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Desiccating Stress Induces T Cell-Mediated Sjögren’s Syndrome-Like Lacrimal Keratoconjunctivitis

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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.

Materials and Methods

Mice

Female BALB/c, T cell-deficient nude BALB/c (BALB/cByJ-HH11<+/−>), 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 Al- lergan 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 (Cafaro) 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...
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 E800 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 × 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 × 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 ∼5 × 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 FACSscan 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

Values for cell counts in tissue specimens and tear production were evaluated by either Student’s t test or ANOVA.

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 layer.
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. 2F).

**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.](http://www.jimmunol.org/) 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 (brown stain) in basal conjunctival epithelium. E, CD4+ T cell density in conjunctivae of DS and NS mice. F, Reduced conjunctival GC density in BALB/c mice treated with anti-CD25 before exposure to 5 days of DS. There were three animals in each group in two independent experiments (i.e., n = 6 mice/group).
reduction in tear production (Fig. 3A) 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. 5A). 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. 5D), 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. 5G). 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. 5H), 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 mice...
mice treated with anti-CD25 Ab before receiving adoptively transferred CD4⁺ CLN from DS donors developed severe LKC and a 38% reduction in tear production (Fig. 7). To confirm that the CD4⁺CD25⁺ T cells exerted a mitigating effect on the development of dry eye disease, nude mice were reconstituted with one spleen cell equivalent of either CD4⁺CD25⁺Foxp3⁺ putative regulatory T cells from euthymic, BALB/c donors that had been maintained in an NS environment. On the same day, the nude mice also received pathogenic, LKC-inducing CD4⁺T cells from BALB/c donor mice that had been maintained for 5 days in a DS environment. Nude mice reconstituted with CD4⁺CD25⁺Foxp3⁺ putative regulatory T cells resisted the development of LKC and maintained 90% tear production compared with a 28% reduction in tears in nude mice not receiving CD4⁺CD25⁺ T cells (Fig. 8A). Moreover, CD4⁺CD25⁺Foxp3⁺ reconstituted mice resisted inflammation and expressed a 60% reduction in the number of

FIGURE 5. DS-induced LKC is mediated by T cells. BALB/c mice were placed in DS for 5 days, and CLN and spleen cells were collected. CLN cells were treated with anti-Thy 1.2 Ab and complement to deplete T cells. One donor-equivalent of unfractionated or CD4⁺ T cell-enriched CLN or spleen cells was injected i.p. into each BALB/c nude mouse. Nude mice were kept under NS conditions. Tissues were collected for histology 48 h later. A. Histopathology of conjunctiva, cornea, and LG after adoptive CLN cell transfer. Note the absence of GC in conjunctivae of recipients of untreated or complement-treated CLN cells. Normal GC (short arrows) in hosts receiving T cell-depleted CLN cells. Corneal inflammation (long arrow) is absent in recipients of anti-Thy 1.2-treated CLN cells. B. Mononuclear cells infiltrate the conjunctivae of CLN cell recipients. C. Reduced tear production in CLN cell recipients. D. Intense LG inflammation (arrow) in recipients of CD4⁺ T spleen cells from DS donors. E. Normal LG. F. Isotype control Ab (background) staining of LG from a recipient of CD4⁺ spleen cells from DS donors. G. Anti-CD4 immunostaining (brown) of LG from same mouse as that in F. H. Accumulation of CD4⁺ T cells in the conjunctiva after adoptive transfer of CFSE-labeled, CD4⁺ T cell-enriched spleen cells. There were five animals in each group.

FIGURE 6. Absence of conjunctival and corneal inflammation in BALB/c nude mice that received CFSE-labeled CD4⁺ spleen cells from NS BALB/c donor mice. There were five animals in each group.
mononuclear cells and a 97% reduction in the number of neutrophils that infiltrated the conjunctiva (Fig. 8, B and C). Histopathological analysis confirmed the mitigating effects of adoptively transferred CD4+CD25+Foxp3+ cells. Conjunctival GC were virtually eliminated in recipients of CD4+ T cells from DS donors, but were spared in nude mice that were reconstituted with CD4+CD25+Foxp3+ cells from NS donors (Fig. 8, D–F). Likewise, corneal and LG inflammation was extinguished in CD4+CD25+Foxp3+ cell recipients (Fig. 8, G–L).

Discussion

The causes of dry eye diseases, including SS, are multifactorial and involve immunogenetic, hormonal, and environmental factors (18, 19). In this study we demonstrated the isolated effect of a single environmental factor, DS, on the development of immunemediated dry eye disease. Animals subjected to DS developed ocular surface lesions that mimicked those found in dry eye patients. The preponderance of CD4+ T cells in LG inflammation bore a striking resemblance to the lesions found in SS patients and in animal models of SS (19–21). More important, the results indicate that tear insufficiency alters the function of the corneal epithelium and elicits inflammation of the ocular surface. The DS-induced inflammation elicited the generation of autoreactive CD4+ T cells that infiltrated all three components of the lacrimal functional unit (i.e., cornea, conjunctiva, and LG). Once generated, pathogenic CD4+ T cells produced ocular surface and LG inflammation, even in animals housed in an NS environment.

The milder ocular surface inflammation induced by DS in euthymic BALB/c mice compared with C57BL/6 mice is consistent with the hypothesis that DS-induced LKC is mediated by CD4+ T cells, but is mitigated by CD4+CD25+ natural regulatory T cells, because BALB/c mice have a better developed repertoire of CD4+CD25+ regulatory T cells than C57BL/6 mice (17). Moreover, elimination of CD25+ T cells in euthymic mice increased the severity of LKC, whereas reconstitution of athymic mice with CD4+CD25+ regulatory T cells mitigated LKC.

A phenomenon similar to what we have reported also occurs in some patients who are exposed to ocular surface dryness from any number of mechanisms. It is not unusual for patients who take medications with anticholinergic side effects, such as antidepresants, to develop a permanent dry eye despite cessation of the offending medication. These patients frequently respond to immunomodulatory therapies such as topical corticosteroids and cyclosporine. This phenomenon is also observed when there is incomplete lid closure after blepharoplasty surgery where there is exposure and desiccation of a portion of the ocular surface. Patients with well-documented normal tear production before this surgery may develop a marked depression in tear production that persists after the exposure is treated. However, these patients frequently respond to immunomodulatory therapies. We believe that persistent DS results in the up-regulation of ocular surface Ags that initiate an autoimmune LKC. This is influenced by a variety of host susceptibility factors, including age, gender, and systemic immune dysregulation (e.g., rheumatoid arthritis or graft-vs-host disease).

The present findings also suggest that mild inflammation produced by transient DS is brought under control by CD4+CD25+Foxp3+ regulatory T cells. However, in systemic autoimmune diseases, such as SS, ocular surface inflammation occurs in conjunction with inflammation in multiple organ systems and might be more resistant to immune regulation by CD4+CD25+Foxp3+ regulatory T cells. In SS, it is widely believed that inflammation of the LG compromises tear production, which culminates in desiccation of the ocular surface. The desiccated ocular surface, in turn, provokes inflammation and might elicit the generation of a danger signal, which breaks tolerance to self epitopes expressed on cells on the ocular surface. Our results indicate that desiccation of the ocular surface induces an immune response not only to the corneal and conjunctival epithelium, but also to elements in the LG, suggesting that there are shared epitopes among the components of the LFU. Identifying these epitopes will be important for devising therapeutic strategies that may be useful in the management of >10 million dry eye patients.

Disclosures

The authors have no financial conflict of interest.
Figure 8. CD4+CD25+Foxp3+ T cells mitigate LKC. BALB/c nude mice were not reconstituted (none) or were reconstituted with CD4+CD25+Foxp3+ T cells (putative suppressor cells) from euthymic BALB/c mice maintained in NS conditions. Pathogenic CD4+ T cells were isolated from euthymic BALB/c mice subjected to DS and were adoptively transferred to nude mice on the same day as the reconstitution with CD4+CD25+Foxp3+ T cells. A, Reduced tear production. B, Mononuclear cell infiltrates into the conjunctivae. C, Neutrophil infiltration into conjunctivae. Histopathology of conjunctiva (D–F), cornea (G–I), and LG (J–L) is shown. Groups consisted of untreated controls (D, G, and J), recipients of pathogenic CD4+ T cells from DS donors (E, H, and K), and recipients of pathogenic CD4+ T cells from DS donors and CD4+CD25+ T cells from NS donors. Note the loss of conjunctival GC (arrows; E) and intense LG inflammation (K) in a nude mouse that received CD4+ T cells from a DS mouse, but was not reconstituted with normal CD4+CD25+Foxp3+ T cells. There were two animals in E, H, and K; all other panels had five animals in each group.

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