

# Expression of Th-1 Chemokines and Chemokine Receptors on the Ocular Surface of C57BL/6 Mice: Effects of Desiccating Stress

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**PURPOSE.** To evaluate the effects of desiccating ocular surface stress on the expression of chemokines and their receptors by the corneal epithelium and conjunctiva of C57BL/6 and BALB/c mice.

**METHODS.** Experimental dry eye was created in C57BL/6 and BALB/c mice. The concentrations of macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), MIP-1 $\beta$ , monokine induced by interferon (MIG)- $\gamma$ , and interferon- $\gamma$ -inducible protein (IP)-10 in the corneal epithelia and conjunctiva were measured by a multiplex immunobead assay. Expression of MIP-1 $\alpha$ ; MIP-1 $\beta$ ; regulated on activation, normal T-cell expressed and secreted (RANTES), MIG, IP-10; monocyte chemoattractant protein (MCP)-3; eotaxin-1; CCR5; CXCR3; and CCR3 in the cornea and conjunctiva were evaluated by real-time PCR and immunostaining.

**RESULTS.** Desiccating stress significantly increased concentrations of MIP-1 $\alpha$ , MIP-1 $\beta$ , IP-10, and MIG proteins in the corneal epithelium and conjunctiva of C57BL/6 mice. Furthermore, it increased levels of MIP-1 $\alpha$ , MIP-1 $\beta$ , and CCR5 transcripts in the cornea and conjunctiva and RANTES, MIG, IP-10, and CXCR3 transcripts in the conjunctiva of C57BL/6 mice. In contrast, levels of MCP-3, eotaxin-1, and CCR3 transcripts increased in both tissues of BALB/c mice. In situ immunodetection of chemokines and their receptors was similar to their pattern of gene expression.

**CONCLUSIONS.** Specific patterns of Th-1 and -2 chemokines and their receptors are induced in the mouse ocular surface by desiccating stress in a strain-related fashion. Desiccating stress potentially stimulates the expression of Th-1 cell-attracting chemokines and chemokine receptors on the ocular surface of C57BL/6 mice. (*Invest Ophthalmol Vis Sci.* 2007;48:2561–2569) DOI:10.1167/iovs.07-0002

Dry eye has been demonstrated to cause inflammation on the ocular surface, evidenced by increased levels of inflammatory cytokines (interleukin [IL]-1, IL-6, IL-8, and tumor

necrosis factor [TNF]- $\alpha$ ) in the tear fluid and conjunctival epithelium, increased expression of immune activation and adhesion molecules (HLA-DR and intercellular adhesion molecule-1) by the conjunctival epithelium and an increased number of T lymphocytes in the conjunctiva.<sup>1–6</sup> Another pathologic finding in dry eye is an increased concentration and activity of matrix metalloproteinases (MMPs), such as MMP-9 in the tear fluid and corneal epithelium and increased apoptosis or programmed cell death in the ocular surface epithelium.<sup>6–11</sup> The desiccating and hyperosmolar stress in dry eye has been shown to activate mitogen-activated protein kinase (MAPK) signaling pathways in the ocular surface epithelium. These cellular signaling pathways regulate the expression of cytokines, chemokines, MMPs, and epithelial differentiation proteins, such as involucrin.<sup>12–16</sup>

Chemokines are 8- to 10-kDa secreted proteins that play a key role in the recruitment of immune and inflammatory cells to sites of inflammation. Chemokines are produced by a variety of cell types and are subclassified into four groups, based on their structures: CXC ( $\alpha$ ), CC ( $\beta$ ), C ( $\gamma$ ), and CXXXC ( $\delta$ ).<sup>17</sup> Chemokines shows specificity in attracting cell subtypes based on their complement of chemokine receptors. For example, the receptors CCR5 and CXCR3 are expressed on T-helper type 1 (Th-1) cells and natural killer (NK) cells that participate in cell-mediated immune response, whereas others such as CCR3 and CCR4 are found on eosinophils, basophils, and Th-2 lymphocytes, which modulate allergic response.<sup>18,19</sup>

Because T cells are recruited to the conjunctiva in response to the stress of dry eye, chemokines and their receptors may participate in this immunopathologic response.<sup>20,21</sup> We hypothesize that desiccating stress increases the expression of chemokines and their receptors in the ocular surface tissues of mice relative to the naïve condition. Although increased expression of CCR5 in conjunctival epithelium has been reported in patients with dry eye, a comprehensive evaluation of chemokines and their receptors on the ocular surface in dry eye has not been performed.<sup>21,22</sup>

The purpose of the present study was to investigate the expression of CCR5 and its ligands, macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ /CCL3), MIP-1 $\beta$ /CCL4, and RANTES/CCL5; CXCR3 and its ligands, monokine induced by interferon- $\gamma$  (MIG/CXCL9) and interferon- $\gamma$ -inducible protein 10 (IP-10/CXCL10); and CCR3 and its ligands, monocyte chemoattractant protein 3 (MCP3/CCL7) and eotaxin-1/CCL11, in the cornea and conjunctiva after exposure to desiccating ocular surface stress in C57BL/6 and BALB/c mouse strains that have been documented to manifest different immune responses to infectious and inflammatory stimuli.<sup>23,24</sup>

## METHODS

### Mouse Model of Dry Eye

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the stan-

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TABLE 1. Oligonucleotide Primers Used for Real-Time PCR

Gene Name	Symbol	Assay ID*
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	Mm99999915_gl
CC chemokine receptor 5	<i>CCR5</i>	Mm01216171_ml
CXC chemokine receptor 3	<i>CXCR3</i>	Mm00438269_ml
CC chemokine receptor 3	<i>CCR3</i>	Mm00515543_ml
Macrophage inflammatory protein 1 $\alpha$	<i>MIP-1<math>\alpha</math>/CCL3</i>	Mm00441258_ml
Macrophage inflammatory protein 1 $\beta$	<i>MIP-1<math>\beta</math>/CCL4</i>	Mm00443111_ml
Regulated upon activation, normal T-cell expressed and secreted	<i>RANTES/CCL5</i>	Mm01302428_ml
Monokine induced by interferon- $\gamma$	<i>MIG/CXCL9</i>	Mm00434946_ml
Interferon- $\gamma$ -inducible protein 10	<i>IP-10/CXCL10</i>	Mm00444235_ml
Monocyte chemoattractant protein 3	<i>MCP-3/CCL7</i>	Mm00443113_ml
Eotaxin-1	<i>Eotaxin-1/CCL11</i>	Mm00441238_ml

\* Identification number from Applied Biosystems.

dards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental dry eye (EDE) was induced in 6- to 8-week-old C57BL/6 and BALB/c mice of both genders (Jackson Laboratories, Bar Harbor, ME), by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) in alternating hindquarters four times a day (8 AM, 11 AM, 2 PM, and 5 PM) with exposure to an air draft and <40% ambient humidity for 18 hours per day, as previously reported.<sup>15,25</sup> Mice were euthanized after 5 or 10 days (5D and 10D in figures and tables) of this treatment. A group of age- and gender-matched mice that did not receive any treatment to induce dry eye served as untreated (UT) control subjects.

### Multiplex Immunobead Cell-Signaling Assay

The levels of MIP-1 $\alpha$ , MIP-1 $\beta$ , MIG, and IP-10 in the corneal epithelium and conjunctiva were measured with a sensitive, fluorescent multiplex immunobead assay (Luminex; BioSource-Invitrogen, Carlsbad, CA). Corneal epithelia and conjunctival tissues were collected at each time point, and 10 samples of each tissue (cornea or conjunctiva) from each mouse strain were pooled in extraction reagent (Tissue Extraction Reagent I; BioSource-Invitrogen) supplemented with a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN) for 15 minutes. The cell extracts were centrifuged at 14,000g for 15 minutes at 4°C, and the supernatants were stored at -80°C before use. The protein concentration of the cell extracts was measured with a protein assay kit (Micro BCA; Pierce, Rockford, IL).

Each sample (10  $\mu$ g/25  $\mu$ L) was added to wells of a 96-well plate and incubated overnight at 4°C in the dark, with 25  $\mu$ L of 1 $\times$  beads coupled to mouse chemokine-specific antibodies. Serial dilutions of each chemokine were also added to wells in the same plate, to generate a standard curve. The next day, the beads were washed and mixed with 25  $\mu$ L of 1 $\times$  biotinylated secondary chemokine antibody mixture for 1 hour at room temperature, followed by washing and subsequent incubation with 25  $\mu$ L of streptavidin-phycoerythrin for 30 minutes (both steps performed in the dark). After a final wash, the wells were resuspended in 100  $\mu$ L of assay buffer. Chemokine concentrations were measured with a multiplex analysis system (model 100 IS 2.3; Luminex, Austin, TX). Chemokine concentrations were measured in samples from four different sets of experiments, and the results were averaged. The minimum detectable concentrations of MIP-1 $\alpha$ , MIP-1 $\beta$ , MIG, and IP-10 were 15, 25, 15, and 40 pg/mL, respectively.

### RNA Isolation and Real-Time PCR

Total RNA was isolated from the corneal epithelia or conjunctiva that were collected from 10 eyes and pooled at each time point by using acid guanidium thiocyanate-phenol-chloroform extraction.<sup>26</sup> The RNA concentration was measured by its absorption at 260 nm, and the samples were stored at -80°C until use.

First-strand cDNA was synthesized from 1  $\mu$ g of total RNA with random hexamers and M-MuLV (Moloney murine leukemia virus) reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Piscataway, NJ) as previously described.<sup>14,15,27</sup> Real-time PCR was performed by using specific probes (*TaqMan* MGB; Applied Biosystems, Inc. [ABI], Foster City, CA; Table 1), with a master mix (*TaqMan* CR Universal PCR Master Mix AmpErase UNG; ABD), in a commercial thermocycling system (Mx3005P QPCR System; Stratagene, La Jolla, CA), according to the manufacturer's recommendations. Assays were performed in duplicate in each experiment and were repeated in four sets of mice. A nontemplate control was included in all the experiments to evaluate DNA contamination of the reagent used. The *GAPDH* gene was used as an endogenous reference for each reaction, to correct for differences in the amount of total RNA added. The results of quantitative PCR were analyzed by the comparative  $C_T$  method,<sup>14</sup> where target change =  $2^{-\Delta\Delta C_T}$ . The cycle threshold ( $C_T$ ) was determined using the primary (fluorescent) signal as the cycle at which the signal crosses a user-defined threshold. The results were normalized by the  $C_T$  of *GAPDH* and the relative mRNA level in the C57BL/6 untreated group was used as the calibrator.

### Immunodetection of Chemokines and Chemokine Receptors

Immunofluorescent staining to evaluate expression of chemokine and chemokine receptor proteins in corneal and conjunctival tissue sections was performed by a previously reported method.<sup>28</sup>

Eyes and adnexa from three mice in each group (four different sets of experiments) were surgically excised, embedded in OCT compound (VWR, Suwanee, GA), and flash frozen in liquid nitrogen. Sagittal 8- $\mu$ m sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at -80°C. The tissue sections (two slides per animal) for immunofluorescent staining were fixed in acetone at -20°C (for CCR5 and CXCR3) or 4% paraformaldehyde at 4°C (for MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, MIG, IP-10, MCP-3, eotaxin-1, and CCR3) for 10 minutes. After blocking with 20% normal horse (for MIP-1 $\alpha$ , MIP-1 $\beta$ , MIG, IP-10, MCP-3, CCR5, and CXCR3) or goat (for RANTES, eotaxin-1, and CCR3) serum in PBS for 45 to 60 minutes, primary antibodies against the chemokines or chemokine receptors listed in Table 2 were applied, and the sections were incubated for 1 hour at RT. Secondary antibodies, Alexa-Fluor 488-conjugated donkey anti-goat IgG, Alexa-Fluor 488-conjugated goat anti-rabbit IgG, or Alexa-Fluor 488-conjugated goat anti-rat IgG (1:300 dilution) were then applied, and the sections were incubated in a dark chamber for 1 hour, followed by counterstaining with propidium iodide (PI; 2  $\mu$ g/mL in PBS) for 10 minutes. Secondary antibody alone and goat anti-mouse isotype, rabbit anti-mouse isotype, or rat anti-mouse isotype (BD PharMingen, San Jose, CA) controls were also performed.

TABLE 2. Primary Anti-mouse Antibody Used for Immunofluorescent Staining and Immunohistochemistry

Primary Antibody	Category	Dilution	Source*
CCR5	Goat polyclonal	1:200	Santa Cruz
CXCR3	Goat polyclonal	1:200	Santa Cruz
CCR3	Rabbit polyclonal	1:100	BD Pharmigen
MIP-1 $\alpha$ /CCL3	Goat polyclonal	1:7	R&D Systems
MIP-1 $\beta$ /CCL4	Goat polyclonal	1:7	R&D Systems
RANTES/CCL5	Rat monoclonal	1:20	R&D Systems
MIG/CXCL9	Goat polyclonal	1:10	R&D Systems
IP-10/CXCL10	Goat polyclonal	1:7	R&D Systems
MCP-3/CCL7	Goat polyclonal	1:50	Santa Cruz
Eotaxin-1/CCL11	Rat monoclonal	1:20	R&D Systems

\* Santa Cruz Biotechnology, Santa Cruz, CA; BD PharMingen, San Jose, CA; R&D Systems, Minneapolis, MN.

Digital confocal images (512  $\times$  512 pixels) were captured with a laser scanning confocal microscope (LSM 510, with krypton-argon and He-Ne laser; Carl Zeiss Meditec, Inc., Thornwood, NY) with 488-nm excitation and 543-nm emission filters (LP505 and LP560, respectively; Carl Zeiss Meditec, Inc.) and were acquired with a 40/1.3 $\times$  oil-immersion objective. Images from untreated control and EDE samples were captured with identical photomultiplier tube gain settings and were processed with the microscope system (LSM-PC software; Carl Zeiss Meditec, Inc.) and image-analysis (Adobe Photoshop 7.0; Adobe Systems, San Jose, CA) software. Staining intensities were graded by consensus of two masked observers who used a previously reported scale: grade 0, no different from the secondary antibody control; +, slightly greater than the secondary antibody control; ++, moderate staining; and +++, intense staining.<sup>29</sup>

Immunohistochemistry was performed to detect and count stromal cells stained positively for the chemokine receptors CCR5 and CXCR3, as previously described.<sup>30</sup> After fixation in acetone at  $-20^{\circ}\text{C}$  for 10 minutes, endogenous peroxidases were quenched

with 0.3%  $\text{H}_2\text{O}_2$  in PBS for 10 minutes. The sections were sequentially blocked with avidin/biotin block (Vector Laboratories, Burlingame, CA) for 10 minutes each. After the sections were blocked with 20% normal rabbit serum in PBS for 45 minutes, primary antibodies were applied and incubation continued for 1 hour at RT. After they were washed, the sections were incubated with biotinylated rabbit anti-goat secondary antibodies (Vectastain Elite ABC Kit; Vector Laboratories) according to the manufacturer's protocol. The samples were finally incubated with 3,3'-diaminobenzidine (DAB; NovaRed; Vector Laboratories) peroxidase substrate to give a red stain (2 to 10 minutes, optimized for each antibody) and counterstained with Mayer's hematoxylin. Secondary antibody alone and goat anti-mouse and rabbit anti-mouse isotype (BD PharMingen) control experiments were also performed. The sections were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DXM 1200 camera; Nikon, Garden City, NY). The number of cells staining positively for CCR5 or CXCR3 was counted in conjunctival tissue sections. Three sections from each animal were evaluated. Positively stained cells were

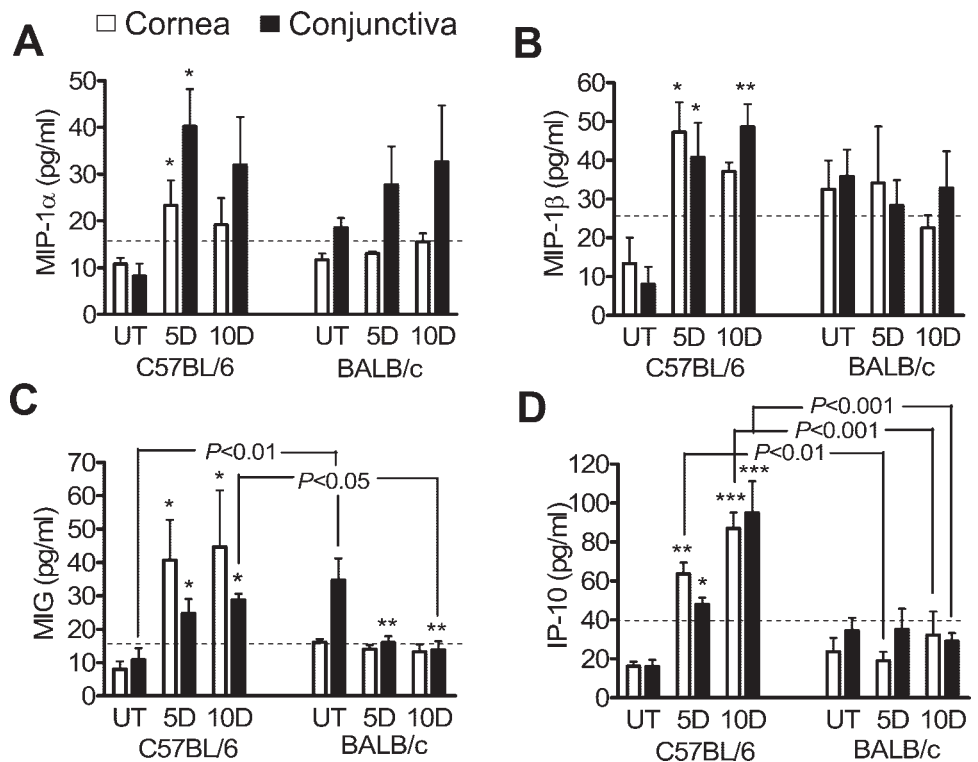
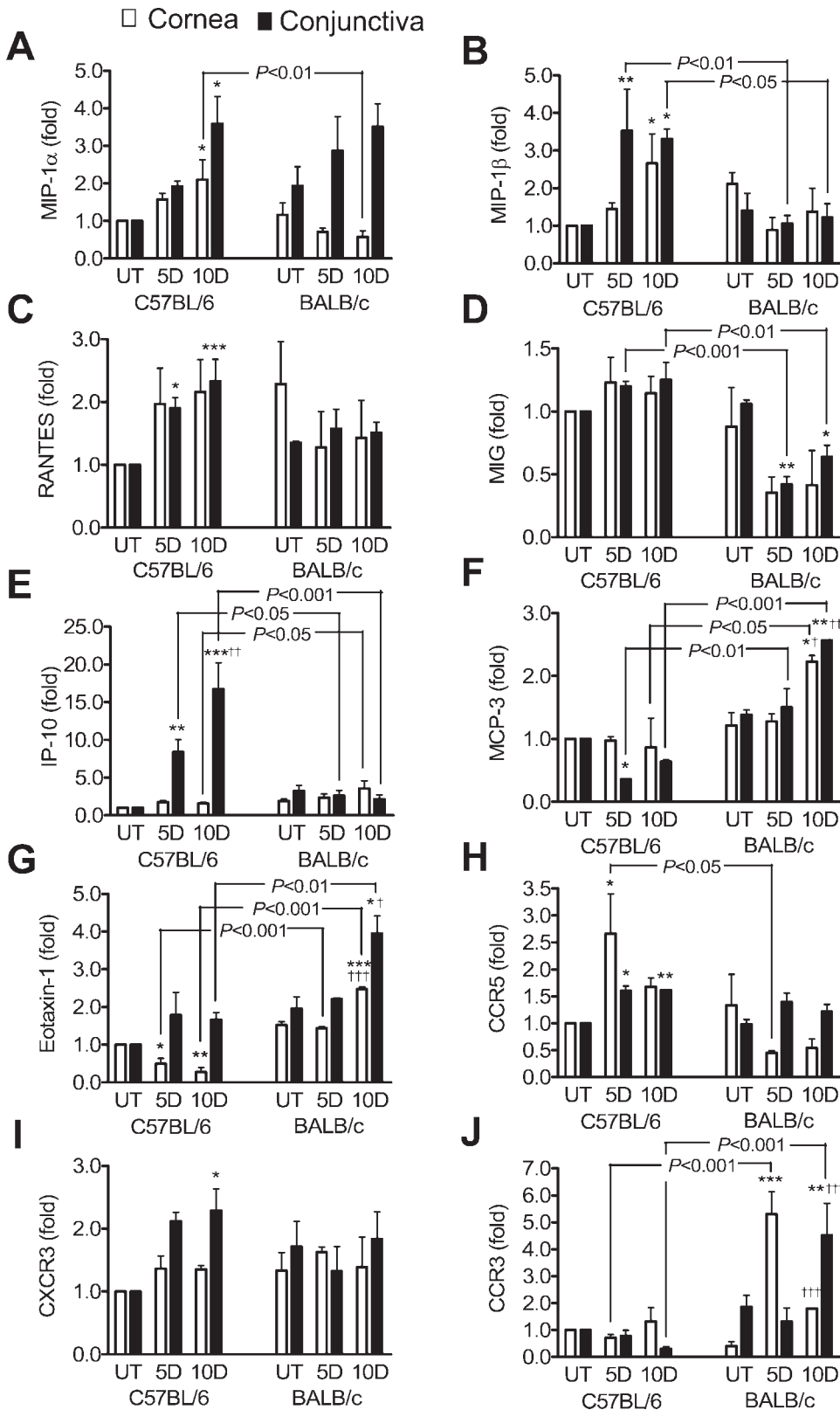


FIGURE 1. Concentrations of MIP-1 $\alpha$  (A), MIP-1 $\beta$  (B), MIG (C), and IP-10 (D) in the corneal epithelium and conjunctiva of UT mice and mice with experimental dry eye for 5 (5D) and 10 (10D) days in the C57BL/6 and BALB/c strains, as determined by immunobead assay. Data are expressed as the mean  $\pm$  SEM. Dotted line: lowest value in the linear portion of the curve generated from the observed mean fluorescence intensities versus the observed concentrations. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. C57BL/6-UT or BALB/c-UT.



**FIGURE 2.** Real-time PCR data showing the relative expression (x-fold) of MIP-1 $\alpha$  (A), MIP-1 $\beta$  (B), RANTES (C), MIG (D), IP-10 (E), MCP-3 (F), eotaxin-1 (G), CCR5 (H), CXCR3 (I), and CCR3 (J) in the corneal (CN) and conjunctival (CJ) epithelium of untreated (UT) mice and mice with experimental dry eye for 5 (5D) and 10 (10D) days in both C57BL/6 and BALB/c strains. Data are expressed as the mean  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 vs. C57BL/6-UT or BALB/c-UT. † $P$  < 0.05, †† $P$  < 0.01, ††† $P$  < 0.001 vs. C57BL/6-5D or BALB/c-5D.

counted over a length of at least 500  $\mu$ m in the conjunctival epithelium and to a depth of 75  $\mu$ m below the epithelial basement membrane in the stroma for a distance of 500  $\mu$ m, with image-analysis software (Metavue 6.2r4 software; Molecular Devices, Sunnyvale, CA). Results were expressed as the number of positive cells per 100  $\mu$ m.

**Statistical Analysis**

Results are presented as the mean  $\pm$  SEM of at least four separate experiments. Statistical differences in the immunobead assay and real-time PCR results were evaluated by two-way ANOVA, with post hoc analysis with the Bonferroni test. The Mann-Whitney test was used to



TABLE 3. Quantitation of Immunostaining Intensity for Chemokines and Their Receptors in Untreated and Experimental Mice

	Tissue	C57BL/6 Mice			BALB/c Mice		
		UT	EDE-5D	EDE-10D	UT	EDE-5D	EDE-10D
MIP-1 $\alpha$	CN	0	++	++	0	0	0
	CJ	0	++	++	0	0	+
MIP-1 $\beta$	CN	0	++	++	0	0	0
	CJ	0	+++	+++	0	0	0
RANTES	CN	0	++	+	0	0	0
	CJ	0	++	++	0	+	+
MIG	CN	0	++	+++	0	0	0
	CJ	0	++	+++	0	0	0
IP-10	CN	0	+++	+++	0	0	+
	CJ	0	++	+++	0	0	0
MCP-3	CN	0	0	0	0	++	++
	CJ	0	0, +	0, +	0	++	++
Eotaxin-1	CN	0	0	0	0	++	++
	CJ	0	0, +	0, +	0	++	++
CCR5	CN	0, S*+	0, S++	+, S++	+, S+	0, S+	0, S+
	CJ	+	++	++	+	+	+
CXCR3	CN	0	0, S+	+	0	0	0
	CJ	0	++	+++	0	0	0
CCR3	CN	0	0	0	0	++	+++
	CJ	0	0, +	0, +	0	++	+++

Data were collected in the corneal (CN) and conjunctival (CJ) epithelium of untreated (UT) mice and mice with experimental dry eye (EDE) for 5 (5D) and 10 (10D) days. Level: 0, no different than the secondary antibody control; +, slightly greater than the secondary antibody control; ++, moderate staining; +++, intense staining.

\* S, stomal staining.

compare the number of CCR5- or CXCR5-positive cells between untreated mice and mice with EDE for 5 or 10 days.  $P < 0.05$  was considered significant.

## RESULTS

### Th-1 Attracting Chemokines in the Cornea and Conjunctiva

The results of the immunobead chemokine detection assays are presented in Figure 1. The MIP-1 $\alpha$  and -1 $\beta$  concentrations in the corneal epithelium of C57BL/6 mice significantly increased from  $10.80 \pm 2.29$  and  $13.37 \pm 11.59$  pg/mL in untreated controls to  $23.33 \pm 9.31$  ( $P < 0.05$ ) and  $47.27 \pm 13.34$  pg/mL ( $P < 0.05$ ) after EDE for 5 days, respectively. Although the difference was not statistically significant when compared with the control samples, the respective concentrations after 10 days of EDE were  $19.17 \pm 9.90$  and  $37.1 \pm 4.02$  pg/mL in C57BL/6 mice. The concentrations of MIG and IP-10 in the C57BL/6 corneal epithelium significantly increased respectively from  $7.99 \pm 4.10$  and  $16.23 \pm 3.95$  pg/mL in untreated control animals to  $40.70 \pm 20.90$  ( $P < 0.05$ ) and  $63.63 \pm 10.13$  pg/mL ( $P < 0.01$ ) after 5 days of EDE and to  $44.67 \pm 29.41$  ( $P < 0.05$  compared with the control) and  $87.00 \pm 14.08$  pg/mL ( $P < 0.001$  compared with the control) after 10 days of EDE. In contrast, there were no changes in MIP-1 $\alpha$ , MIP-1 $\beta$ , MIG, and IP-10 concentrations in the BALB/c corneal epithelium at either 5 or 10 days of EDE. The IP-10 concentration in the corneal epithelium was significantly lower in BALB/c than in C57BL/6 mice after 5 and 10 days of EDE ( $P < 0.01$  and  $P < 0.001$ , respectively).

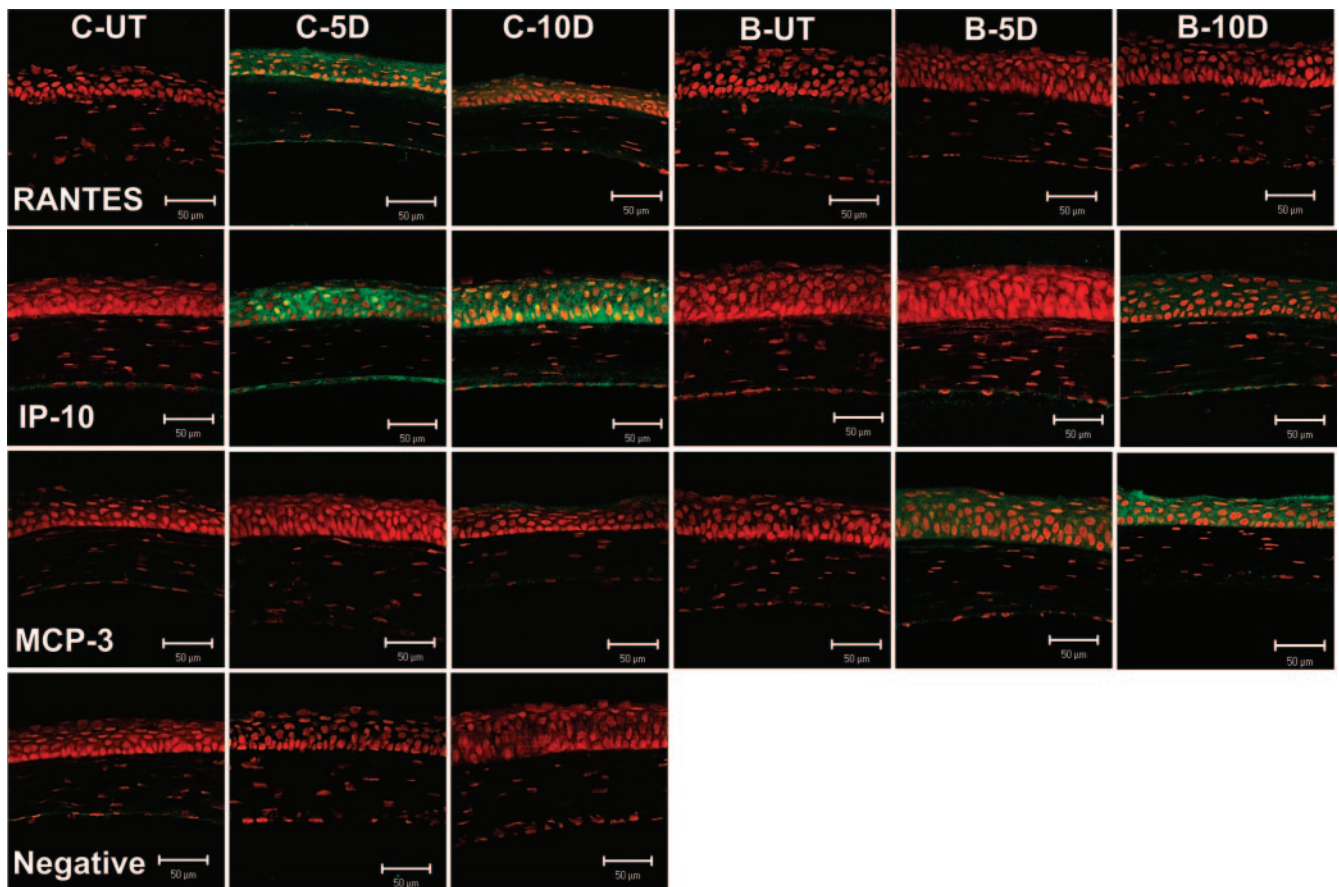
The MIP-1 $\alpha$  and -1 $\beta$  concentrations in the conjunctiva of C57BL/6 mice increased from  $8.18 \pm 5.45$  and  $8.02 \pm 5.05$  pg/mL in untreated controls to  $40.23 \pm 15.90$  ( $P < 0.05$ ) and  $40.80 \pm 15.41$  pg/mL ( $P < 0.05$ ) after EDE for 5 days and to  $36.98 \pm 15.48$  ( $P > 0.05$  compared with the control) and  $48.63 \pm 10.21$  pg/mL ( $P < 0.01$  compared with the control) after EDE for 10 days, respectively. The concentrations of MIG

and IP-10 in the C57BL/6 conjunctiva significantly increased from  $10.84 \pm 6.01$  and  $16.03 \pm 6.04$  pg/mL in untreated control subjects to  $24.70 \pm 7.50$  ( $P < 0.05$ ) and  $47.93 \pm 6.13$  pg/mL ( $P < 0.05$ ) after 5 days of EDE and to  $28.77 \pm 3.19$  ( $P < 0.05$ , compared with the control) and  $94.93 \pm 28.31$  pg/mL ( $P < 0.001$ , compared with the control) after 10 days of EDE, respectively. In contrast, there were no changes in MIP-1 $\alpha$ , MIP-1 $\beta$ , and IP-10 concentrations in the BALB/c conjunctiva at either 5 or 10 days of EDE. The MIG concentration in the BALB/c conjunctiva significantly decreased at 5 and 10 days of EDE compared with the control ( $P < 0.01$  for both). The MIG concentration in the conjunctiva of untreated mice was higher in BALB/c than in C57BL/6 mice ( $P < 0.01$ ). However, after 10 days of EDE, the conjunctiva of BALB/c had lower MIG and IP-10 concentrations ( $P < 0.05$  and  $P < 0.001$ , respectively) than did those of C57BL/6 mice.

### Effects of Desiccating Stress on Levels of Chemokine and Chemokine Receptor Transcripts in the Cornea and Conjunctiva

The effect of desiccating stress on levels of chemokine and chemokine receptor transcripts in the cornea and conjunctiva was evaluated by real-time PCR (Fig. 2). In the corneal epithelium of C57BL/6 mice, the level of CCR5 transcript at 5 days of EDE and MIP-1 $\alpha$  and -1 $\beta$  transcripts at 10 days of EDE significantly increased compared with those in untreated control mice. In the conjunctiva of C57BL/6 mice, the levels of MIP-1 $\beta$ , RANTES, IP-10, and CCR5 transcripts at 5 days of EDE and MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, IP-10, CCR5, and CXCR3 transcripts at 10 days of EDE significantly increased compared with baseline. There was a decrease in eotaxin-1 (5 and 10 days of EDE) and MCP-3 (5 days of EDE) transcript levels in the corneal and conjunctival tissues of C57BL/6 mice, respectively.

In contrast, in the BALB/c corneal epithelium, the levels of CCR3 transcript at 5 days of EDE and MCP3 and eotaxin-1 transcripts at 10 days of EDE significantly increased compared with those in untreated control animals. In the BALB/c con-



**FIGURE 3.** Immunofluorescent staining and laser scanning confocal microscopy in corneal tissue sections stained with polyclonal antibodies (green) to RANTES, IP-10, and MCP-3. Nuclei were counterstained with propidium iodide (red), and merged images were produced. Negative control images are also shown. Representative staining in the corneal epithelium in untreated controls (UT) and after 5 (5D) and 10 (10D) days of experimental dry eye in C57BL/6 (C) and BALB/c (B) mice. Scale bar, 50  $\mu$ m.

junctiva, the levels of MCP3, eotaxin-1, and CCR3 transcripts at 10 days of EDE significantly increased compared with baseline. There was no change in the levels of Th-1 chemokines or their ligands in the corneal and conjunctival tissues of BALB/c mice in response to desiccating stress, except for MIG, which significantly decreased in the conjunctiva at 5 and 10 days of EDE.

The corneal epithelium of BALB/c mice had lower CCR5 at 5 days, lower MIP-1 $\alpha$  and IP-10 at 10 days, higher eotaxin-1, and CCR3 at 5 days, and higher MCP-3 and eotaxin-1 transcript levels at 10 days of EDE than C57BL/6 mice. The conjunctiva of BALB/c mice had lower MIP-1 $\beta$ , MIG, and IP-10 at 5 and 10 days; higher MCP-3 at 5 days; and higher MCP-3, eotaxin-1, and CCR3 transcript levels at 10 days of EDE than C57BL/6 mice.

### Immunodetection of Chemokines and Chemokine Receptors

Chemokine and chemokine receptor antigens were immunodetected in corneal and conjunctival tissue sections obtained from C57BL/6 and BALB/c mice (Table 3; Figs. 3, 4, 5).

Staining for MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, MIG, and IP-10 increased after 5 and 10 days of EDE in the corneal and conjunctival epithelium of C57BL/6 mice. Although weak staining for MIP-1 $\alpha$  (10 days of EDE) and RANTES (5 and 10 days of EDE) in the conjunctival epithelium and IP-10 (10 days of EDE) in the corneal epithelium of BALB/c mice was noted, most of the Th-1 cell-attracting chemokines were undetectable in the corneal and conjunctival tissues of BALB/c mice. MCP-3, eotaxin-1, and CCR3 were barely detected in the cornea and conjunctiva of

C57BL/6 mice, but there was a moderate to marked increase in immunoreactivity in both tissues of BALB/c mice.

CCR5 and CXCR3 (not shown) staining increased in the subepithelial corneal stroma after induction of EDE in C57BL/6 mice (Fig. 5A). There was no change in the level of staining for these chemokine receptors in the corneal epithelia of BALB/c mice; however, weak superficial stromal staining was noted in the BALB/c corneas after induction of EDE. In conjunctival sections stained by an immunohistochemical technique, rare mononuclear cells stained positively for CCR5 ( $n = 3.5 \pm 2.6/100 \mu\text{m}$ ) or CXCR3 ( $n = 1.0 \pm 2.2/100 \mu\text{m}$ ) in the conjunctival stroma of untreated C57BL/6 mice. After 5 and 10 days of EDE, the number of CCR5-positive cells ( $n = 9.8 \pm 1.7/100 \mu\text{m}$  and  $n = 12.8 \pm 2.2/100 \mu\text{m}$ , respectively;  $P < 0.01$  for both) as well as the number of CXCR3-positive cells ( $n = 7.0 \pm 2.1/100 \mu\text{m}$  and  $n = 10.3 \pm 1.7/100 \mu\text{m}$ , respectively;  $P < 0.01$  for both) significantly increased (Fig. 5B). However, the number of CCR5- or CXCR3-positive cells remained unchanged in the conjunctival stroma of BALB/c mice.

### DISCUSSION

We evaluated the effect of desiccating stress on the expression of chemokines and their receptors in the ocular surface tissues of C57BL/6 and BALB/c mice. In the C57BL/6 strain which has a predilection for a Th-1 immune response to proinflammatory stress,<sup>23</sup> there was a significant increase in



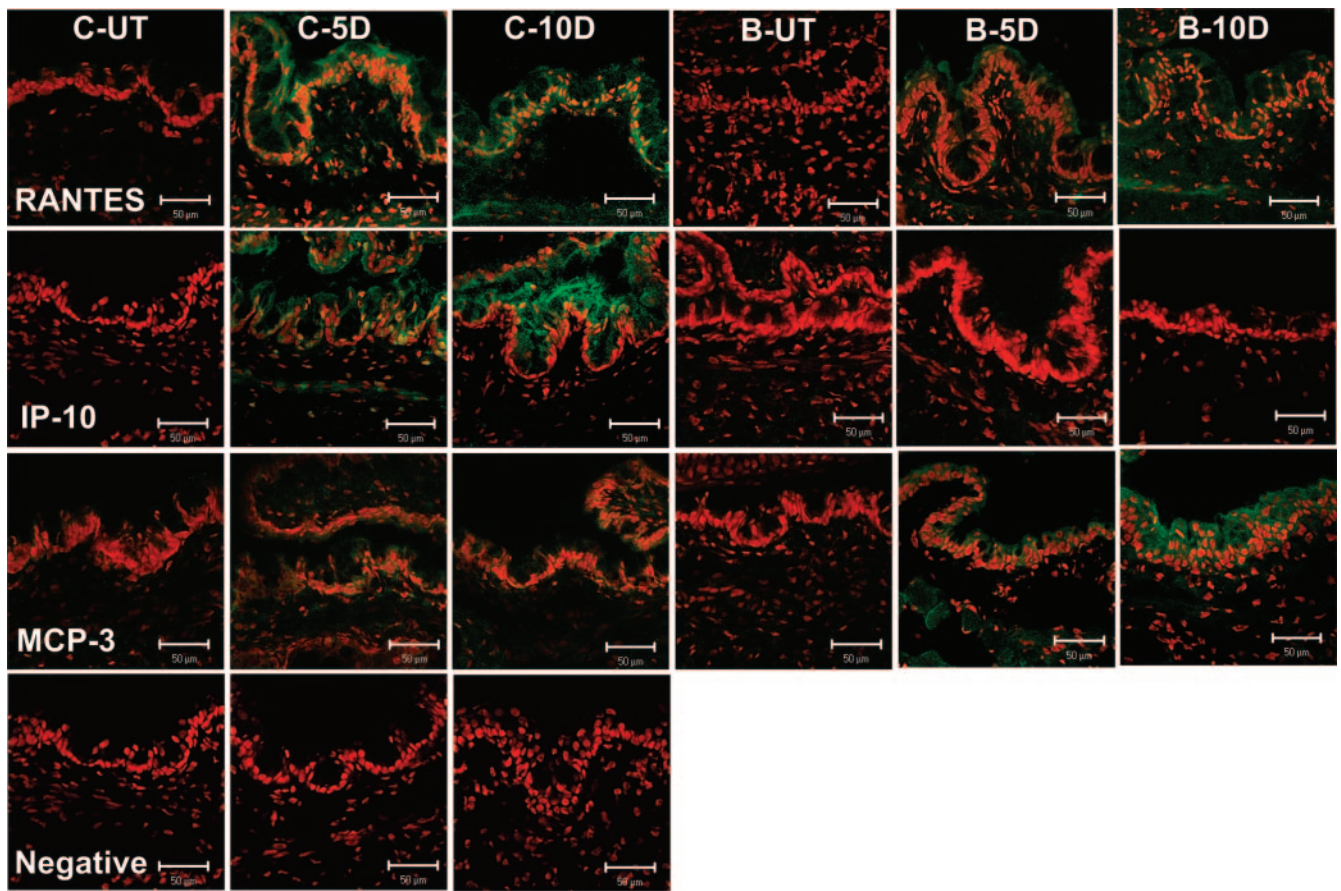


FIGURE 4. Immunofluorescent staining and laser scanning confocal microscopy in conjunctival tissue sections. The remaining description is as in Figure 3.

concentrations of MIP-1 $\alpha$ , MIP-1 $\beta$ , MIG, and IP-10 proteins in the corneal epithelium and conjunctiva. Desiccating stress also increased MIP-1 $\alpha$ , MIP-1 $\beta$ , and CCR5 transcripts in the cornea and conjunctiva and RANTES, MIG, IP-10, and

CXCR3 transcripts in the conjunctiva of C57BL/6 mice. In contrast, the levels of MCP-3, eotaxin-1, and CCR3 transcripts increased in the tissues of BALB/c mice which manifest a Th-2 immune response.<sup>31</sup> Immunofluorescent staining

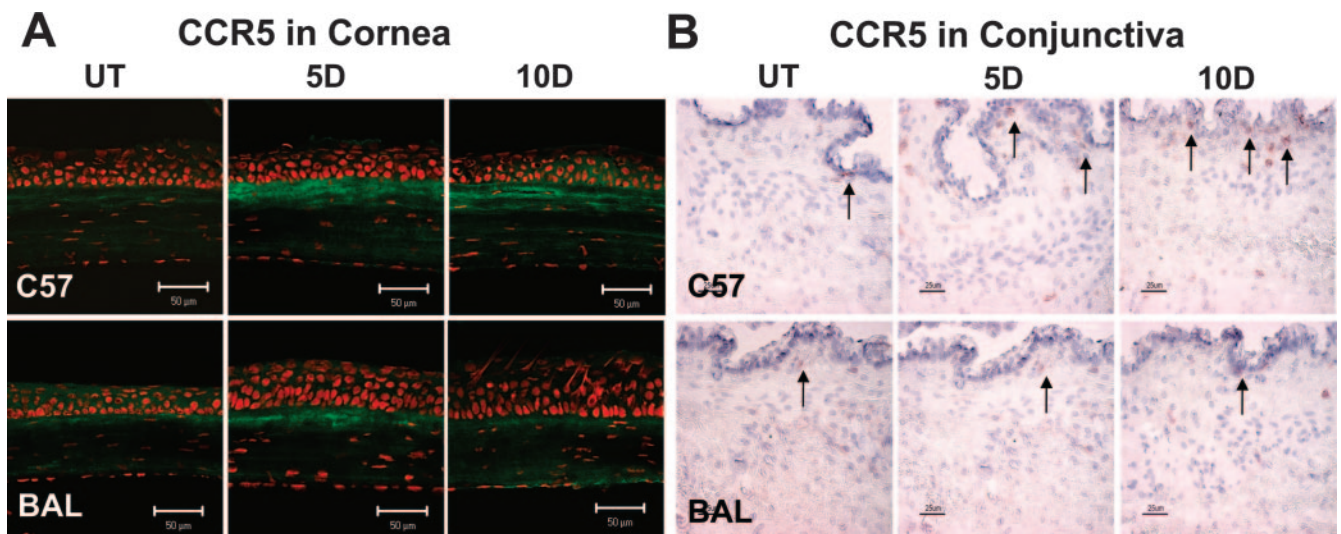


FIGURE 5. (A) Laser scanning confocal microscopy of immunofluorescent staining in the cornea stained for CCR5 (green) with propidium iodide (red) nuclear counterstaining in untreated controls (UT) and after 5 (5D) and 10 (10D) days of experimental dry eye in C57BL/6 (C57) and BALB/c (BAL) mice. Staining in the subepithelial corneal stroma increased after induction of dry eye. (B) Immunohistochemistry for CCR5 in the conjunctiva of C57BL/6 and BALB/c mice in untreated controls and after 5 and 10 days of experimental dry eye. The CCR5-positive cells (arrows) increased in the conjunctival stroma of C57BL/6 mice after induction of dry eye. Scale bar: (A) 50  $\mu$ m; (B) 25  $\mu$ m.

supported these results. Although weak immunostaining for MIP-1 $\alpha$ , RANTES, and IP-10 was observed in the ocular surface epithelia of BALB/c mice, there was no significant change in the levels of immunoreactivity for these Th-1 chemokines. These findings suggest that these chemokines may be a key component of the ocular surface inflammatory response to desiccating stress.

Chemokines play a role in leukocyte movement, inflammation, response to infection, angiogenesis, tumor growth, and stem cell proliferation.<sup>18</sup> They induce Th-1 or -2 immune responses through the complement of their receptors that are expressed on different types of leukocytes. In particular, CCR5 and its ligands MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, as well as CXCR3 and its ligands MIG, IP-10, interferon-inducible T-cell  $\alpha$  chemoattractant (ITAC) induce a cell-mediated Th-1 immune response, whereas CCR3 and its ligands MCP-2, -3 and -4; eotaxin-1 and -2; and RANTES induce a Th-2 response that is characteristic of allergy or atopy.<sup>19</sup>

There is considerable evidence that production of chemokines and their receptors increases in a variety of ocular surface diseases, especially allergy and infection. Increased levels of CCR3 and eotaxin were noted in tears of patients with allergic keratoconjunctivitis.<sup>32-34</sup> MCP-1, MCP-3, and RANTES were also highly expressed in the conjunctiva of patients with vernal keratoconjunctivitis.<sup>35</sup> In addition, several chemokines, including IP-10, were noted to participate in the pathogenesis of herpes simplex keratitis.<sup>36</sup> In vitro stimulation of human conjunctival epithelial cells or keratocytes with inflammatory mediators has been shown to stimulate production of chemokines such as RANTES, IL-8, IP-10, MIG, and ITAC.<sup>37,38</sup>

We have reported that hyperosmolar stress induced inflammation in human limbal epithelial cells by increasing expression and production of IL-1 $\beta$ , TNF- $\alpha$ , and the CXC chemokine IL-8, a process that was mediated through activation of the MAPK signaling pathways.<sup>13</sup> Therefore, it is likely that the increased production of chemokines and chemokine receptors by the corneal and conjunctival epithelia that we observed in this study may occur in response to stimulation of these proinflammatory cytokines. Although there is no clinically evident ocular surface inflammation in our mouse model of dry eye, we noted CD4<sup>+</sup> T-cell infiltration of the epithelium in the goblet-cell-rich area of the conjunctiva of C57BL/6 mice after exposure to desiccating stress.<sup>25</sup> We found an inverse correlation between the number of CD4<sup>+</sup> T cells and conjunctival goblet cells (De Paiva CS, et al. *IOVS* 2006;47:ARVO E-Abstract 5579). In addition, desiccating stress increased corneal permeability, corneal surface irregularity, and expression of cornified envelope protein, such as involucrin and small proline-rich protein-2 by the corneal epithelium of C57BL/6 mice.<sup>39</sup> These phenomena are similar to human dry eye, where T-cell infiltration of the conjunctiva has also been noted, even in eyes with aqueous tear deficiency and no inflammatory signs.<sup>4</sup>

Our study indicates that dry eye increases the production of Th-1 cell-attracting chemokines in the ocular surface epithelium of C57BL/6 mice. These chemokines can attract Th-1 cells, NK cells, macrophages, and immature dendritic cells that express a specific complement of chemokine receptors, such as CCR5 and CXCR3, from the blood vessels to the corneal and conjunctival tissues. Previous studies have reported that CCR5 was overexpressed by the conjunctival epithelium of patients with dry eye; however, little else has been described about changes in levels of chemokines and their receptors in the ocular surface in dry eye.<sup>21,22</sup> CCR5 has been shown to play a critical role in mediating recruitment and mobilization of MHC class II<sup>+</sup> Langerhans cells into the corneal epithelium.<sup>40</sup> Therefore, the ocular surface epithelium is not simply a target of the disease process, but appears to be an active participant in the local immune responses to dry eye.

Immunofluorescent staining showed an increase in CCR5 or CXCR3 staining in the subepithelial corneal stroma of C57BL/6 mice after induction of EDE. The identity of these cells was not established, but this stromal staining may indicate infiltration by Th-1 or NK cells expressing these chemokine receptors. Immunohistochemistry also demonstrated an increase in CCR5- and CXCR3-positive cells in the conjunctival stroma of C57BL/6 mice.

Our findings that chemokines and their receptors are up-regulated in our mouse model of dry eye can be extrapolated to the pathogenesis of human dry eye disease, in which inflammation has been found to play an important role.<sup>41</sup> Certain patients may display a more robust cell-mediated response to dry eye based on their immunogenetic make-up. For example, patients with Sjögren's syndrome and Stevens-Johnson syndrome, conditions in which genetic predisposition has been established,<sup>42,43</sup> have more severe keratoconjunctivitis sicca, with greater goblet cell loss and greater ocular dye staining, than other dry eye conditions with a similar low level of tear production.<sup>44,45</sup> Further studies are needed, to determine whether increased production of certain chemokines or their receptors is responsible for the greater disease severity in these patients.

In conclusion, specific Th-1 and -2 immune responses are induced after desiccating stress in a strain-dependent fashion. Desiccating stress stimulates expression of Th-1 cell attracting chemokines and chemokine receptors on the ocular surface of C57BL/6 mice. These findings suggest that blocking production of certain chemokines or their receptors may modulate the ocular surface immune-inflammatory response in dry eye disease.

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