Ocular Surface APCs Are Necessary for Autoreactive T Cell-Mediated Experimental Autoimmune Lacrimal Keratoconjunctivitis


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Ocular Surface APCs Are Necessary for Autoreactive T Cell-Mediated Experimental Autoimmune Lacrimal Keratoconjunctivitis

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As specialized sentinels between the innate and adaptive immune response, APCs are essential for activation of Ag-specific lymphocytes, pathogen clearance, and generation of immunological memory. The process is tightly regulated; however, excessive or atypical stimuli may ignite activation of APCs in a way that allows self-Ag presentation to autoreactive T cells in the context of the necessary costimulatory signals, ultimately resulting in autoimmunity. Studies in both animal models and patients suggest that dry eye is a chronic CD4+ T cell-mediated ocular surface autoimmune-based inflammatory disease. Using a desiccating stress-induced mouse model of dry eye, we establish the fundamental role of APCs for both the generation and maintenance of ocular-specific autoreactive CD4+ T cells. Subconjunctival administration of liposome-encapsulated clodronate efficiently diminished resident ocular surface APCs, inhibited the generation of autoreactive CD4+ T cells, and blocked their ability to cause disease. APC-dependent CD4+ T cell activation required intact draining cervical lymph nodes, as cervical lymphadenectomy also inhibited CD4+ T cell-mediated dry eye disease. In addition, local depletion of peripheral conjunctival APCs blocked the ability of dry eye-specific CD4+ T cells to accumulate within the ocular surface tissues, suggesting that fully primed and targeted dry eye-specific CD4+ T cells require secondary activation by resident ocular surface APCs for maintenance and effector function. These data demonstrate that APCs are necessary for the initiation and development of experimental dry eye and support the standing hypothesis that dry eye is a self-Ag-driven autoimmune disease.

Dry eye, also known as dysfunctional tear syndrome, is a common ocular surface disease with high prevalence and significant morbidity worldwide (1, 2). Patients experience a variety of symptoms including ocular discomfort, fatigue, and chronic pain, accompanied by blurred and fluctuating vision. In the most severe cases, corneal opacification or ulceration may result in reduced vision and blindness. Emerging evidence suggests that dry eye is an ocular surface autoimmune-based inflammatory disease. Environmental and/or microbial stress, combined with genetically predisposed factors, is thought to perpetuate chronic autoreactive T cell-mediated inflammation and dysfunction of the lacrimal function unit (LFU; cornea, conjunctiva, lacrimal glands, and meibomian glands) (3). Inflammatory cell infiltration within the LFU tissues correlates with elevated proinflammatory cytokine levels, increased epithelial cell apoptosis, and diminished mucin-secreting goblet cell numbers, coupled with decreased tear production in animal models (4) and patients with dry eye (5).

CD4+ T cells make a prominent contribution to chronic inflammation during the immunopathogenesis of dry eye, under-scoring its basis as an autoimmune-based inflammatory disease. This notion is supported by several lines of evidence: 1) activated CD4+ T cells are localized within the ocular surface tissues of dry eye patients (5, 6); 2) CD4+ T cells are sufficient to induce dry eye in mice (4); and 3) compounds that inhibit T cells (e.g., cyclosporine A) attenuate dry eye disease in both animals and humans (5, 7). Dry eye disease, induced in a mouse model of autoimmune lacrimal keratoconjunctivitis (ALKC), is a valuable tool for defining the underlying mechanisms of CD4+ T cell-mediated disease (4). Mice exposed to environmental stress (i.e., desiccating stress [DS]) and administered scopolamine develop clinical and histopathological similarities to the human disease, including a robust CD4+ T cell infiltrate into the LFU (4). Moreover, CD4+ T cells isolated from the cervical lymph nodes (CLNs) and/or spleen of experimental dry eye mice readily traffic to the ocular surface, where they mediate full-blown dry eye disease when adoptively transferred to athymic T cell-deficient nude recipient mice (4). CD4+ T cell homing and pathogenesis was restricted to the ocular surface tissues of the nude recipient mice, indicating that autoreactive CD4+ T cells are targeted to the ocular surface during activation within the CLN and spleen of donor mice exposed to DS.

Engagement with APCs is necessary for activation and differentiation of CD4+ T cells within the secondary lymphoid organs (e.g., interaction with dendritic cells [DCs]) and reactivation and maintenance of activated T cells at sites of inflammation (e.g., interaction with DCs and/or macrophages). Activation of naïve T cells by competent APCs is well defined (8), but much less is known regarding the requirements of APC-dependent reactivation of fully primed, activated T cells. DCs are among the most potent professional APCs and in the presence of the proper costimulatory signals may effectively present self-Ag to autoreactive T cells during the development and progression of autoimmune disease (9, 10). In the eye, DCs and macrophages are present within the ocular surface tissues (11). For example, DCs are present in the healthy...
corneal stroma (12, 13), increasing in number and with expression of costimulatory molecules following inflammatory insults (14). MHC class II (MHC II) molecules are required for presentation of antigenic epitopes to CD4+ T cells and are also upregulated on APCs localized within the ocular surface tissues in animal models (15) and in patients with dry eye (16). Homing of CD11c+CD11b+MHC II+ APCs from the ocular surface to the CLN is dependent on CCR7–CCL21 signaling (13); more recently, homing of mature MHC II+ APCs to the draining CLNs was shown to be associated with enhanced lymphoangiogenesis on the ocular surface during DS-induced experimental dry eye (17). Nonetheless, the absolute role of APCs in the initiation and progression of dry eye has not been evaluated.

In this report, we demonstrate for the first time, to our knowledge, that APCs are necessary for both the generation and maintenance of ocular-specific autoreactive CD4+ T cells during the immunopathogenesis of experimental dry eye. Local depletion of APCs in the conjunctiva of mice exposed to DS inhibited the generation of autoreactive CD4+ T cells and blocked the ability to adoptively transfer disease to T cell-deficient nude recipient mice. Similarly, surgical removal of the CLN by cervical lymphadenectomy inhibited CD4+ T cell activation and T cell-mediated dry eye disease. In addition, fully primed and targeted dry eye-specific CD4+ T cells did not readily accumulate and cause disease in APC-depleted mice, implying that secondary activation by resident ocular surface APCs is required for T cell maintenance and effector function within the ocular surface tissues. These data demonstrate that ocular surface APCs are necessary for the initiation and development of dry eye in a mouse model of autoreactive T cell-mediated experimental ALKC and support the paradigm that dry eye is a self-Ag-driven autoimmune disease.

Materials and Methods

Dry eye mouse model of ALKC

Female C57BL/6 mice (6–8 wk old) were purchased from Taconic Farms (Oxnard, CA). Induction of dry eye using the mouse model of ALKC was performed as previously described (4, 18–20). In brief, mice were exposed to DS in perforated cages with constant airflow from fans positioned on both sides and room humidity maintained at 30–35%. Injection of scopolamine hydrobromide (0.5 mg/0.2 ml; Sigma-Aldrich, St. Louis, MO) was administered three to four times a day (three times a day: 08:00, 12:00, and 17:00 h; four times a day: 08:00, 12:00, 14:00, and 17:00 h) on alternating hind flanks to augment disease. DS was induced for 5 consecutive d, whereas control mice were maintained at the same conditions corresponding to the 3/9 and 6/12 positions on a clock.

Adoptive transfer

CD4+ T cells were purified from CLNs and spleen isolated from control or DS mice (5 d) and were adoptively transferred (5 × 10^6 cells suspended in 100 µl sterile PBS) via i.p. injection into nude recipient mice as previously described (4, 21). Mice were sacrificed 3 d posttransfer, and tears and ocular surface tissues were collected for analysis.

Preparation of dichloromethylene diphosphonate (clodronate) liposomes

Liposome-encapsulated clodronate has been widely used to deplete phagocytic APCs; ingestion of clodronate liposomes leads to intracellular release and accumulation of clodronate, which induces apoptosis (22). Clodronate liposomes (C12MDP-LIP) were prepared as previously described (23). Briefly, 8 mg cholesterol (Sigma-Aldrich) and 86 mg phosphatidylcholine (Sigma-Aldrich) were dissolved in 10 ml chloroform in a round-bottom flask. After low-vacuum rotary evaporation at 40°C, a thin film formed on the inner surface of the flask. Either 10 ml PBS, to make PBS containing liposomes, or 2.5 g C12MDP (clodronate; Sigma-Aldrich) dissolved in 10 ml PBS, to make C12MDP-LIP, was added to the flask. The film was then dispersed by gentle rotation for 18 h and sonicated for 3 min at room temperature to disperse the liposomes. Liposomes were washed twice by centrifugation in PBS at 10,000 × g for 20 min and resuspended in 10 ml PBS. C12MDP-LIP and PBS containing liposomes were stored at 4°C and used within 14 d of preparation. All liposomes ranged in size from 100 nm to 3 µm in diameter. Each 100 µl C12MDP-LIP suspension contained 1.0 mg clodronate.

Depletion of APCs on the ocular surface using liposome-encapsulated clodronate

Donor mice received subconjunctival injections of 3 µl (two injections/eye) on three separate occasions starting 4 d before exposure to DS and then again on days 1 and 3 of DS. Where indicated, nude recipient mice were treated with clodronate or PBS-loaded liposomes on days −4 and −1 prior to receiving DS-specific CD4+ T cells from dry eye mice according to the following permutations: ocular dexter (OD), clodronate; ocular sinister (OS), PBS; OD, clodronate; OS, no treatment; OD, PBS; and OS, no treatment. Injections were placed within the subconjunctival space; the injections alternated between temporal-nasal and inferior-superior placement corresponding to the 3/9 and 6/12 positions on a clock.

Cervical and axillary lymphadenectomy

Cervical or axillary lymph nodes were surgically excised from C57BL/6 mice (n = 5/group) under general anesthesia with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich) 7 d prior to induction of DS. Sham surgery was performed in a separate group of mice and consisted of surgical incision and wound closure with staples. All mice were allowed to fully recover before being returned to their cage.

Cytokine and chemokine levels in tears during experimental ALKC

Relative levels of select cytokines and chemokines present in the tears were evaluated over the course of experimental ALKC using Luminex analysis (Luminex, Austin, TX) as previously described (21). In brief, tears were collected at various time points (e.g., 0 or 5 d post-DS); 1.5 µl Beadlyte assay buffer (Millipore, Bellerica, MA) was placed on each eye, and 1 µl/eye was collected and combined with 8 µl Beadlyte buffer. Buffer and tear fluid were collected by capillary action using a 1-ml volume glass capillary tube (Drummond Scientific, Broomall, PA) positioned in the tear meniscus of the lateral canthus. Samples were frozen at −80°C until the time of assay. Protein levels were assessed using the appropriate Millipore beads (Millipore) and analyzed on a Luminex 100 or Luminex FLEXMAP 3D (Luminex).

T cell isolation and flow cytometry

Control and DS mice were sacrificed at various time points (e.g., days 0, 1, 3, 6, 7, and 10) following sustained exposure to DS. CLNs and spleen were harvested, RBCs were lysed, and single-cell suspensions were generated according to standard protocol. Cells were phenotyped using FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD69, and APC-conjugated rat anti-mouse MHC II, CD83, CD86, and CCR7. In all cases, isotype-matched conjugated Abs were used as controls. Cells were blocked with CD16/32 Ab for 10 min and then incubated with primary Abs for 20–40 min at 4°C, washed, and analyzed using an FACStar flow cytometer (BD Biosciences, Mountain View, CA) and FlowJo software (Tree Star, Ashland, OR). Frequency data are presented as the percentage of positive cells within the gated population.

Histology

Whole eyes including lids were surgically excised, fixed in 10% formalin, and embedded in paraffin. Eight-micrometer sections were stained with H&E or periodic acid–Schiff (PAS) reagent to evaluate gross inflammatory cell infiltration; and 3, intense inflammatory cell infiltration. Whole eyes including lids were surgically excised, embedded, and flash frozen in optimal cutting temperature (OCT) compound; VWR, Suwanee, GA). Eight-micrometer sagittal sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at

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CD4+ T cell activation, indicated by an increased frequency of mice (day 0) (Fig. 1). Accumulation of mature DCs preceded the temporal activation of CD4+ T cells (15.1% to 6 d DS (16.8% to 6 d DS)). Among ocular surface monocytes, the numbers of CD11c+ DCs (1.5% versus 0.2%) compared with naive mice (day 0) (A), which correlated with activation of CD4+ T cells during initiation and development of experimental dry eye (B). Data are represented as average frequency ± SEM. Statistically significant values (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) are indicated relative to naive (day 0) control mice.

**Results**

**Accumulation of mature DCs correlates with CD4+ T cell activation within the regional CLNs**

Previous studies demonstrated that exposure to DS induces production of proinflammatory cytokines and chemokines (e.g., IL-1β, TNF-α, CCL2, CCL3, CCL5, and CXCL10) (4, 20, 25). Acute-response cytokine production was observed early following exposure to DS; for example, TNF-α was significantly (*p ≤ 0.05) elevated in the tears of dry eye mice by 24 h (44.1 ± 9.8 pg/ml) following sustained exposure to DS compared with naive mice (16.2 ± 1.5 pg/ml). As DCs are potent APCs that function at the interface of the innate and adaptive immune response, the kinetics of CD11c+ DC activation was evaluated within the regional draining CLN. Acute cytokine production was associated with an increased percentage of CD11c+ DCs (1.95 ± 0.3% versus 0.91 ± 0.20%; p ≤ 0.05) within the draining CLNs (Fig. 1A). Moreover, the CD11c+ DCs displayed elevated expression of activation/maturation markers, including MHC II+ (0.94 ± 0.21% versus 0.55 ± 0.11%), CD83+ (1.52 ± 0.36% versus 0.38 ± 0.10%; p ≤ 0.05), CD86+ (1.32 ± 0.20% versus 0.62 ± 0.13%; p ≤ 0.05), and CCR7+ (0.90 ± 0.15% versus 0.48 ± 0.14%) compared with naive mice (day 0) (Fig. 1A). Accumulation of mature DCs preceded CD4+ T cell activation, indicated by an increased frequency of CD4+ T cells bearing the early activation marker CD69+. A significant increase (p ≤ 0.001) in the frequency of CD4+CD69+ T cells was observed as early as day 3 (15.1 ± 1.0%) and peaked by 6 d DS (16.8 ± 0.6%) as compared with control (day 0) mice (10.5 ± 0.5%) (Fig. 1B). These data are consistent with the temporal framework indicative of an Ag-specific immune response and support the hypothesis that the DS-induced DC activation/maturation mediates expansion of autoreactive lymphocytes within the regional lymph nodes during the immunopathogenesis of experimental dry eye.

**Liposome-encapsulated clodronate effectively depletes APCs within the ocular surface tissues**

To determine the functional role of APCs during the initiation and development of experimental dry eye, APCs present within the ocular surface tissues were depleted using liposome-encapsulated clodronate (22), which has been used successfully to deplete APCs in models of viral infection (26), corneal transplant (27, 28), and autoimmunity (29). Liposomes loaded with clodronate or PBS were injected (3 µl × two injections/eye) into the conjunctival space (alternating between opposing temporal-nasal and inferior-superior injections, corresponding to the 3/9 and 6/12 positions on a clock, respectively) 4 d before exposure to DS and then again on days −1 and 3 of DS. Mice treated with clodronate displayed a significant (p ≤ 0.001) reduction in the number of CD11b+ monocytes within the conjunctiva (12.5 ± 2.1) compared with mice that received PBS liposomes as a control (45.6 ± 3.9), without apparent toxicity to the surrounding epithelial cells (Fig. 2A, 2B). Among ocular surface monocytes, the numbers of CD11c+ DCs (1.5 ± 0.9) were significantly (p ≤ 0.01) decreased within the conjunctiva of clodronate-treated mice relative to mice that received PBS liposomes (4.2 ± 0.9) (Fig. 2C, 2D); the total number of Iba1+ macrophages followed a similar trend, with significantly (p ≤ 0.01) less cells in clodronate-injected mice (0.5 ± 0.2) compared with control (3.7 ± 1.0) (data not shown). Therefore, subconjunctival instillation of liposome-encapsulated clodronate is effective in reducing the overall number of ocular surface APCs, which includes a broad population of monocytes/macrophages (CD11b+/Iba1+) and DCs (CD11c+).

**APC depletion mutes ocular surface inflammation in mice following exposure to DS**

To determine if depletion of conjunctival APCs impacted the peripheral inflammatory response, proinflammatory cytokine/chemokine production, CD4+ T cell infiltration, and goblet cell numbers were evaluated. Mice treated with liposome-encapsulated clodronate showed a slight trend toward decreased levels of APC-derived cytokines (e.g., TNF-α, IL-6, and IL-12) (Fig. 3A) and
prominent T cell-derived cytokines (e.g., IL-2, IFN-γ, IL-17, and IL-10) compared with PBS controls, although the change was not significant, indicating that the ocular surface tissues were able to mount an acute proinflammatory response despite reduced APC counts. However, mice exposed to DS and treated with clodronate displayed a significant (p ≤ 0.01) decrease in the number of infiltrating CD4+ T cells (7.9 ± 2.0) within the conjunctiva compared with dry eye mice treated with PBS (20.1 ± 2.8) as a control (Fig. 3B, 3C). Reduced CD4+ T cell infiltration correlated with preservation of ocular surface tissues, assessed by a significant increase (p ≤ 0.001) in the numbers of goblet cells in clodronate-treated mice (77.9 ± 6.6) relative to PBS control (47.3 ± 5.1), which was not significantly different compared with goblet cell numbers found in naive mice (91.8 ± 7.0) (Fig. 3E). These results demonstrate that the absence of a fully intact population of resident ocular surface APCs significantly impacts T cell-mediated immunopathogenesis in dry eye mice, without disrupting the DS-induced acute proinflammatory cytokine response.

**APC depletion attenuates the generation of DS-specific autoreactive T cells and the development of dry eye following adoptive transfer to T cell-deficient mice**

We hypothesized that the absence of a complete repertoire of APCs within the ocular surface tissues would inhibit generation of ocular surface-specific autoreactive CD4+ T cells within the regional draining CLNs. To this end, the pathogenic capacity of CD4+ T cells isolated from APC-depleted donor mice and exposed to DS for 5 d was evaluated following adoptive transfer to T cell-deficient nude recipient mice. The current study shows that CD4+ T cells are activated by 5 d of DS (Fig. 1B), and our previous work demonstrated that CD4+ T cells from untreated DS mice were sufficient to mediate experimental dry eye in the nude

**FIGURE 2.** Local delivery of clodronate liposomes depletes ocular surface APCs. A and C, Immunohistochemistry (IHC) using anti-CD11b+ or anti-CD11c+ Abs indicated that subconjunctival injections of liposome-encapsulated clodronate reduced the overall number of APCs within the conjunctiva, without apparent toxicity to the surrounding epithelial cells. Original magnification ×200. B and D, Quantification showed a significant decrease in CD11b+ and CD11c+ cells (*p ≤ 0.05, **p ≤ 0.001) compared with mice that received PBS-loaded liposomes as a control. Arrows indicate examples of CD11b+ and CD11c+ cells. Data are shown as average conjunctival cell counts ± SEM and representative of three independent experiments, with an n = 5 to 6 mice/group.

**FIGURE 3.** Depletion of APCs decreases CD4+ T cell accumulation within the ocular surface tissues of mice exposed to DS. Proinflammatory cytokine/chemokine production, CD4+ T cell infiltration, and goblet cell numbers were evaluated on the ocular surface between mice exposed to DS and treated with either clodronate- or PBS-loaded liposomes. A, Mice treated with clodronate displayed a slight, but nonsignificant, decrease in the levels of select epithelial cell, APC-derived (e.g., TNF-α, IL-6, and IL-12), and predominant T cell-derived cytokines (e.g., IL-2, IFN-γ, IL-17, and IL-10) within the tears compared with PBS controls at 5 d of DS. By contrast, clodronate-treated DS mice displayed a decrease in the number of infiltrating conjunctival CD4+ T cells by IHC (B) that was statistically significant compared with PBS controls (C). Decreased accumulation of CD4+ T cells correlated with increased numbers of PAS+ cells within the conjunctiva (D), indicating goblet cells were preserved in APC-depleted mice (E). A, Average concentration of protein (pg/ml) within a 2 μl tear volume ± SEM. B, CD4+ T cell staining in the conjunctiva. Original magnification ×200. C, Average conjunctival (Conj.) CD4+ T cell counts ± SEM. D, PAS+ goblet cell staining in the conjunctiva. Original magnification ×200. E, Average conjunctival goblet cell counts ± SEM. The data are representative of three independent experiments, with an n = 5 to 6 mice/group. Statistically significant values (**p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001) are indicated.
Recipients by 3 d posttransfer (4). By contrast, nude recipient mice receiving CD4+ T cells from clodronate-treated donor mice exposed to DS displayed a marked reduction ocular surface inflammation compared with recipients of CD4+ T cells from DS mice treated with PBS as a control (Figs. 4, 5). For example, a significant decrease ($p \leq 0.05$) in overall inflammatory cell infiltration was observed within the ocular surface tissues (Fig. 4A), reported as a lower severity score in recipients of CD4+ T cells from clodronate-treated donor mice (0.3 ± 0.1) compared with PBS controls (1.2 ± 0.4) (Fig. 4B). Moreover, CD4+ T cells isolated from clodronate-treated donor mice displayed a reduced capacity to accumulate within the ocular surface tissues of nude recipients (9.8 ± 2.3) relative to PBS controls (58.3 ± 12.6) (Fig. 4C, 4D). The absence of a CD4+ T cell accumulation correlated with preservation of ocular surface tissues (Fig. 4E); goblet cell counts were significantly ($p \leq 0.01$) higher in the conjunctiva of nude mice receiving CD4+ T cells from clodronate-treated donor mice (91.5 ± 0.4) compared with controls (42.0 ± 9.5) (Fig. 4F). Muted inflammatory cell infiltration was also associated with a striking reduction in the proinflammatory cytokine/chemokine response, indicated by decreased IL-1β, TNF-α, IL-2, IL-6, CCL5, CXCL10, IFN-γ, and IL-17 levels within the tears of nude recipients of CD4+ T cells isolated from clodronate-treated donor mice (Fig. 5). Moreover, there was also a decrease in IL-10 (112 ± 18 pg/ml) in nude mice receiving CD4+ T cells from APC-depleted mice exposed to DS compared with those receiving cells from PBS-liposome–treated controls (282 ± 86 pg/ml), suggesting that regulatory T cells derived from donor mice were not involved in dampening the pathogenic CD4+ T cell response. Taken together, these results suggest that the absence of APCs in mice exposed to DS inhibits generation of ocular-specific autoreactive CD4+ T cells, which otherwise readily traffic to the ocular surface tissues of nude recipient mice and mediate robust pathological changes resembling dry eye disease.

**Local depletion of APCs prevents accumulation of autoreactive T cells within the ocular surface tissues**

To determine if peripheral maintenance of fully primed and targeted DS-specific CD4+ T cells requires secondary activation by resident ocular surface APCs, nude recipient mice were treated with clodronate on days −4 and −1 prior to receiving pathogenic CD4+ T cells from dry eye mice. Subconjunctival injection of clodronate-loaded liposomes in the OD eye of nude recipient mice significantly reduced ($p \leq 0.001$) the average number of DS-specific CD4+ T cells (4.4 ± 1.4) accumulating within the conjunctiva compared with the contralateral OS eye of the same mice receiving PBS liposomes as an internal control (38.1 ± 7.6) (Fig. 6A). A similar significant ($p \leq 0.001$) decrease in CD4+ T cell

**FIGURE 4.** APC depletion in mice exposed to DS attenuates the generation of autoreactive CD4+ T cells and blocks the ability to adoptively transfer T cell-mediated disease to nude recipient mice. CD4+ T cells were isolated from the CLNs and spleen of clodronate- or PBS-treated mice exposed to DS and adoptively transferred to nude recipient mice to determine if the absence of a full repertoire of APCs within the ocular surface tissues inhibits generation of ocular surface-specific autoreactive CD4+ T cells. H&E staining (A) showed a significant decrease in overall inflammatory cell infiltration within the ocular surface tissues of nude recipients of CD4+ T cells from clodronate-treated donor mice compared with PBS controls (3 d postadoptive transfer) (B). Furthermore, IHC (C) confirmed that CD4+ T cells isolated from clodronate-treated donor mice did not readily accumulate within the conjunctiva, as there was only trace CD4+ staining, which accounted for a significant decrease in CD4+ T cells (D) and protection from the loss of PAS-positive goblet cells (E, F). A, H&E staining. Original magnification ×200. B, Overall inflammatory score ± SEM (scale 0–3). C, CD4+ T cell staining in the conjunctiva. Original magnification ×200. D, Average conjunctival (Conj.) CD4+ T cell counts ± SEM. E, PAS+ goblet cell staining in the conjunctiva. Original magnification ×200. F, Average conjunctival goblet cell counts ± SEM. The data are representative of three independent experiments, with $n = 5$ to 6 mice/group. Statistically significant values (*$p \leq 0.05$, **$p \leq 0.01$) are indicated relative to nude recipients of CD4+ T cells from PBS liposome-treated mice.
FIGURE 5. Depletion of APCs in mice exposed to DS inhibits the CD4+ T cell-mediated proinflammatory cytokine response following adoptive transfer to nude recipient mice. CD4+ T cells isolated from APC-depleted donor mice exposed to DS did not induce robust production of select acute response proinflammatory cytokines IL-1β (A), TNF-α (B), IL-6 (C); chemokines CCL5 (D) and CXCL10 (E); or predominant T cell-derived cytokines IL-2 (F), IFN-γ (G), and IL-17 (H) compared with mice receiving CD4+ T cells from DS mice treated with PBS liposomes at 3 d post-adoptive transfer. Data are shown as average concentration of protein (pg/ml) within a 2 μl tear volume ± SEM and representative of three independent experiments, with an n = 5 to 6 mice/group. Statistically significant values (p ≤ 0.05) are indicated relative to tear values of nude mice receiving CD4+ T cells from donor mice exposed to DS and treated with PBS liposomes as a control.

Accumulation was also observed when clodronate-treated OD eyes (7.1 ± 0.9) were compared with untreated OS eyes (36.9 ± 7.1) (Fig. 6B). By contrast, there was no difference between the number of infiltrating CD4+ T cells between control PBS-treated OD eyes (25.1 ± 3.2) and untreated OS eyes (30.0 ± 5.3), confirming that there is not a bias between right and left eyes with respect to T cell-mediated inflammation (Fig. 6C). Attenuated accumulation of DS-specific CD4+ T cells within the ocular surface tissues of the OD eyes from clodronate-liposome-treated nude recipient mice correlated with preservation of conjunctival goblet cells indicated by increased numbers compared with PBS-liposome–treated (49.7 ± 6.8 versus 33.8 ± 7.6) or untreated (44.8 ± 7.0 versus 18.8 ± 3.5; p ≤ 0.01) internal OS control eyes (Fig. 7). Average goblet cell counts for healthy untouched nude mouse eyes were 80.1 ± 9.7, indicating that APC depletion in nude recipient mice preserves goblet cells, but not completely to normal levels. Collectively, these results suggest that DS-specific CD4+ T cells infiltrating the ocular surface tissues need to reencounter their cognate APCs for peripheral maintenance and effector function.

Lymphadenectomized mice do not develop T cell-mediated experimental dry eye

To further evaluate the role of APC-dependent autoreactive CD4+ T cell activation during the immunopathogenesis of dry eye, a bilateral surgical cervical lymphadenectomy (CLN SX) was performed 7 d prior to induction of DS. A separate group of control mice received either sham surgery (Sham SX) or axillary lymphadenectomy (AX SX). In cervical lymphadenectomized mice exposed to DS, there were significantly (p ≤ 0.05) less CD4+ T cells (68.6 ± 6.6) in the conjunctiva compared with Sham SX controls (90.1 ± 6.7) (Fig. 8A, 8B). The change correlated with preservation of goblet cell density in cervical lymphadenectomized mice (79.3 ± 1.5), which was significantly (p < 0.001) higher relative to Sham SX mice (73.9 ± 2.1) and comparable to untreated naive mouse control levels (80.6 ± 2.9) (Fig. 8C). By contrast, removal of the axillary lymph nodes did not result in a significant decrease in CD4+ T cells or increase in goblet cell density (Fig. 8D, 8E).

We have previously shown that both CLN-derived or splenic CD4+ T cells from dry eye mice were sufficient to cause dry eye disease in T cell-deficient nude recipient mice (4). To establish if removal of the CLN impacted CD4+ T cell activation in the spleen, CD4+ T cells from all experimental groups were adoptively transferred to T cell-deficient mice, and CD4+ T cell infiltration and PAS+ goblet cell density was assessed at 3 d post-transfer. As noted in the donor mice, nude recipients of CD4+ T cells from DS mice with CLN excision also displayed significantly (p ≤ 0.001) less infiltration of CD4+ T cells and retention of live goblet cells (Fig. 9A–C). However, excision of axillary nodes in donor mice exposed to DS also influenced splenic CD4+ T cell activation; there was significantly (p ≤ 0.05) less infiltration of donor-derived CD4+ T cells following adoptive transfer in nude recipients, which also correlated with a significant (p ≤ 0.001) increase in goblet cell counts (Fig. 9A, 9B), suggesting that the axillary nodes may also support a degree of Ag and/or APC lymphatic drainage from the ocular surface.

To resolve and support the finding that removal of axillary lymph nodes in donor mice exposed to DS resulted in reduced CD4+ T cell infiltration in nude recipient mice, subconjunctival administration of 1% Evans blue was used to trace lymphatic drainage from the
nodes. In addition, the face and fore and hind paws also accurately lymphatic drainage exists from the ocular surface to the axillary control eyes that were either untreated or treated with PBS liposomes.

The current findings prompted us to hypothesize that APCs provide the fundamental link between the DS-induced innate response and a self-Ag-driven autoreactive T cell response to ocular surface tissues during the immunopathogenesis of dry eye.

The current study supports and expands on our earlier work to demonstrate that APCs are necessary for the initiation and development of dry eye in the mouse model of ALKC. CD11c⁺DCs have long been known to play a predominant role activation of naïve T cells within the regional lymphoid organs, and CD11c⁺CD11b⁺MHC II⁺ cells have previously been shown to traffic from the ocular surface to the draining CLNs during inflammation (13).

In the context of experimental dry eye, exposure to DS resulted in accumulation of mature CD11c⁺DCs bearing costimulatory molecules within the draining CLNs by 24 h that correlated with subsequent activation of CD4⁺ T cells, with the kinetics indicative of an Ag-driven T cell response. Furthermore, this report establishes that subconjunctival injection of liposome-encapsulated clodronate is a sufficient method to diminish the population of an Ag-driven T cell response. Furthermore, this report establishes that subconjunctival injection of liposome-encapsulated clodronate is a sufficient method to diminish the population of APCs, and not regulatory T cells, is directly responsible for attenuating pathogenic T cells. In addition, there are several studies demonstrating that clodronate liposomes do not directly affect T cell function and proliferation in vitro and in vivo (32–34), further supporting the requirement for Ag presentation during the development of experimental dry eye. The observation that cervical lymphadenectomized mice exposed to DS also displayed significantly less infiltrating CD4⁺ T cells and higher goblet cell density corroborates the hypothesis that activation of autoreactive CD4⁺ T cells occurs in the draining CLNs via cell-to-cell contact with ocular surface-derived APCs bearing self-Ag.

The current findings are also consistent with similar studies using other inflammatory models with a high degree of peripheral

FIGURE 6. APCs are necessary for maintenance of autoreactive T cells within the ocular surface tissues following adoptive transfer of dry eye-specific autoreactive CD4⁺ T cells. To determine if peripheral maintenance of fully primed and targeted DS-specific CD4⁺ T cells requires secondary activation by resident ocular surface APCs, DS-specific CD4⁺ T cells were adoptively transferred to nude recipients treated with clodronate- or PBS-loaded liposomes according to the following permutations: OD, clodronate; OS, PBS; OD, clodronate; OS, no treatment; OD, PBS; and OS, no treatment. Subconjunctival injection of clodronate-loaded liposomes in the OD eye of nude recipient mice resulted in a dramatic reduction in the average number of DS-specific CD4⁺ T cells compared with the contralateral internal control OS eye treated with PBS liposomes (A), which was similar when clodronate-treated OD eyes were compared with untreated OS eyes at 3 d postadoptive transfer (B). By contrast, there was no difference between the number of infiltrating CD4⁺ T cells between control PBS-treated OD eyes and untreated OS eyes (C). Data are shown as average conjunctival CD4⁺ T cell counts ± SEM and representative of two independent experiments, with an n = 5 to 6 mice/group. Statistically significant values (**p = 0.001) are indicated relative to internal OS control eyes that were either untreated or treated with PBS liposomes.

FIGURE 7. The absence of DS-specific CD4⁺ T cells within the ocular surface tissues of APC-depleted nude recipient mice correlates with preservation of conjunctival goblet cells. Adoptive transfer of pathogenic DS-specific T cells to nude recipient mice treated with liposome-encapsulated clodronate did not result in a significant loss in conjunctival goblet cells compared with contralateral internal control eyes, which were either treated with PBS-loaded liposomes or left untreated. Data are shown as average conjunctival goblet cell counts ± SEM quantified at 3 d postadoptive transfer. The data are representative of two independent experiments with an n = 5 to 6 mice/group. Statistically significant values (**p ≤ 0.01) are indicated relative to OS internal control eyes that were either untreated or treated with PBS liposomes.
lymphatic drainage to the CLNs. For example, surgical excision of the CLNs delayed the onset and reduced clinical disease in the neuroinflammatory model of experimental autoimmune encephalomyelitis (35, 36), and cervical lymphadenectomy dramatically reversed corneal allograft rejection to a survival rate of 92% compared with 0% survival in the eyes of mice with intact CLNs (37).

The absence of APCs within the ocular surface tissues of clodronate-treated mice did not dramatically affect the acute proinflammatory cytokine response to DS. These data have several implications in the face of reduced CD4+ T cell infiltration and goblet cell preservation are inversely proportional during the experimental dry eye (43), and although a significant decrease in IFN-γ was not observed in clodronate-treated mice, it is possible that IFN-γ levels are below the threshold required to compromise goblet cell integrity after 5 d of DS, or perhaps IFN-γ derived from infiltrating CD4+ T cells has a greater impact on goblet cells than, for instance, NK cell-derived IFN-γ (41). The 5-d time point was chosen because this is the period when maximal CD4+ T cell activation was observed within the draining CLNs; therefore, it is also possible that Th1- and Th17-derived cytokines may continue to increase on the ocular surface of dry eye mice, further augmenting the difference between PBS and clodronate-treated mice at later time points.

Adoptive transfer studies were used to confirm an absolute role of APCs in priming and targeting autoreactive CD4+ T cells to the ocular surface during experimental dry eye. APC-depleted donor mice exposed to DS were unable to transfer CD4+ T cell-mediated disease to athymic nude recipient mice, whereas CD4+ T cells from PBS-treated control mice maintained pathogenicity and

Along these lines, the mild and nonsignificant decrease in prominent Th1- and Th17-derived cytokines (e.g., IL-2, IFN-γ, IL-17) in mice exposed to DS suggests other resident and/or infiltrating cell types are a reservoir for IFN-γ (e.g., NK cells) (41) and IL-17 (e.g., γδ T cells) (42) and may contribute to steady-state levels of tear cytokines during the DS-induced inflammatory response. We have previously shown that Th1 CD4+ T cell infiltration and goblet cell preservation are inversely proportional during the experimental dry eye (43), and although a significant decrease in IFN-γ was not observed in clodronate-treated mice, it is possible that IFN-γ levels are below the threshold required to compromise goblet cell integrity after 5 d of DS, or perhaps IFN-γ derived from infiltrating CD4+ T cells has a greater impact on goblet cells than, for instance, NK cell-derived IFN-γ (41). The 5-d time point was chosen because this is the period when maximal CD4+ T cell activation was observed within the draining CLNs; therefore, it is also possible that Th1- and Th17-derived cytokines may continue to increase on the ocular surface of dry eye mice, further augmenting the difference between PBS and clodronate-treated mice at later time points.

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**FIGURE 9.** Intact CLNs are required for pathogenicity of DS-specific splenic CD4+ T cells. To further determine if CLN removal impacted splenic CD4+ T cell activation, CD4+ T cells from naive mice or those exposed to DS with Sham SX, AX SX, or CLN SX were adoptively transferred to T cell-deficient mice. Nude recipients of CD4+ T cells from DS mice with CLN SX (A) displayed significantly decreased CD4+ T cell infiltration (B) and increased the number of goblet cells (C). A–C. A similar, but intermediate, response was also observed in nude recipient mice that received CD4+ T cells from DS donor mice with AX SX. CD4+ T cell staining in the conjunctiva (A); average conjunctival CD4+ T cell counts ± SEM (B); and average conjunctival goblet cell counts ± SEM (C), quantified at 3 d post-adoptive transfer. Original magnification ×400. The data are representative of two independent experiments with an n = 5 mice/group. Statistically significant values (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) are indicated.

**FIGURE 8.** Cervical lymphadenectomized mice do not develop full-blown T cell-mediated experimental dry eye. To evaluate the role of the draining CLNs in APC-dependent autoreactive CD4+ T cell activation, CLN SX was performed before exposing mice to DS. A separate group of DS mice received either Sham SX or AX SX. CLN excision (A) resulted in significantly less CD4+ T cells in the conjunctiva compared with Sham SX (B); AX SX did not result in a significant decrease in CD4+ T cells. C. Goblet cell density was also preserved in CLN SX mice, but was not maintained in AX SX controls. CD4+ T cell staining in the conjunctiva (A); average conjunctival CD4+ T cell counts ± SEM (B); and average conjunctival goblet cell counts ± SEM (C), quantified at 3 d post-adoptive transfer. Original magnification ×400. The data are representative of two independent experiments with an n = 5 mice/group. Statistically significant values (*p ≤ 0.05, ***p ≤ 0.001) are noted.
caused full-blown dry eye disease. It is interesting to note that excision of axillary nodes did not influence CD4+ T cell counts or goblet cell density in donor mice, but rather exerted moderate disease when the CD4+ T cells were transferred to nude recipient mice. During the development of experimental dry eye, it is possible that ocular surface Ags gain access to other lymph nodes, for instance: 1) by soluble transport of Ags into the conduit system of other lymph nodes, where resident DCs may gain access for presentation to Ag-specific T cells (44); or 2) DCs activated within the ocular surface tissues may transport the Ag to other lymph nodes, or to other DCs, to fuel the autoimmune response (45). As noted, DS induces lymphangiogenesis on the ocular surface, which likely facilitates APC and/or Ag drainage during experimental dry eye (17). Indeed, subconjunctival administration of Evans blue showed intense lymphatic drainage from the ocular surface to the CLNs, but also mild drainage to axillary nodes, supporting a role for other lymph nodes in the priming of ocular surface-specific CD4+ T cells. However, the exact mechanisms by which axillary lymph nodes influence activation of DS-specific CD4+ T cells in the spleen is not known.

This study also suggests that secondary activation or triggering of DS-specific CD4+ T cells by APCs residing locally within the ocular surface is required for peripheral maintenance and effector function. APC-depleted nude recipient mice showed a marked reduction in number of pathogenic DS-specific CD4+ T cells accumulating within the conjunctiva compared with internal control eyes of the same mice, which also correlated with decreased pathology. These results suggest that DS-specific CD4+ T cells infiltrating the ocular surface tissues need to re-encounter their cognate APCs, including DCs and/or macrophages, for peripheral maintenance and effector function. In support, Goyal et al. (46) used a topical CCR2 antagonist to demonstrate that infiltrating CD11b+ cells were required for T cell-mediated disease in a similar mouse model of DS-induced dry eye. The role of secondary T cell activation at peripheral inflammatory sites is further supported in other animal models. Resident macrophages isolated from the CNS were capable of stimulating MBP-reactive T cells ex vivo (47). During the immunopathogenesis of experimental autoimmune encephalomyelitis, infiltrating encephalitogenic Th cells required restimulation by their cognate APCs to identify their target (48, 49), and whereas activated OVA-specific or purified protein derivative of tuberculin-specific T cell lines also migrated into CNS, only activated MBP-specific T cells were maintained within in the spinal cord and caused neurologic impairment, including decreased motor function and partial to complete hind limb paralysis (50). By and large, compared with the extensive body of evidence defining the interactions between naïve Th cells and APCs (i.e., DCs within the secondary lymphoid organs), there is still a limited understanding of the peripheral APC requirements of fully primed, activated Th cells.

These data establish an essential role of APCs in initiating and maintaining chronic activation of CD4+ T cells during experimental dry eye and support the paradigm that it is a self-Ag-driven autoimmune-based inflammatory disease. Regarding the autoantigen itself, little is known. Type 3 muscarinic acetylcholine receptor was proposed based on the presence of autoreactive serum from dry eye patients (51), and excessive acetylcholine receptor stimulation was demonstrated to expose cryptic type 3 muscarinic acetylcholine receptor epitopes to be sampled by APCs (52). Putative autoantigens were also identified from the kalli- krein family, namely Klk13 and Klk1b22 (53, 54). Nonetheless, identifying the specific initiating autoantigen is fundamentally difficult and still remains elusive among the vast spectrum of organ-specific autoimmune diseases. Studies focused on defining the influence of inflammatory stimuli on activation of different APC subsets and how these subsets direct activation and differentiation of pathogenic lymphocytes will be valuable in developing a greater understanding of the complex mechanisms orchestrating the immunopathogenesis of dry eye disease.

Disclosures


References


