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# Desiccating Stress Induces T Cell-Mediated Sjögren's Syndrome-Like Lacrimal Keratoconjunctivitis[1]

**Jerry Y. Niederkorn,[2,3]* Michael E. Stern,[2†] Stephen C. Pflugfelder,[2‡] Cintia S. De Paiva,[‡] Rosa M. Corrales,[‡] Jianping Gao,[†] and Karyn Siemasko[†]**

**Chronic dry eye syndrome affects over 10 million people in the United States; it is associated with inflammation of the lacrimal gland (LG) and in some cases involves T cell infiltration of the conjunctiva. We demonstrate that environmental desiccating stress (DS) elicits T cell-mediated inflammation of the cornea, conjunctiva, and LG, but not other organs in mice. The lacrimal keratoconjunctivitis (LKC) was mediated by CD4$^+$ T cells, which, when adoptively transferred to T cell-deficient nude mice, produced inflammation in the LG, cornea, and conjunctiva, but not in any other organ. Adoptively transferred CD4$^+$ T cells produced LKC even though recipients were not exposed to DS. LKC was exacerbated in euthymic mice depleted of CD4$^+$CD25$^+$forkhead/winged helix transcription factor$^+$ regulatory T cells. The results suggest that DS exposes shared epitopes in the cornea, conjunctiva, and LG that induce pathogenic CD4$^+$ T cells that produce LKC, which under normal circumstances is restrained by CD4$^+$CD25$^+$forkhead/winged helix transcription factor$^+$ regulatory T cells.** *The Journal of Immunology,* **2006, 176: 3950–3957.**

Dry eye is a common ocular disease and is one of the leading causes of patients seeking ophthalmic care. Dry eye or lacrimal keratoconjunctivitis (LKC)[4] has a tremendous impact on quality of life and can cause debilitating eye pain and loss of vision. In animal models and humans, chronic dry eye disease is often associated with T cell infiltration of the conjunctiva (1–3). T cell infiltration has also been observed in dry eye patients with or without a systemic autoimmune disease, such as Sjogren's syndrome (SS) (3). It has been proposed that in some forms of dry eye disease, autoreactive T cells alter the function of the ocular surface epithelium and also cause reduced tear secretion (4). The presence of CD4$^+$ T cells in the epithelium and stroma of the conjunctiva and the salutary effect of the T cell-immunosuppressive drug, cyclosporine, in some patients support the proposition that dry eye is an immune-mediated disease (2, 5). However, others have argued that inflammation of the cornea and conjunctiva is the result, not the cause, of dry eye disease.

It is now recognized that the ocular surface and lacrimal glands function as an integrated unit, termed the lacrimal functional unit (LFU), which is linked by their sensory and autonomic nerves (4). Inflammation in any component of the LFU has the capability to compromise the function of other components through soluble mediators, generation of autoreactive T cells to shared Ags, and inhibition of neural transmission (4). SS causes profound dysfunction of the LFU, which includes decreased tear secretion, loss of conjunctival goblet cells (GC) transformation of the ocular surface epithelium to a keratinized surface. Immunopathological changes, including immune-mediated inflammation and increased production of inflammatory cytokines and chemokines, have been detected in the dysfunctional lacrimal glands and ocular surface tissues in SS (6–8).

It has been proposed that a wide range of factors that alter the integrity of tissues create danger signals that activate APCs and promote the generation of immune responses to self Ags (9). The danger hypothesis has been implicated in the pathogenesis of SS (10) and is consistent with the observation that desiccating stress (DS) to the ocular surface induces the expression of proinflammatory cytokines (11). With this in mind, we tested the hypothesis that environmental DS to the ocular surface would break self-tolerance and induce the development of T cell-mediated inflammation directed against epitopes expressed on the ocular surface and the lacrimal gland (LG).

## Materials and Methods

### *Mice*

Female BALB/c, T cell-deficient nude BALB/c (BALB/cByJ-HfH11<ν>), and C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory or Charles River Laboratories. All animal experiments were approved by the institutional animal care and use committees at Allergan and Baylor College of Medicine. All studies adhered to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

### *Induction of DS in mice*

DS was induced by s.c. injection of scopolamine hydrobromide (0.5 mg/0.2 ml; Sigma-Aldrich) four times a day (0800, 1200, 1400, and 1700 h), alternating between the left and right flanks of 4- to 6-wk-old mice, as previously described (12). Up to five mice were placed in a cage with a perforated plastic screen on one side to allow airflow from a fan (Cafrano) placed 6 in. in front of it for 16 h/day. Room humidity was maintained at 30–35% and temperature at 80°F. DS was induced for either 5 or 12 consecutive days. This model of dry eye disease has been used previously with no discernible ill effects from the scopolamine treatment or low humidity (11, 12). Changes in corneal permeability were assessed by measuring corneal staining by Oregon Green dextran (OGD; 70,000 m.w.; Molecular Probes) as previously described (13). Aqueous tear production was assessed

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[4] Abbreviations used in this paper: LKC, lacrimal keratoconjunctivitis; CLN, cervical lymph node; DS, desiccating stress; FoxP3, forkhead/winged helix transcription factor; GC, goblet cell; LFU, lacrimal functional unit; LG, lacrimal gland; NS, nonstressed; OGD, Oregon Green dextran; SS, Sjogren's syndrome.

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using cotton thread (Quick Thread; FCI Ophthalmics) as previously described (14). Control mice were maintained in a nonstressed (NS) environment containing 50–75% relative humidity without exposure to forced air. Tear production was measured with phenol red-impregnated cotton threads (Zone-Quick; Oasis) placed into the tear meniscus of the lateral canthus for 30 s.

### *Measurement of GC density*

Surgically excised eyes were fixed in 10% formalin and embedded in paraffin. Sections (6 μm) were stained with periodic acid-Schiff reagent. Sections from three eyes in each group (in two different sets of experiments) were examined and photographed with a Nikon Eclipse E400 microscope equipped with a Nikon DXM 1200 digital camera. GC density was measured in the superior and inferior bulbar and tarsal conjunctiva and expressed as number per 100 μm using Metavue 6.24r software (Molecular Devices).

### *In vivo analysis of adoptively transferred CFSE-labeled lymphocytes*

Spleen cells ($1 \times 10^7$/ml) were collected from BALB/c mice subjected to DS and were labeled with 10 μM CFSE (Molecular Probes) for 15 min at room temperature. Cells were washed three times in RPMI 1640 and were injected i.p. into BALB/c mice ($1 \times 10^7$ spleen cells/0.1 ml/mouse).

### *Immunohistochemistry*

OCT-embedded globes with attached lids were sectioned at 10-μm thickness and stained for the expression of mouse CD4 (rat anti-mouse CD4; rat IgG2a, k; clone H129.19; BD Pharmingen) using Vectastain Elite ABC reagents (Vector Laboratories).

### *Adoptive cell transfer and anti-Thy 1.2 Ab treatment*

Spleens and cervical lymph nodes (CLN) were collected from mice subjected to DS and NS, and one donor-equivalent of either spleen or CLN cells was transferred i.p. to syngeneic nude mice. One donor-equivalent is defined as the number of cells remaining after the respective in vitro manipulation (e.g., anti-Thy 1.2 Ab treatment or $CD4^+$ T cell enrichment) of a single spleen or CLN from a single donor. The remaining cells represent the total lymphocyte population for that spleen cell category for a single donor. One splenic equivalent of T cells was equal to $\sim 5 \times 10^7$ cells. Spleen cells were depleted of T cells by in vitro treatment with anti-Thy 1.2 Ab (BD Pharmingen) in the presence of complement. Control aliquots of spleen cells were treated with complement alone. Cells were washed with RPMI 1640 medium before being adoptively transferred in 0.1 ml of RPMI 1640 medium.

### *$CD4^+$ and $CD25^+$ T cell enrichment*

BALB/c CLN cell suspensions were enriched for $CD4^+$ T cells by positive selection using rat anti-mouse CD4-conjugated magnetic microbeads (MACS system; Miltenyi Biotec) as described previously (15). The CD4-enriched cell suspensions contained >87% $CD4^+$ T cells as determined by flow cytometry. $CD4^+CD25^+$ T cells were enriched using a mouse $CD4^+CD25^+$ regulatory T cell magnetic isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Permeabilized $CD4^+CD25^+$ cells were tested for the expression of cytoplasmic forkhead/winged helix transcription factor (Foxp3) protein. Briefly, $CD4^+CD25^+$ cells were washed three times in HBSS, resuspended in Cytofix/Cytoperm solution (BD Pharmingen), and incubated for 30 min at room temperature. Cells were washed three times in Perm/Wash buffer and resuspended with an FITC-labeled rat anti-mouse Foxp3 Ab (eBiosciences) at a concentration of 1 μg/ml for 30 min on ice. Cells were washed in Perm/Wash buffer three times, incubated with PE-labeled secondary Ab for 20–30 min at 4°C, washed three additional times in Perm/Wash buffer, fixed in 1% paraformaldehyde, and assessed for fluorescence in a FACScan flow cytometer (BD Biosciences). The results were analyzed using CellQuest version 3.1f software (BD Biosciences). $CD4^+CD25^+$ cells were found to be 89% $Foxp3^+$ (data not shown).

### *In vivo depletion of $CD25^+$ cells*

In vivo depletion of $CD25^+$ regulatory T cells was achieved as described previously (16). Briefly, anti-CD25 Ab (hybridoma PC61; American Type Culture Collection) was administered i.p. to normal BALB/c mice 7 days before and on the day DS was initiated.

### *Statistics*

## Results

### *DS induces immune-mediated inflammation of the ocular surface and lacrimal gland*

C57BL/6 mice exposed to DS displayed altered corneal epithelial barrier function, which was reflected in an 85% increase in the uptake of the OGD label that was used to measure the integrity of the corneal epithelial barrier (Fig. 1). Histopathological analysis revealed reduced numbers of conjunctival GC, decreased tear production, and $CD4^+$ T cell infiltration of the conjunctival basal

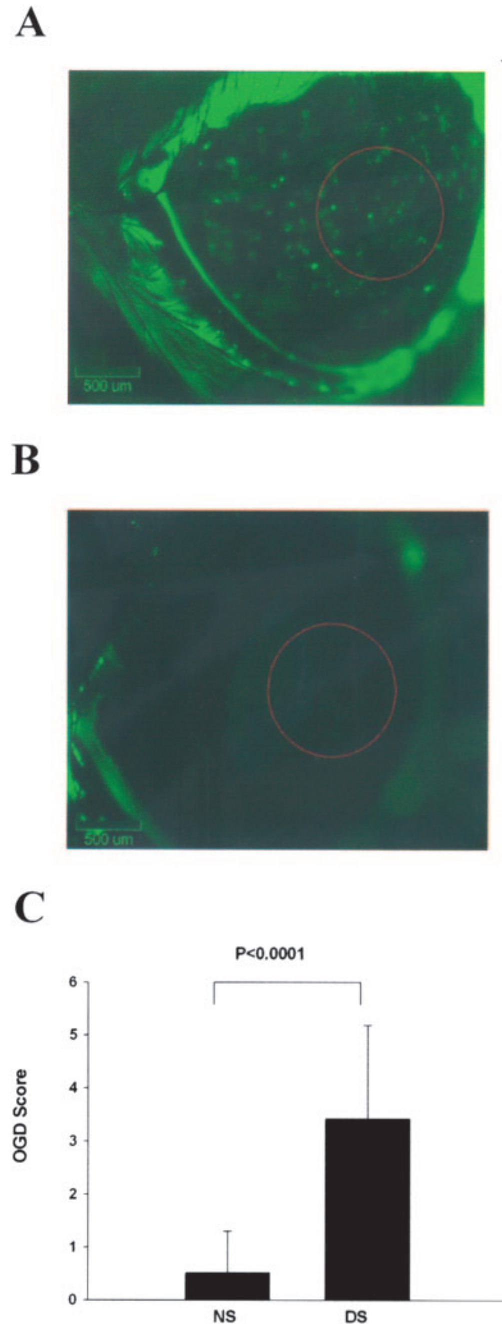


**FIGURE 1.** Increased corneal permeability induced by DS. C57BL/6 mice were subjected to 5 days of DS or NS conditions. Mouse corneas were viewed and photographed under cobalt blue light 10 min after application of 1 μl of the fluorescent molecule OGD to the ocular surface. *A*, Ocular surface of mouse subjected to 5 days of DS. *B*, Ocular surface of untreated,

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epithelium (Fig. 2, *A–E*). The greater severity of keratoconjunctivitis in C57BL/6 mice than BALB/c mice is consistent with the observation that BALB/c mice have a larger repertoire of $CD4^{+}CD25^{+}$ regulatory T cells compared with C57BL/6 mice (17). The role of $CD4^{+}CD25^{+}$ regulatory T cells was tested by treating BALB/c mice with anti-CD25 Ab to deplete natural regulatory T cells before subjecting the mice to DS. Anti-CD25-treated BALB/c mice developed significant keratoconjunctivitis and displayed a steep reduction in the density of conjunctival GC compared with the isotype control mice and mice subjected to DS without Ab treatment (Fig. 2*F*).

*DS-induced lacrimal keratoconjunctivitis can be adoptively transferred with $CD4^{+}$ T cells*

Adoptive transfer experiments were performed to determine the role of T cells in the ocular inflammation elicited by DS. Draining CLN cells were collected from BALB/c mice after a 5-day exposure to DS and were adoptively transferred to athymic (nude) BALB/c mice that were maintained under NS conditions (50–75% relative humidity). Even though the adoptive cell transfer recipients were NS, they had reduced tear production and developed LKC after receiving CLN cells from donors treated with DS (Fig. 3). Inflammation was immune mediated, because the nude mouse recipients of CLN cells from NS donors maintained normal tear production and did not develop inflammation of the conjunctiva (Fig. 3, *B* and *C*). Importantly, inflammation in recipients of CLN cells from DS donors was restricted to the lacrimal functional unit (cornea, conjunctiva, and LG), and no significant inflammatory cell infiltrates were found in the salivary gland, oral mucosa, thyroid, heart, lung, colon, spleen, adrenal gland, or vagina (data not shown). Histopathological analysis revealed significant LG inflammation and loss of conjunctival GC, which coincided with a steep

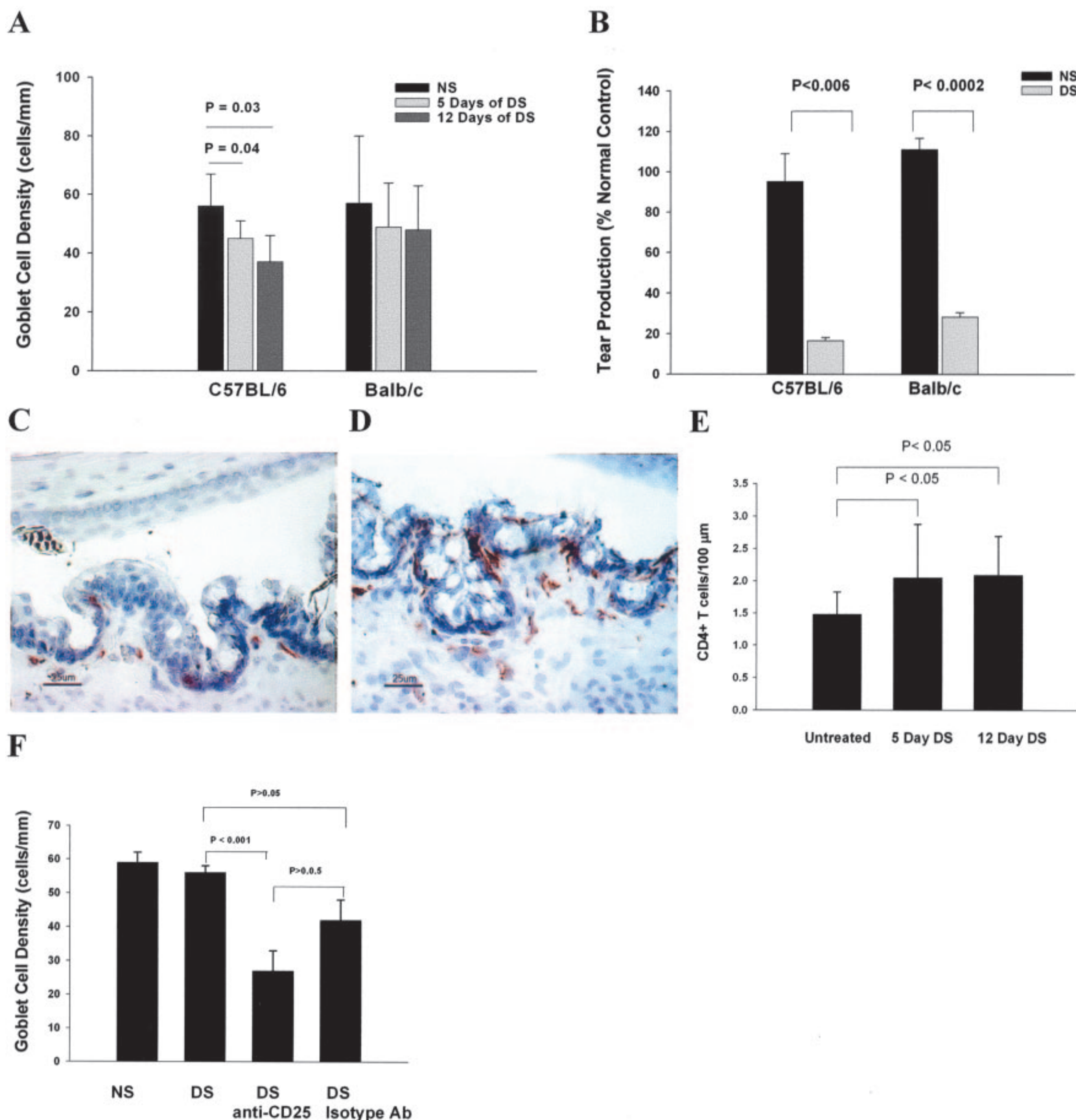


**FIGURE 2.** DS induces LKC. C57BL/6 mice (*A–E*) and BALB/c mice (*A*, *B*, and *F*) were maintained in either NS conditions (50–75% relative humidity) or DS (30–35% relative humidity) for either 5 days (*A–E*) or 12 days (*A* and *C*). Five days of DS consistently produced LKC in C57BL/6 mice. *A*, Reduced numbers of conjunctival GC; *B*, diminished tear production; *C*, $CD4^{+}$ cell density in conjunctivae of NS mice. *D*, Mice treated with DS; $CD4^{+}$ T cells

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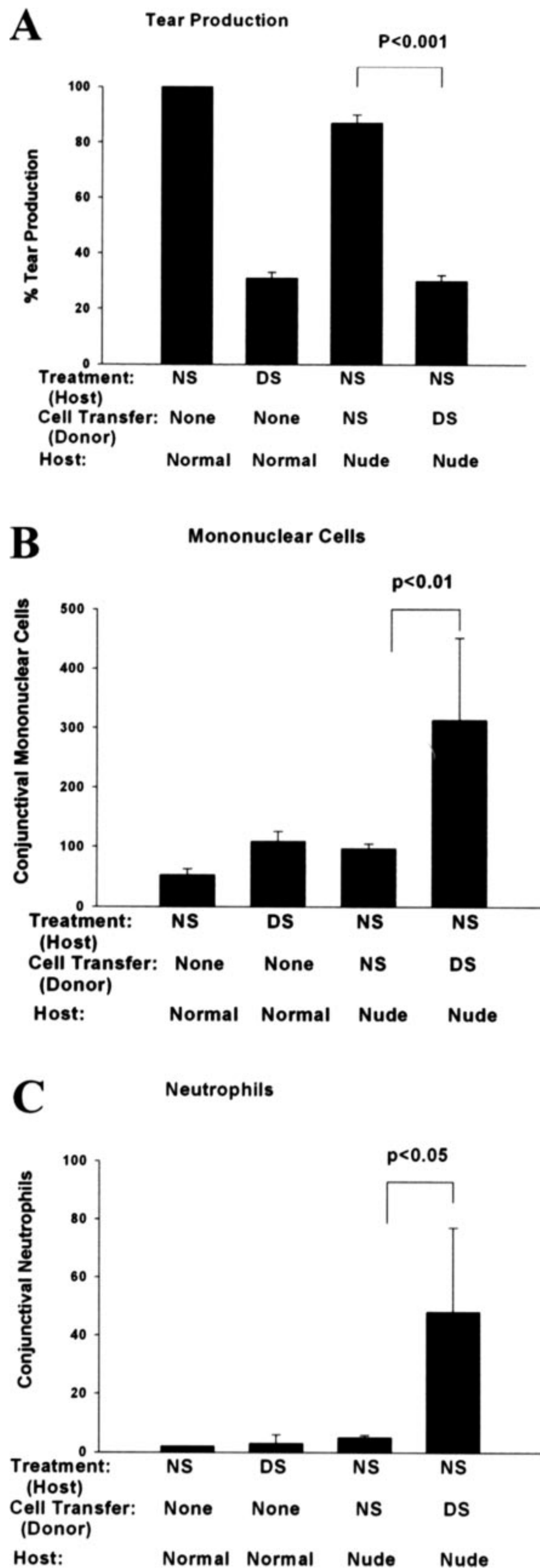


**FIGURE 3.** Adoptive T cell transfer of LKC. Euthymic BALB/c mice (normal) were subjected to either DS or NS conditions for 5 days, and one donor-equivalent of CLN cells was adoptively transferred to each athymic (nude) BALB/c nude mouse recipient. Nude mice were maintained under NS conditions after adoptive cell transfer and were assessed for tear production

reduction in tear production (Fig. 3*A*) and the appearance of ocular surface inflammation (Fig. 3, *B* and *C*). Immunohistochemical staining demonstrated a significant infiltrate of $CD4^+$ T cells in the LG in the adoptive cell transfer recipients (Fig. 4). Inflammation was T cell dependent, because in vitro treatment of CLN cell suspensions with anti-Thy 1.2 Ab plus complement before adoptive transfer prevented the development of LKC (Fig. 5*A*). Moreover, histopathological examination of the eyes and LGs of recipients of CLN cell suspensions depleted of T cells demonstrated significantly reduced mononuclear cellular infiltrates in the conjunctivae and the sparing of 80% of the normal tear production (Fig. 5, *B* and *C*). To determine whether the $CD4^+$ T cells in the conjunctivae and LGs contributed to LKC, adoptive transfer experiments were performed in which either $CD4^+$ T cell-enriched or $CD4^+$ T cell-depleted CLN cell suspensions were collected from mice subjected to DS and transferred to syngeneic BALB/c nude mice. Recipients of $CD4^+$ T cell-enriched CLN cells developed intense LG inflammation (Fig. 5*D*), with significant increases in the number of both conjunctival mononuclear cells and neutrophils (data not shown). Moreover, $CD4^+$ T cells were detected in the LG of the adoptive transfer recipients (Fig. 5*G*). Additional evidence of $CD4^+$ T cell involvement was demonstrated in experiments in which $CD4^+$ T cells were collected from mice subjected to DS, labeled with CFSE, and adoptively transferred to nude mice that were maintained in an NS environment. Accumulations of CFSE-labeled $CD4^+$ T cells were found in the conjunctival epithelium of the nude mice that received adoptive cell transfers (Fig. 5*H*), but were absent in nonocular organs (data not shown). By contrast, adoptive transfer of CFSE-labeled $CD4^+$ T cells from normal donors did not preferentially localize in the corneas or conjunctivae of nude mouse recipients (Fig. 6), suggesting that mice subjected to DS develop $CD4^+$ T cells that preferentially localize in the eye and LGs, where they produce inflammatory disease, GC loss, and LG dysfunction.

### *Role of $CD4^+CD25^+$ T cells in mitigating LKC*

The inflammation produced in the adoptive cell transfer recipients was consistently more severe in nude mice than euthymic mice, suggesting the possible mitigating effects of one or more regulatory T cell populations in the euthymic host. To examine the role of natural regulatory T cells in LKC, euthymic BALB/c mice were treated with anti-CD25 mAb to deplete putative natural regulatory T cells before receiving adoptively transferred T cells from BALB/c donors treated with DS. Euthymic BALB/c

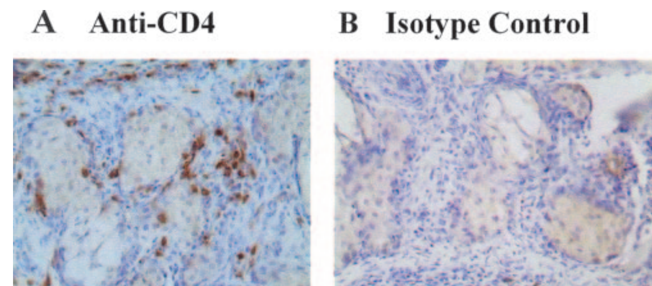


**FIGURE 4.** $CD4^+$ T cell infiltrate in lacrimal glands in recipients of adoptively transferred $CD4^+$ T cells from mice subjected to DS. *A*, $CD4^+$

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