both ocular surface inflammation and the resultant release of inflammatory cytokines that in turn may up-regulate the expression of chemokine ligands specific for CCR5 receptor.

Using 2-color flow cytometry, we found a small population of CCR5-positive cells that were bone marrow–derived (CD45 positive), although their number in the patients with dry eye was not significantly different from that in the normal subjects. However, the majority of CCR5-positive cells were CD45 negative (non–bone marrow–derived) resident epithelial cells of the conjunctiva. To our knowledge, this is the first study to demonstrate that resident epithelial cells of the conjunctiva express chemokine receptor CCR5, and this expression is markedly up-regulated in both of the principal types of dry eye syndrome. Our findings suggest that conjunctival epithelium is not simply the target of the disease process but may also participate in the local immune response in dry eye syndrome. In the presence of proinflammatory cytokines, certain nonimmune (including epithelial) cells can potentially function as "nonprofessional" antigen-presenting cells as reflected by their up-regulated expression of major histocompatibility complex class II antigen and costimulatory molecules.[34] Recently, Zhan et al[35] reported up-regulation in expression of HLA-DR (human major histocompatibility class II) as well as CD80 on the human conjunctival epithelial cells after treatment with interferon γ. Additionally, it has been shown that by binding to their cognate receptors on antigen-presenting cells, CCR5 ligands can induce expression of polarizing cytokines such as interleukin 12,[36] which can promote T-cell differentiation to T_H1 cells.[37] Although we have no direct data to prove it at this point, we can speculate that in the presence of the inflammatory microenvironment of dry eye syndrome, the epithelial cells of the conjunctiva acquire
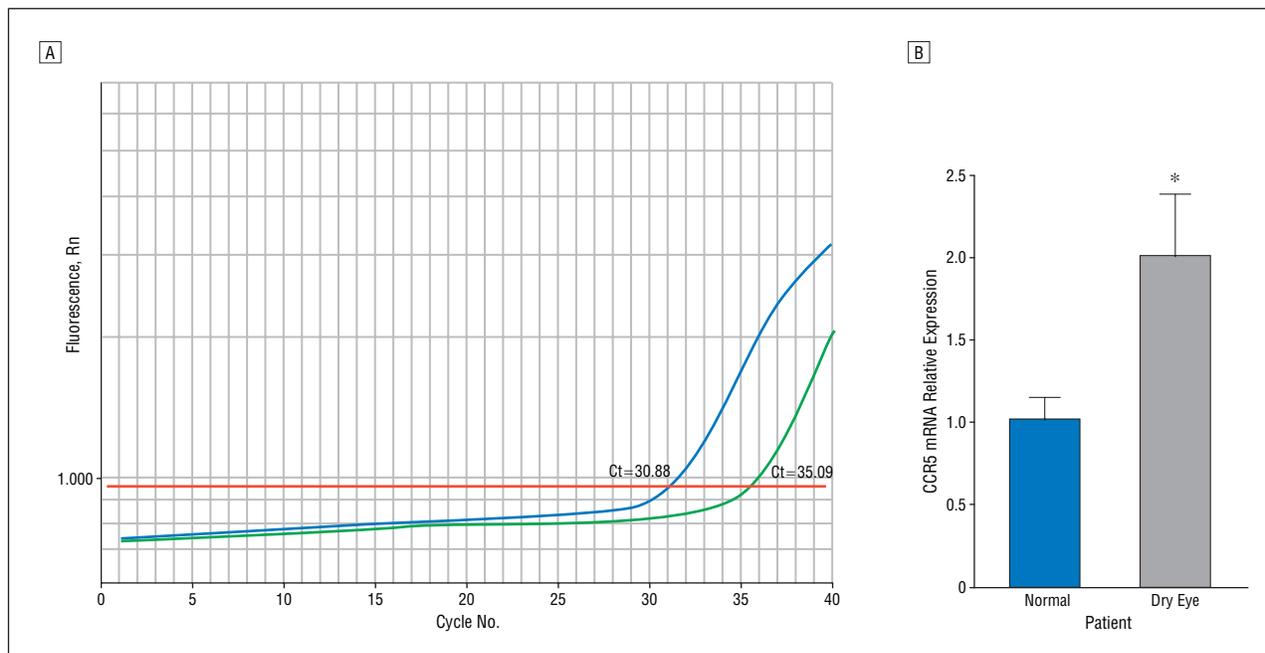
Figure 4. Real-time PCR analysis of CCR5 from RNA isolated from conjunctival impression cytology samples. A, Amplification plot showing *CCR5* gene amplification from a patient with dry eye (blue curve) and a normal subject (green curve). Ct indicates the threshold cycle (red line). B, Mean relative expression of CCR5 messenger RNA (mRNA) in normal subjects and patients with dry eye. Error bars indicate SEM. *$P<.05$, Mann-Whitney U test.

cell surface phenotypic characteristics of antigen-presenting cells, including chemokine receptor expression that may hence contribute to T-cell differentiation.

Our study has shown that CCR5 protein and transcript overexpression is seen in a variety of dry eye syndromes. Thus, it is possible to hypothesize that by blocking CCR5 receptor-ligand interaction, it may be possible to modulate the manifestations of immunoinflammatory responses at the ocular surface in dry eye syndrome. Further studies are needed to precisely define the role of CCR5 receptor and its ligands in mediating ocular surface immune responses and their functional relevance in dry eye syndrome.

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