Tear Production and Ocular Surface Changes in Experimental Dry Eye after Elimination of Desiccating Stress

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Purpose. To investigate the severity and duration of desiccating stress-induced dry eye disease between mice with and without a genetic predisposition to spontaneous autoimmunity.

Methods. Experimental dry eye was induced in 12- to 16-week-old wild-type C57BL/6 and autoimmune NOD.B10.$H2^b$ mice by subcutaneous injection of scopolamine with exposure to an air draft for 10 days. Tear volume and corneal smoothness were measured at baseline, 5 and 10 days after desiccating stress, and 3, 7, 14, and 28 days after the removal of desiccating stress. Periodic acid-Schiff staining and immunohistochemistry were performed to evaluate the densities of conjunctival goblet cells and $CD4^+$ T cells in each group. Interleukin (IL)-1β and IL-6 concentrations in conjunctival tissues were measured by multiplex immunobead assay.

Results. Signs of experimental dry eye were noted at 5 and 10 days after desiccating stress in both strains. After the removal of desiccating stress, in C57BL/6 mice, tear production and corneal smoothness improved at 3 and 7 days, respectively, and conjunctival goblet cells and $CD4^+$ T-cell densities and cytokine levels returned to baseline levels at 14 days. In contrast, in NOD.B10.$H2^b$ mice, none of the parameters recovered to baseline levels during a period of 28 days after the removal of desiccating stress.

Conclusions. After the removal of desiccating stress in experimental dry eye, tear volume and ocular surface parameters recovered within 2 weeks in C57BL/6 mice, whereas they remained unchanged in NOD mice. In contrast to autoimmune mice, experimental dry eye can be reversed after the elimination of desiccating stress in nonsusceptible mice. (*Invest Ophthalmol Vis Sci.* 2011;52:7267–7273) DOI:10.1167/iovs.11-7231

Dry eye disease is a multifactorial disorder of the tears and ocular surface, resulting in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface.[1] Inflammation of the ocular surface and increased osmolarity of the tear film play an important role in the pathogenesis of dry eye. Immunopathologic changes in the conjunctival epithelium of dry eye include inflammatory cell infiltration, increased expression of immune activation and adhesion molecules, apoptosis markers, matrix metalloproteinases, inflammatory cytokines, and T-helper type 1 (Th-1)–attracting chemokines and their receptors.[2–6]

A dry eye animal model mimicking human dry eye disease is a valuable tool for use in investigation of the multiple factors that have been implicated in the pathogenesis of this condition and for investigation of the efficacy of therapeutic agents. Desiccating stress by environmental and pharmacologic means has been used for the induction of dry eye in several different mouse strains, including C57BL/6, BALB/c, CBA, and 129SvEc/CD-1.[7–10] Desiccating ocular surface stress has been known to induce autoreactive T cells that, when adoptively transferred to naive immunodeficient hosts, cause Sjögren's syndrome-like inflammation in the lacrimal gland, cornea, and conjunctiva, but not in other organs, suggesting the existence of shared epitopes among the components of the lacrimal functional unit.[10] In contrast to these strains, one of the nonobese diabetic (NOD) mouse strains, NOD.B10.$H2^b$, was found to develop spontaneous dry eye and Sjögren's syndrome-like autoimmune inflammation in the ocular surface and lacrimal gland with age.[11,12] Desiccating environmental stress can worsen this process, indicating that environmental factors may exacerbate autoimmunity in susceptible persons.[12] Several immunoregulatory defects have been identified in the NOD mouse.[13–15]

Although experimental dry eye can be induced several days after the initiation of desiccating stress and can continue during a period of stress, the extent of maintenance of experimental dry eye after termination of the stress is unknown. In the present study, we investigated the effects of elimination of desiccating stress on tear production and ocular surface changes in a mouse model of experimental dry eye. Experimental dry eye was induced by pharmacologic inhibition of aqueous tear production and desiccating environmental stress in the C57BL/6 and NOD.B10.$H2^b$ strains. After elimination of desiccating environmental and pharmacologic stress, changes of tear production, ocular surface irregularities, conjunctival goblet cell and $CD4^+$ T-cell densities, and interleukin (IL)-1β and IL-6 concentrations in conjunctival tissue were analyzed in both strains.

Materials and Methods

Mouse Model of Dry Eye and Experimental Procedure

This research protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Maintenance of animals and all in vivo experiments were performed in accordance with institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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Twelve- to 16-week-old male C57BL/6 and NOD.B10.$H2^b$ mice were used in these experiments. Experimental dry eye was induced 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 a standard desiccating environment created by placing the mice between two fans to obtain a continuous air flow (15 L/min) in a room at 25°C with an ambient humidity of 35% for 18 hours per day, as previously reported.[5,8–10,12] During these experiments, the animals' behavior, food, and water intake were not restricted.

Ten days after the initiation of experimental dry eye, elimination of desiccating stress was made by discontinuing scopolamine injection and placing the mice in a normal humidity and temperature environment. Mice were euthanatized at baseline, at 5 and 10 days after desiccating stress, and at 3, 7, 14, and 28 days after the removal of desiccating stress. Measurement of tear volume, corneal smoothness, conjunctival goblet cell and $CD4^+$ T-cell densities, and IL-1β and IL-6 levels in conjunctival tissue was performed in four animals per time point per strain, and all experiments were repeated. Both eyes were examined for the measurement of tear volume, corneal smoothness, and cytokine concentration. Right eyes were used for histology, and left eyes were used for immunohistochemistry.

In addition, to investigate sexual differences in the resolution rate of experimental dry eye in C57BL/6 mice, tear volume, ocular surface irregularities, and conjunctival goblet cell and $CD4^+$ T-cell densities after elimination of desiccating stress were compared between the male and female C57BL/6 stains.

Measurement of Tear Production

Tear volume was measured with phenol red–impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA), as previously described.[16] The threads were held with jeweler forceps and placed in the lateral canthus for 20 seconds. The tear volume, expressed in millimeters of thread wet by the tear and turned red, was read under a microscope (SMZ 1500; Nikon, Melville, NY). The measured uptake of tear fluid in millimeters was compared with a standard curve prepared from cotton threads of known uptake volume of a stock basic solution (1500 mL of 0.9% saline and 5 mL of 5 N NaOH) over 20 seconds, with volumes in the range that would be expected in mouse tears.[17,18]

Evaluation of Corneal Smoothness

Reflected images of a white ring from the fiberoptic ring illuminator of the stereoscopic zoom microscope (SMZ 1500; Nikon) were taken immediately after euthanasia. Corneal smoothness was assessed by two masked observers who graded the distortion of a white ring reflected off the corneal epithelium in digital images, as previously described.[19] The corneal irregularity severity score was calculated using a five-point scale based on the number of distorted quarters in the reflected ring: 0, no distortion; 1, distortion in one quarter; 2, distortion in two quarters; 3, distortion in three quarters; 4, distortion in all four quarters; 5, severe distortion, in which no ring could be recognized.

Histology

Eyes and adnexa were surgically excised, fixed in 4% paraformaldehyde, and embedded in paraffin. Six-micrometer sections were stained with periodic acid-Schiff reagent. Sections from four mice of each group were examined and photographed with a microscope (Olympus, Tokyo, Japan) equipped with a digital camera. Goblet cell density in the superior and inferior conjunctiva was measured in three sections of each eye using image analysis software (Media Cybernetics, Silver Spring, MD) and was expressed as the number of goblet cells per 100 μm.

Immunohistochemistry

Immunohistochemistry was performed to detect and count cells that stained positively for CD4 in the conjunctiva and lacrimal glands. Eyes, adnexa, and lacrimal glands were surgically excised, embedded in OTC compound (VWR, Suwanee, GA), and flash frozen in liquid nitrogen. Eight-micrometer sections were cut with a cryostat. Cryosections from four mice per group were fixed in acetone at −20°C for 10 minutes. After fixation, endogenous peroxidases were quenched with 0.3% H_2O_2 in PBS for 10 minutes. The sections were sequentially blocked with avidin/biotin block (Vector Laboratories, Burlingame, CA) for 10 minutes each. After blocking with 20% normal serum in PBS for 45 minutes, rat monoclonal antibody against CD4 (clone H129.9, 10 μg/mL; BD Biosciences, San Jose, CA) was applied and incubated for 1 hour at room temperature. After washing, the sections were incubated with biotinylated goat anti-rat antibody (BD Biosciences). The samples were then incubated with 3,3′-diaminobenzidine (NovaRed; Vector Laboratories) peroxidase substrate and counterstained with Mayer's hematoxylin. Secondary antibody alone and rat anti-mouse isotype (BD Biosciences) controls were also included. Three sections from each animal were examined and photographed using a microscope (Olympus) equipped with a digital camera. $CD4^+$ cells were counted over a length of at least 500 μm in the conjunctival epithelium using image analysis software (Media Cybernetics).[12,19] Results were expressed as the number of positive cells per 100 μm.

Multiplex Immunobead Assay

The levels of IL-1β and IL-6 in the conjunctiva were measured using a multiplex immunobead assay (Luminex 200; Luminex Corp., Austin, TX). Conjunctival tissues were collected from each group and pooled in lysis buffer containing protease inhibitors for 30 minutes. The cell extracts were centrifuged, and the supernatants were stored at −70°C until use. The supernatants were added to wells containing the appropriate cytokine bead mixture that included mouse monoclonal antibodies specific for IL-1β and IL-6 (Panomics, Santa Clara, CA) for 60 minutes. Serial dilutions of cytokines were also added to wells in the same plate as the supernatant samples to generate a standard curve. The plate was incubated for 30 minutes at room temperature by biotinylated detection antibody. After three washes with assay buffer, the reactions were detected after the addition of streptavidin-phycoerythrin using an analysis system (xPONENT, Austin, TX).

Statistical Analysis

Results are presented as the mean ± SEM. Nonparametric comparisons were made with the Wilcoxon signed-rank test, and parametric comparisons were made with the paired *t*-test. $P < 0.05$ was considered statistically significant.

Results

Changes in Tear Production

In C57BL/6 mice, tear volume significantly decreased at 5 days ($P < 0.01$) and 10 days ($P = 0.01$) after desiccating stress compared with baseline. After the removal of desiccating stress, the volume improved to baseline levels at 3 days, 7 days, 14 days, and 28 days ($P > 0.05$ compared with baseline) (Fig. 1A). In NOD.B10.$H2^b$ mice, tear volume also decreased at 5 days ($P = 0.01$) and 10 days ($P = 0.01$) after desiccating stress compared with baseline. After the removal of desiccating stress, the volume remained decreased at 3 days ($P = 0.01$), 7 days ($P = 0.02$), 14 days ($P = 0.02$), and 28 days ($P = 0.02$) (Fig 1B).

Changes in Corneal Surface Irregularities

In C57BL/6 mice, corneal smoothness score significantly increased at 5 days ($P = 0.01$) and 10 days ($P = 0.01$) after desiccating stress compared with baseline. After the removal of desiccating stress, the score improved to baseline levels at 7 days, 14 days, and 28 days ($P > 0.05$ compared with baseline) (Figs. 2A, 2B). In NOD.B10.$H2^b$ mice, corneal smoothness score also increased at 5 days ($P = 0.01$) and 10 days ($P < 0.01$) after desiccating stress compared with baselines. After the removal of desiccating stress, the score remained unchanged at 3 days ($P = 0.01$), 7 days ($P = 0.02$), 14 days ($P = 0.03$), and 28 days ($P = 0.04$) (Figs. 2C, 2D).

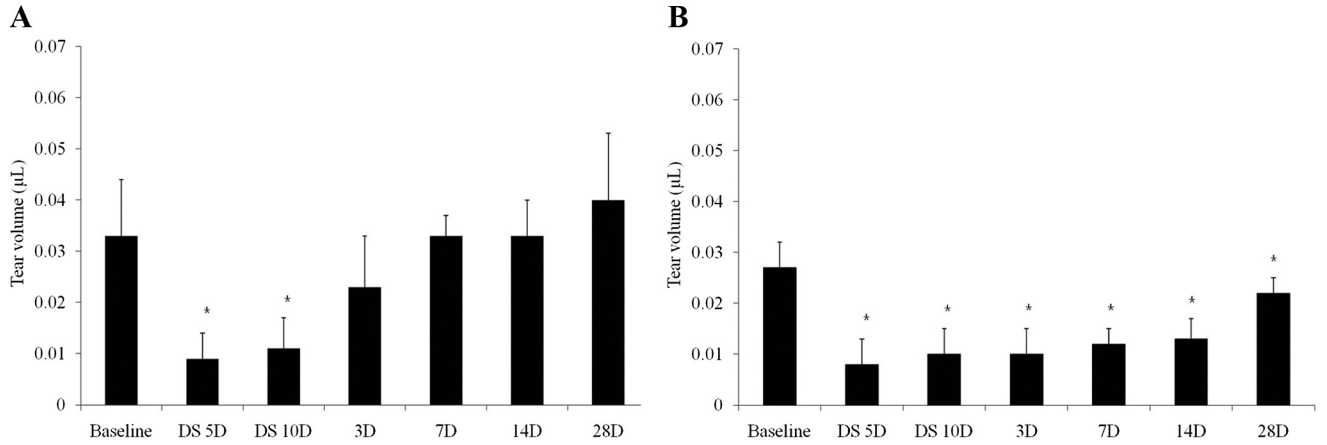

FIGURE 1. Changes in tear volume in experimental dry eye after the elimination of desiccating stress (DS) in C57BL/6 (**A**) and NOD.B10.$H2^b$ (**B**) mice. $^*P < 0.05$ compared with baseline.

Changes in Conjunctival Goblet Cell Density

In C57BL/6 mice, the mean density of conjunctival goblet cells significantly decreased after 5 days ($P < 0.01$) and 10 days ($P < 0.01$) of desiccating stress. After stress removal, the cell density returned to baseline levels at 14 days and 28 days ($P > 0.05$ compared with baseline) (Figs. 3A, 3B). In NOD.B10.$H2^b$ mice, the mean density of conjunctival goblet cells also decreased after 5 days ($P = 0.01$) and 10 days ($P = 0.01$) of desiccating stress. Despite stress removal, the cell density remained decreased at 3 days ($P = 0.01$), 7 days ($P = 0.01$), 14 days ($P = 0.02$), and 28 days ($P = 0.02$) (Figs. 3C, 3D).

Changes in $CD4^+$ T-Cell Density in the Conjunctiva and Lacrimal Glands

In C57BL/6 mice, the mean density of conjunctival $CD4^+$ T cells significantly increased after 5 days ($P < 0.01$) and 10

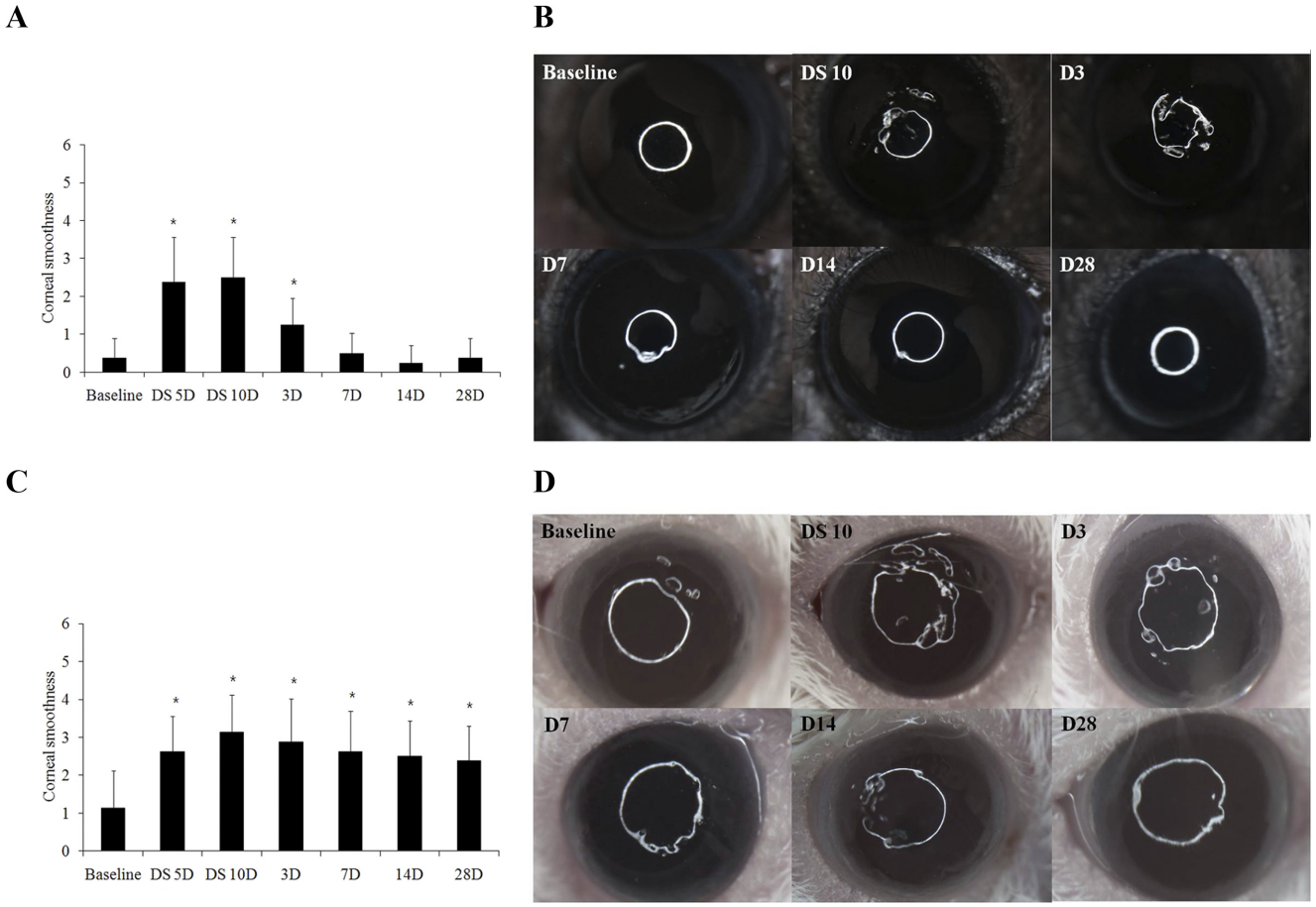

FIGURE 2. Changes in corneal smoothness score in experimental dry eye after the elimination of desiccating stress (DS) in C57BL/6 (**A**, **B**) and NOD.B10.$H2^b$ (**C**, **D**) mice. $^*P < 0.05$ compared with baseline.

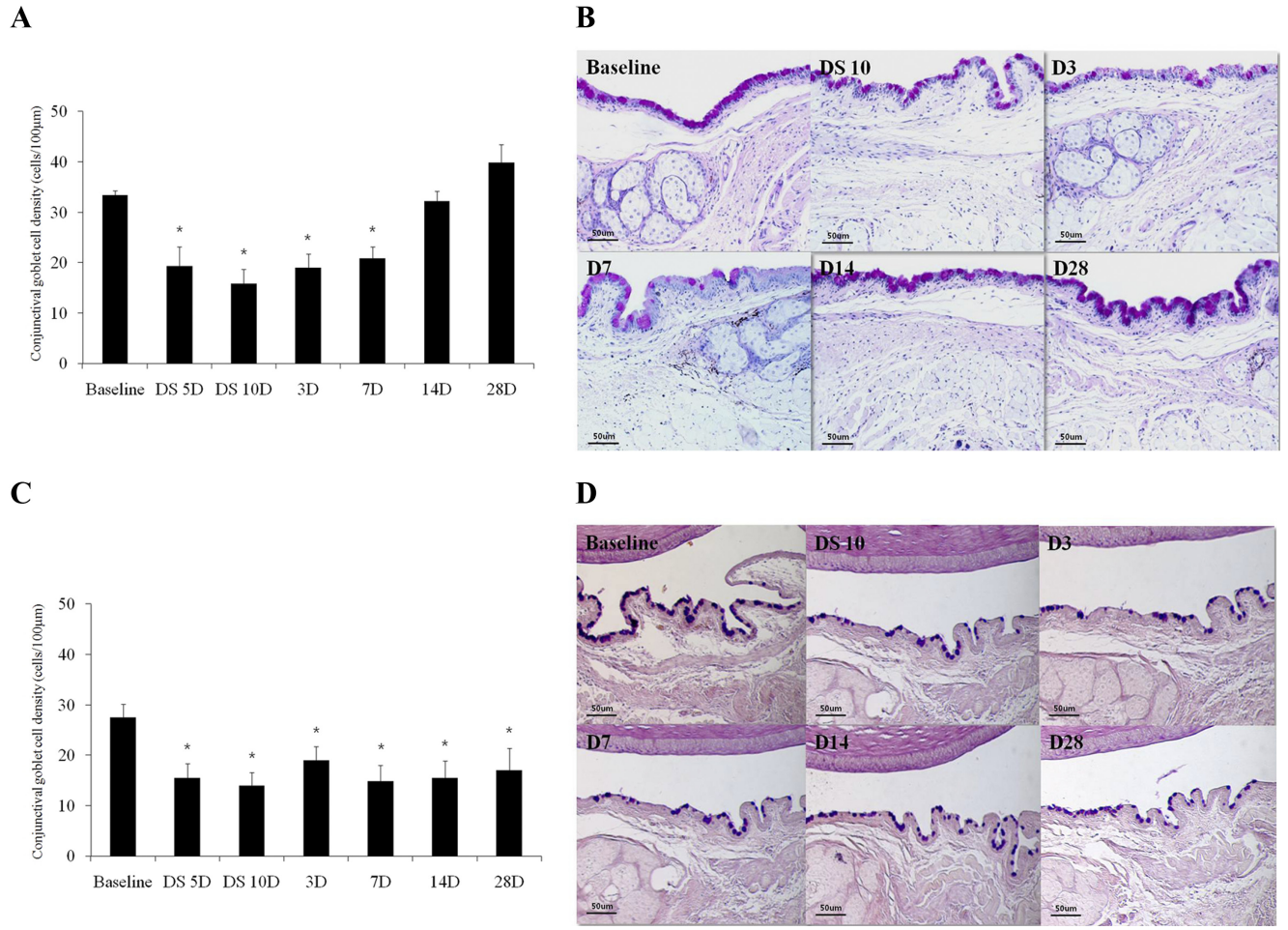

FIGURE 3. Changes in conjunctival goblet cell density in experimental dry eye after the elimination of desiccating stress (DS) in C57BL/6 (**A**, **B**) and NOD.B10.$H2^b$ (**C**, **D**) mice. *$P < 0.05$ compared with baseline.

days ($P < 0.01$) of desiccating stress. After stress removal, cell density returned to baseline levels at 14 days and 28 days ($P > 0.05$ compared with baseline) (Figs. 4A, 4B). In NOD.B10.$H2^b$ mice, the mean density of conjunctival $CD4^+$ T cells also increased after 5 days ($P = 0.02$) and 10 days ($P = 0.01$) of desiccating stress. Despite stress removal, the cell density remained increased at 3 days ($P = 0.02$), 7 days ($P = 0.02$), 14 days ($P = 0.02$), and 28 days ($P = 0.04$) (Figs. 4C, 4D).

$CD4^+$ T-cell infiltration in the lacrimal glands showed findings similar to those in the conjunctiva. $CD4^+$ T-cell infiltration increased markedly in both strains after desiccating stress. After stress removal, infiltration returned to baseline levels from 14 days in the C57BL/6 strain, whereas it was unchanged during a period of 28 days in the NOD strain (Fig. 5).

Changes in IL-1β and IL-6 Levels in Conjunctiva Tissues

Conjunctival IL-1β and IL-6 concentrations in both C57BL/6 and NOD.B10.$H2^b$ mice significantly increased at 5 days and 10 days after desiccating stress compared with baseline ($P < 0.05$). After stress removal, the concentrations lowered to baseline levels at 14 days and 28 days in the C57BL/6 strain, whereas they remained increased during a period of 28 days in the NOD strain (Figs. 6A–D).

Comparison between Male and Female C57BL/6 Strains

There were no significant differences in tear volume, ocular surface irregularities, and conjunctival goblet cell and $CD4^+$ T-cell densities between male and female C57BL/6 mice (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7231/-/DCSupplemental).

DISCUSSION

Several experimental dry eye models have been used for investigation of the pathogenesis of dry eye and the efficacy of therapeutic agents. Among these, desiccating stress with a dry environment and pharmacologic inhibition of aqueous tear production is the most commonly used model that can result in dry eye signs, including reduced tear volume and tear turnover rate, increased corneal surface irregularity and corneal permeability, disruption of corneal epithelial barrier function, decreased conjunctival goblet cell density, and conjunctival squamous metaplasia.[8,9,12,19,20]

Exposure to a desiccating environment can induce strain-specific responses in mice. The C57BL/6 strain expresses increased levels of Th-1 cytokines in their tears, whereas the BALB/c strain expresses increased levels of Th-2 cytokines.[21,22] Desiccating stress potently stimulates the expression of Th-1 chemokines and chemokine receptors on the ocular surface of C57BL/6 mice.[5]

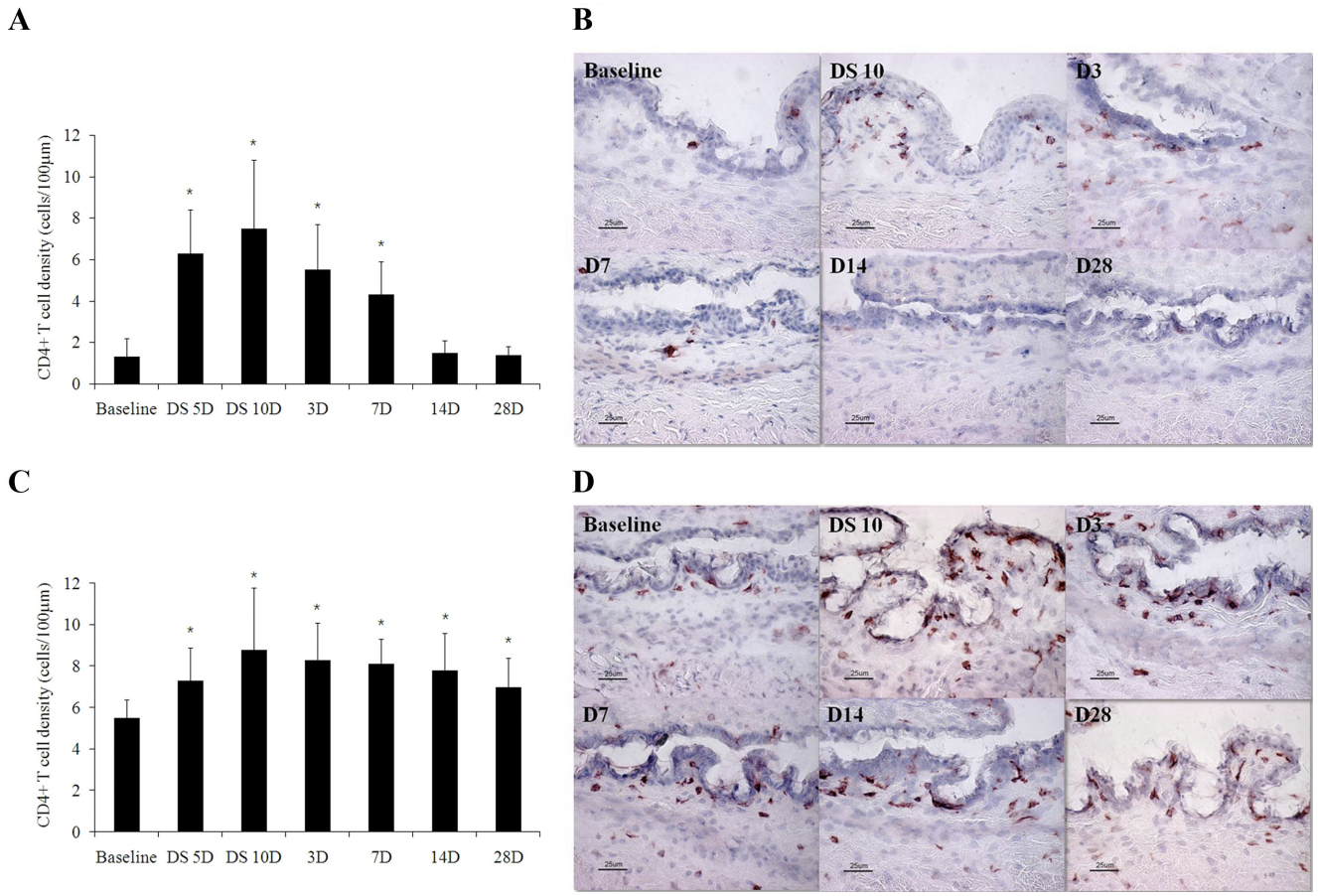

FIGURE 4. Changes in conjunctival $CD4^+$ T-cell density in experimental dry eye after the elimination of desiccating stress (DS) in C57BL/6 (**A**, **B**) and NOD.B10.$H2^b$ (**C**, **D**) mice. $^*P < 0.05$ compared with baseline.

In a previous study, the time course of ocular surface and lacrimal gland changes was investigated in a scopolamine-induced dry eye model.[23] Daily scopolamine doses of 12.5 mg and 25 mg were continuously applied for a 28-day period in Lewis rats, resulting in keratitis, decreased Muc5AC staining in the conjunctiva, and modifications in the fatty acid composition of the extraorbital lacrimal gland. Animals treated with a 12.5-mg/d dose of scopolamine exhibited keratitis after 2, 10, and 28 days. All animals showed keratitis after 17 days. Decreased Muc5AC staining and modifications in the fatty acid composition were detected from early (1 day) to late (28 days) time points. However, no study has been published on the time course of ocular surface changes after the elimination of dry eye stress.

In the present study, we investigated the time course of tear production and ocular surface changes in experimental dry eye after the elimination of desiccating stress in C57BL/6 and NOD.B10.$H2^b$ mice. In C57BL/6 mice, tear volumes and corneal smoothness scores showed improvement at 3 and 7 days, respectively, and the densities of goblet cells in the conjunctiva

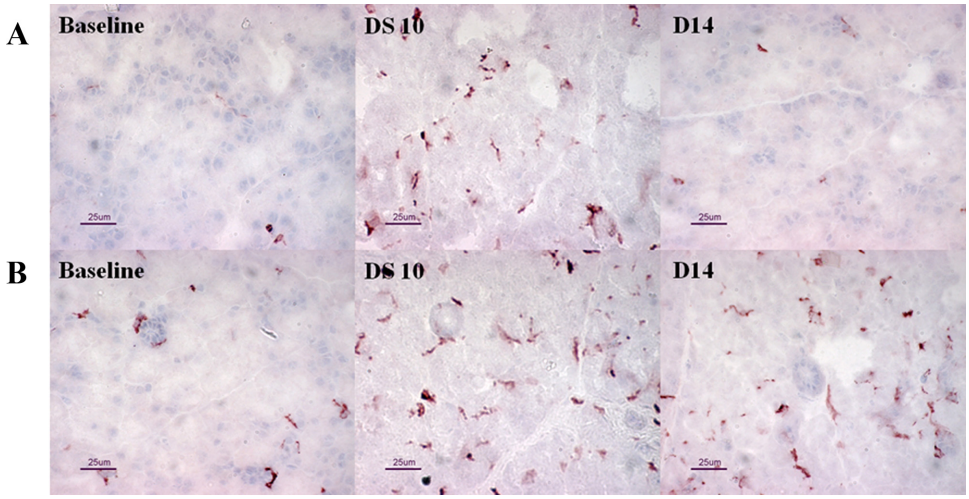

FIGURE 5. Immunohistochemistry showing $CD4^+$ T-cell infiltration in the lacrimal gland after the elimination of desiccating stress (DS) in C57BL/6 (**A**) and NOD.B10.$H2^b$ (**B**) mice.

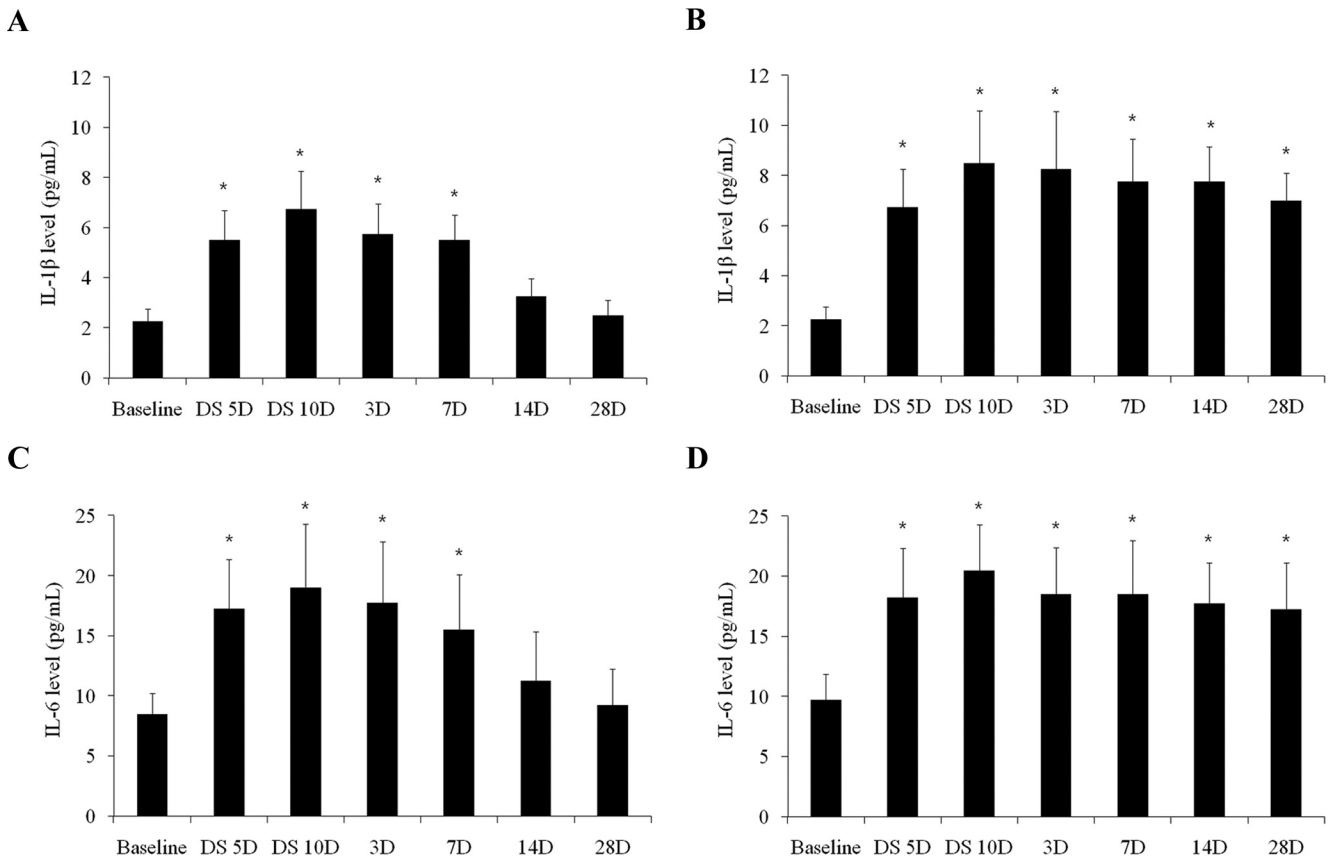

Figure 6. Changes of IL-1β (**A**, **B**) and IL-6 (**C**, **D**) concentrations in the conjunctiva after the elimination of desiccating stress (DS) in C57BL/6 (**A**, **C**) and NOD.B10.*H2*^{b} (**B**, **D**) mice, measured by immunobead assay.

and CD4$^+$ T cells in the conjunctiva and lacrimal glands and the concentrations of conjunctival IL-1β and IL-6 returned to baseline levels at 14 days. In contrast, in NOD.B10.*H2*^{b} mice, none of the parameters recovered to baseline levels during a period of 28 days after the removal of desiccating stress.

Different mouse models, such as the NOD mouse, the MRL/lpr mouse, the NZB/W F1 mouse, the transforming growth factor-β1 knockout mouse, and the NFS/sld mouse, that underwent thymectomy 3 days after birth have been used for the investigation of autoimmune exocrinopathy.[24] Among these, the NOD mouse is the most accurate model for Sjögren's syndrome-like autoimmune inflammation because the development of adenitis is accompanied by decreased secretory function in the lacrimal and salivary glands.[25] Male NOD mice have a tendency to induce more severe inflammation in the lacrimal glands, whereas female mice express more severe sialoadenitis.[26] Lymphocytic infiltration in the lacrimal glands is first detected at approximately 6 to 10 weeks, and secretory dysfunction is detected by 4 months in males.[27] Considering age and sex differences, we selected male NOD mice aged 12 to 16 weeks as an autoimmune dry eye model.

Our study indicates that experimental dry eye can be reversed to the baseline level after the elimination of desiccating environmental and pharmacologic stress in the C57BL/6 mouse, which is not susceptible to spontaneous autoimmunity, whereas it remains unchanged in the autoimmune NOD mouse, which has defective immunoregulation. Identified immunoregulatory defects that are responsible for susceptibility to development of an autoimmune response in NOD mice include impaired negative selection of autoreactive T cells in the thymus, defective apoptosis of effector T cells, and reduced number and function of regulatory T cells.[13–15]

Our findings suggesting that continuous desiccating stress is needed to maintain induced dry eye in mice manifesting a Th-1 response can be extrapolated to the course of the human disease, in which dry eye can be alleviated or treated after modification of desiccating factors. In contrast, in those who are susceptible to autoimmunity or immunoregulatory defects, desiccating factors can trigger or aggravate autoimmune lacrimal keratoconjunctivitis and dry eye. In those cases, dry eye may not be improved by the modification of desiccating factors alone.

In our experiment, male C57BL/6 mice were used in the nonsusceptible dry eye model because of age and sex matching with NOD mice. Although both sexes of C57BL/6 mice or female C57BL/6 mice have been used in the induction of experimental dry eye, no study has been reported about the comparison between male and female mice.[5,9,10,21,22,28] However, we found that there was no significant difference in the induction and resolution rates of experimental dry eye after the induction and elimination of desiccating stress between male and female C57BL/8 mice. The limitation of our study is that a follow-up period of 28 days after the elimination of desiccating stress may be short for the evaluation of long-term changes in tear production and the ocular surface. In our model of experimental dry eye, desiccating environmental stress was induced by air flow only. Further studies with other dry eye animal models and long-term follow-up are needed.

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