Spontaneous Autoimmune Dacryoadenitis in Aged CD25KO Mice

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To investigate time-related immunopathological changes in the lacrimal glands (LGs) of CD25KO mice, we examined LGs of C57BL/6 (wild-type) and CD25KO mice at 8, 12, and 16 weeks of age. T cell infiltration was quantified by flow cytometry, and gland function by tear peroxidase activity and epidermal growth factor mRNA expression. T helper (Th)-1, -2 and -17-associated cytokine expression was evaluated by real-time PCR. Epithelial apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and activated caspase-3 staining. Eight-week-old CD25KO mice demonstrated significantly increased numbers of CD4 and CD8 T cells infiltrating the LGs. This peaked at 12 weeks of age. No peroxidase secretion was detected, and epidermal growth factor mRNA expression was barely detected in CD25KO mice. Ductal epithelial apoptosis was noted in CD25KO mice. Young CD25KO LGs had higher Th-17- (interleukin [IL]-23R, transforming growth factor- β 1, IL-17A, CC chemokine attractant ligand-20) and Th-1-associated cytokine transcripts (interferon- γ , T-bet, IL-12, IL-2, IL-18) than young wild-type LGs. There was also a significant time-related decrease in IL-17A and CC chemokine attractant ligand-20 in CD25KO LGs. Taken together, autoimmune LG infiltration with loss of LG function was observed in CD25KO mice as early as 8 weeks of age. Time-related switch from Th-17 to Th-1 inflammation was noted in CD25KO mice. (*Am J Pathol* 2010, 177:744–753; DOI: 10.2353/ajpath.2010.091116)

Aging has been shown to decrease lacrimal gland (LG) function, with a resultant increase in the incidence of dry eye, affecting 15 to 25% of people over the age of 65 years.¹ Age-related structural changes observed in both murine and postmortem human LG include: acinar atrophy, periductal fibrosis, interlobular duct dilatation, accumulation of lipofuscin granules, and an infiltration of mast cells followed by lymphocytes expressing pro-inflammatory cytokines.^{2–4} An ensuing state of chronic inflammation is believed to contribute to the LG secretory dysfunction that often develops in the elderly.

Dry eye is a common complaint of patients visiting eye care specialists. Patients typically present with irritative symptoms such as burning, dryness, and foreign body sensation. Signs may include decreased tear meniscus, increased tear film debris, corneal irregularity secondary to punctate epithelial erosions, and in more severe cases, corneal filaments and mucous plaques. Its impact on quality of life can be severe, impairing the ability to perform simple daily activities such as driving, reading, and computer use.⁵ Dry eye affects functional vision, with a significant decrease in low contrast acuity vision.⁶ Among dry eye patients, 11% are estimated to have Sjögren's syndrome (SS), a chronic autoimmune disease primarily affecting women (>90%) in their fifth and sixth

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decades of life.⁷ Characterized by a lymphocytic infiltration of the lacrimal and salivary glands, SS clinically manifests with symptoms of dry eye (lacrimal keratoconjunctivitis) and dry mouth (xerostomia).⁷

The mechanisms underlying lacrimal gland dysfunction in SS are not fully understood, but epithelial apoptosis is thought to have a role in the secretory impairment. Studies in SS patients have demonstrated the presence of apoptosis-regulating proteins in the exocrine tissue such as the pro-apoptotic molecules Fas antigen (Apo-1/CD95), Fas ligand (FasL/CD95L), and Bax.^{8–10} Additional studies in animals with SS-like disease suggest T cells are able to induce glandular apoptosis by one of three mechanisms: 1) an interaction between Fas antigen on the epithelial surface and Fas ligand, expressed by the lymphocytes; 2) release of perforins and granzyme B by cytotoxic T cells; and 3) production of pro-apoptotic cytokines such as tumor necrosis factor- α and interferon (IFN)- γ .^{8,10}

The recently identified T-helper (Th)-17 population of CD4⁺ T cells has challenged the Th-1/Th-2 paradigm of T cell development established over 20 years ago. Th-1 cells contribute to host defense of viral, fungal, and intracellular bacteria infections, and are characterized by the production of IFN- γ . Th-2 cells are known to participate in allergic disease, and the eradication of gut parasites and helminthes. This pathway is characterized by the production of interleukin (IL)-4, IL-5, and IL-13 cytokines. The remaining fates for a naïve T cell point toward mutually exclusive development of either Th-17 or regulatory T cell based on the local cytokine milieu. Mice studies have found that transforming growth factor β (TGF- β) in the presence of either IL-6 or IL-21 is sufficient to drive Th-17 differentiation,^{11–13} with its specific transcription factor, retinoid-related orphan receptor- γ t, whereas, the expression of just TGF- β will induce a regulatory T cell development with its specific transcription factor, forkhead box protein 3 (Foxp3).^{11–13} Th-17 committed cells can produce IL-17A, IL-17F, IL-21, IL-22, and CC chemokine attractant ligand-20 (CCL20).^{14,15} IL-17A, the signature cytokine of this pathway, functions as a pro-inflammatory mediator by stimulating the local production of IL-1, IL-6, IL-8, and tumor necrosis factor- α by epithelial cells and fibroblasts, and can further potentiate tissue destruction by inducing the production of matrix metalloproteinases (MMPs).^{16,17}

The Th-17 pathway has been implicated in the development of autoimmunity in humans and animal models, such as inflammatory bowel disease, experimental autoimmune encephalomyelitis, and collagen induced arthritis.^{18–20} IL-17A has been found to be elevated in the synovium of rheumatoid arthritis patients,²¹ sera of patients with systemic lupus erythematosus,²² active brain lesions of multiple sclerosis patients,²³ and vitreous of patients with uveitis.²⁴ IL-17A has also been implicated in the acute corneal barrier dysfunction in an experimental dry eye model, and increased IL-17A mRNA transcript levels were identified in conjunctiva of dry eye patients.²⁵ Furthermore, IL-17A expression has been found in salivary gland ductal epithelial cells of SS patients and nonobese diabetic (NOD) mice.^{26,27}

Numerous animal models of SS have been proposed in an attempt to elucidate the pathogenesis of the disease. Some of the more studied murine models include MRL/lpr, NOD, and NZB/NZW. As the role of the CD4⁺CD25⁺Foxp3⁺ regulatory T cell (Treg) in maintaining peripheral tolerance and controlling organ-specific autoimmunity has expanded,^{28,29} an IL-2R $\alpha^{-/-}$ (CD25KO) mouse model emerged as a model of SS given that it develops a multi-organ inflammatory syndrome involving the exocrine glands, gastrointestinal tract, and a profound antibody-mediated hemolytic anemia.^{30,31} Regulatory T cells normally express a high-affinity form of the IL-2 receptor, composed of the IL-2R β (CD122) and γ chains complexed with the α subunit (CD25). Binding of IL-2 to its receptor in Treg cells induces expression of Foxp3, and promotes cell survival.³² Additionally, IL-2 has been shown to antagonize differentiation of the Th-17 pathway.³² Thus, absence of the high-affinity α subunit could skew the murine naïve cells toward Th-17 and away from regulatory T cell differentiation, promoting inflammation and autoimmunity.

The purpose of this study was to investigate time-related changes in function and immunopathology in the LG of IL-2R $\alpha^{-/-}$ (CD25KO) mice, with emphasis on the T cell-associated cytokine expression. A better understanding of the pathological mechanism behind SS at the molecular level will assist in the development of targeted therapeutics to control the disease process, rather than symptomatic treatments currently available.

Materials and Methods

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the standards in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Heterozygous breeder pairs of CD25^{+/–} mice in a C57BL/6 background (B6.129S4-*Il2ra*^{tm1Dw}/J) mice were purchased from Jackson Laboratories (Bar Harbor, Maine) for establishing breeder colonies. They were housed in our vivarium in a pathogen-free environment. The genotype of CD25KO mice was confirmed according to the Jackson Labs' protocol. C57BL/6 wild-type mice were purchased from Jackson Laboratories.

Mice of both genders were used at 8, 12, and 16 weeks of age. Fifty-two animals per time point (8, 12, and 16 weeks) per strain (CD25KO and wild-type) were used: 5 mice for histological sections, 20 mice for tear collection, and 20 mice for gene analysis, and 6 animals for flow cytometry analysis.

Histology

Extraorbital lacrimal glands were surgically excised, fixed in 10% formalin, paraffin embedded, and 8- μ m sections were cut. Sections were stained with H&E for evaluation of morphology. The area of T cell infiltration was circumscribed in digital images of H&E-stained sections, using

NIS Elements (Nikon, Melville, NY), and the percentage of infiltration calculated using the total area of the LG.

For immunohistochemistry, terminal deoxynucleotidyl transferase-mediated d-UTP nick-end labeling (TUNEL) and immunofluorescent assays, extraorbital lacrimal glands from each strain/time point ($n = 5$) were excised, embedded in optimal cutting temperature compound (VWR, Suwanee, GA), and flash frozen in liquid nitrogen. Sagittal 8- μ m sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at -80°C .

Tear Peroxidase Assay

Tear peroxidase activity was used as a measure of LG function.³³ Tear fluid washings were collected from 20 animals/group, in two independent experiments, using a previously reported method.²⁵ One sample consisted of tear washings from both eyes of two mice pooled (4 μ l) in PBS + 0.1% bovine serum albumin (6 μ l) and stored at -80°C until the assay was performed. Tear peroxidase measurement was performed using an Amplex Red Peroxidase Kit, according to the manufacturer's instructions (Invitrogen-Molecular Probes, Eugene, OR). There were a total of 10 samples from each strain per time point.

Immunohistochemistry

Immunohistochemistry was performed to detect and count the cells in the LG that stained positively for CD4 (clone H129.9, 10 μ g/ml) and CD8 α (clone 53e6.7, 3.125 μ g/ml), [from BD Bioscience, San Diego, CA]. Cryosections were stained with the above mentioned primary antibodies and appropriate biotinylated secondary antibodies (all from BD Pharmingen) and a Vectastain Elite ABC kit using NovaRed reagents (Vector, Burlingame, CA) as previously described.⁹ Secondary antibody alone and appropriate anti-mouse isotype (BD Bioscience) controls were also performed. Three sections from each animal/group/time point were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon).

Flow Cytometry Analysis of Infiltrating Cells

Single-cell suspensions of lacrimal glands of C57BL/6 and CD25KO at 8, 12, and 16 weeks of age ($n = 3$) were prepared by treatment of minced tissue fragments with 0.1% collagenase D (60 minutes at 37°C , Invitrogen-Gibco, Carlsbad, California) and sequentially filtered. Single-cell suspensions were stained with anti-CD16/32 (to block Fc receptors, BD Pharmingen, San Diego, CA, 4°C , 10 minutes), followed by cell surface staining with anti-CD4 (GK1.5; BD Pharmingen), or anti-CD8 (clone 53-6.7, BD Pharmingen). Negative controls were stained with fluorescein isothiocyanate-isotype antibody (BD Pharmingen). Cells were resuspended in violet dye (live/dead cell fixable staining, Invitrogen-Molecular Probes, Carlsbad, CA) and washed. Cells were then resuspended in fixation-permeabilization solution (Cytofix/

Cytoperm; BD Pharmingen) and stored at 4°C until the next day when the analysis was performed. A BD LSRII Benchtop cytometer was used for flow cytometry and data were analyzed using BD Diva Software (BD Pharmingen).

Immunofluorescent Staining and Laser Scanning Confocal Microscopy

Cryosections stained for activated caspase 3 (5 μ g/ml, BD Bioscience) were developed using goat anti-rabbit Alexa-Fluor 488 conjugated IgG antibody as previously described.³⁴ Negative controls performed at the same time consisted of sections incubated with an isotype antibody (BD Bioscience) or sections with the primary antibody omitted.

Digital images (512×512 pixels) of cryosections were captured with a laser-scanning confocal microscope (LSM 510; Zeiss with krypton-argon and He-Ne laser; Carl Zeiss Meditec, Ltd. Thornwood, NY) with 488-excitation and 543-nm emission filters, LP505 and LP560, respectively. They were acquired with a 40/1.3 $\times$ oil-immersion objective.

TUNEL Assay

The TUNEL assay was performed using a kit (ApopTag; Intergen Co., Purchase, NY).³⁵ Cryosections were fixed in 1% paraformaldehyde, and permeabilized with 2:1 ethanol:acetic acid solution. The samples were incubated with TdT enzyme and 11-digoxigenin dUTP at 37°C for 4 hours. After quenching the reaction, samples were blocked with blocking solution and incubated with anti-digoxigenin fluorescein isothiocyanate-conjugated antibody for 60 minutes at room temperature.

RNA Isolation and Real-Time PCR

Total LG RNA from CD25KO and wild-type mice at each time point (8, 12, and 16 weeks) was extracted using an acid guanidium thiocyanate-phenol-chloroform method as previously described.³⁴ Four samples per strain were used, and one sample consisted of pooled LG from five mice per time point. Samples were treated with DNase to prevent genomic DNA contamination according to the manufacturer's instructions (Qiagen, Valencia, CA).

First-strand cDNA was synthesized from 1 μ g of total RNA using random hexamers and M-MuLV reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; GE Health care, Inc., Piscataway, NJ), as previously described.³⁴ Real-time PCR was performed using gene expression assay primers and MGB probes specific for murine target mRNAs (Table 1). The glyceraldehyde-3-phosphate dehydrogenase gene was used as an endogenous reference for each reaction. The results of quantitative PCR were analyzed by the comparative C_T method where target change = $2^{\Delta\Delta C_T}$ (User Bulletin, No. 2, P/N 4303859; ABI). The cycle threshold (CT) was determined using the primary (fluorescent) signal as the cycle at

Table 1. Classification of Target mRNAs Investigated by Real-Time PCR

Target mRNA	Assay IDs
Th-17 pathway	
GAPDH	Mm99999915
MMP-9	Mm00442991
IL-6	Mm00446490
TGF- β 1	Mm00441724
TGF- β 2	Mm00436952
IL-15	Mm00434210
IL-17A	Mm00439619
IL-17R	Mm00434214
IL-17F	Mm00521423
IL-23	Mm00518984
IL-23R	Mm00519942
ROR γ t	Mm00441139
IL-21	Mm00517640
IL-22	Mm00444241
CCL20	Mm00444228
STAT3	Mm00456961
Th-1 pathway	
IFN- γ	Mm00801778
IL-2	Mm00434256
IL-12	Mm00434165
IL-12RB1	Mm00434189
IL-18	Mm00434225
IL-18R	Mm00515180
T-bet	Mm00450960
Th-2 pathway	
IL-4	Mm00445259
IL-13	Mm00434204
GATA-3	Mm00484683

which the signal crossed a user-defined threshold. The results were normalized by the CT value of glyceraldehyde-3-phosphate dehydrogenase and the mean CT of relative mRNA level in the C57BL/6 group at 8 weeks of age was used as the calibrator.

Statistical Analysis

One-way analysis of variance (analysis of variance) with Tukey's post hoc testing was used for statistical comparisons of tear cytokines and gene expression profiles in cornea and conjunctiva. $P \leq 0.05$ was considered statistically significant. These tests were performed using GraphPad Prism 5.0 software (GraphPad Software Incorporation, San Diego, CA).

Results

Spontaneous Dacryoadenitis in the CD25KO Mice

The LG undergoes an array of changes in its structural organization with aging.²⁻⁴ To determine the effect of time-related changes, extra-orbital LG of wild-type and CD25KO mice strains of both genders were excised and prepared for histological examination. During surgical excision, CD25KO LG were noted to be enlarged, red, and inflamed at 8 and 12 weeks of age, while at 16 weeks the LGs were small and atrophic compared with wild-type LGs. At the cellular level, young C57BL/6 wild-type LG

Figure 1. A–D: Representative images of H&E-stained lacrimal gland (LG) sections of C57BL/6 wild-type (A, B) and CD25KO (C, D) mice at 8, 12, and 16 weeks of age. A and C: Images of original magnification $\times 10$. B and D: Higher magnification of white square demarcated area in (A). Note lymphocytic infiltration (circumscribed by black dotted lines) at all time points in CD25KO mice. Openings of epithelial ducts are marked by asterisks. E–H: Immunohistochemistry for CD4 (E–F) and CD8 (G, H) positive T cells in the LG of C57BL/6 wild-type and CD25KO mice at 8, 12, and 16 weeks of age.

exhibited normal gland architecture, with plump acini (Figure 1, A and B), while young CD25KO LG showed acinar atrophy, acinar fibrosis, periductal fibrosis, and intense lymphocytic infiltration occupying up to 70% of the total area of the gland (Figure 1, C and D). These findings in the CD25KO mice further worsened at 12 weeks. At 16 weeks of age, CD25KO LG continued to show lymphocytic infiltration, but greater disarrangement of the gland was noted, with extensive acinar loss and atrophy of the entire gland. LG sections stained for CD4 and CD8 demonstrated a significantly higher density of these T cells in CD25KO mice (Figure 1, F and H) when compared with C57BL/6 wild-type mice (Figure 1, E and G) at all ages. To further quantify the infiltrating immune cells, we performed flow cytometry analysis in LG of both

Figure 2. A: Representative flow cytometry analysis of freshly cells stained for CD4 from LG of CD25KO and C57BL/6 mice at 8, 12, and 16 weeks of age (8W, 12W, and 16W, respectively). Lymphocytes were gated based on characteristic light-scatter properties; single lymphocytes were gated based on forward scatter height versus forward scatter area (FSC-A). Numbers in the quadrants indicate the percentage of cells. **B:** Mean $\pm$ SD of three independent flow cytometry (FACS) experiments of CD4 and CD8+ T cells in LG of CD25KO and C57BL/6 mice at 8, 12, and 16 weeks of age (8W, 12W, and 16W, respectively). ** $P < 0.01$.

strains, at all time points, for CD4 and CD8. Our results, presented in Figure 2, A and B demonstrated that CD25KO mice had significantly higher percentage of CD4 and CD8+ T cells at all ages, compared with C57BL/6 wild-type mice. CD8+ T cells were the dominant cell type. There were no gender-related differences in the amount of infiltrating cells in either strain (data not shown).

These results suggest that time-related structural changes occurred in LG of CD25KO as early as 8 weeks. The ocular surface pathology of these mice were investigated in another study.³⁶

Lack of Gender Differences in CD25KO LG Pathology

Sjogren's syndrome disproportionately affects women over men (9:1 female-to-male ratio).⁷ Previous studies have examined whether gender-related pathological differences also exist in the exocrine tissues of mouse models of this disorder.^{37,38} MRL/lpr and NZB/NZW murine models develop more severe lacrimal gland inflammation in females relative to males, while male NOD mice develop worse LG inflammation than females.³⁷ However, aged female NOD.H2^{h4} mice develop more severe sialoadenitis when compared with age-matched male NOD.H2^{h4} mice, as assessed by histology of the salivary glands and flow cytometry of the infiltrate.³⁸ In addition, female

NOD.H2^{h4} mice have a significantly greater proportion of CD4+ T cells in the infiltrate than males, with no difference in the percentage of CD8+ T Cells.³⁸

To evaluate for any gender-related differences in the immune response of the CD25KO model, male and female CD25KO LG were histologically examined (see Supplemental Figure 1A at <http://ajp.amjpathol.org>). Total T cell infiltration was circumscribed in H&E-stained paraffin sections at all ages and area of infiltration was calculated based on the total area of the LG. We noted that CD25KO of both genders, exhibited similar LG pathology. This was confirmed by measuring the total cell infiltration (see Supplemental Figure 1B at <http://ajp.amjpathol.org>).

We also performed flow cytometry analysis of CD4 and CD8+ T cells in LG of CD25KO mice of both genders at ages 8, 12, and 16 weeks. We did not observe any gender difference in the phenotype of infiltrating cells (data not shown).

Taken together, these results point to a lack of gender differences in the CD25KO LG pathology.

CD25KO LG Exhibit Ductal Epithelial Apoptosis Over Time

Numerous reported studies have suggested the underlying mechanism behind glandular dysfunction in autoimmune-mediated exocrine diseases, such as SS, results from increased levels of epithelial apoptosis.¹⁰⁻¹³ Studies of the salivary glands in the NOD mouse model have demonstrated increased levels of caspase-3, one of the effector caspases essential to carrying out apoptosis, as well as an increase number of cells staining for TUNEL.³⁹ In light of these findings, the presence of caspase-3 and TUNEL staining was evaluated in lacrimal gland specimens.

No activated (Ac)-caspase-3 staining was detected at any age in LG sections of C57BL/6 mice in the acini or epithelial ducts. In contrast, LG CD25KO mice demonstrated immunoreactivity for Ac-caspase-3 in the ductal epithelium at all ages, reaching peak intensity at 12 weeks (Figure 3A).

To confirm the results of Ac-caspase-3 staining, TUNEL staining was performed in sequential LG sections. TUNEL identifies cells that have undergone apoptosis via detection of DNA strand breaks, indicating DNA cleavage. The results of the TUNEL assay confirmed the findings of Ac-caspase-3 staining, with no TUNEL-positive cells at any age in C57BL/6 mice acini or ductal epithelial cells, while intensely positive staining was observed in the ductal epithelia of CD25KO LG at all ages (Figure 3B). It is worth noting that TUNEL immunoreactivity was absent in the infiltrating lymphocytes in the CD25KO mice (Figure 3B, dotted lines).

The absence of caspase-3 and TUNEL staining in acini of CD25KO mice could be due to a lack of acini in the regions of inflammatory cell infiltration. Taken together, these results show that CD25KO LG undergoes significantly elevated levels of ductal epithelial cell apoptosis compared with C57BL/6 wild-type mice, with sparing of the lymphocytic infiltrate.

Figure 3. Merged images of laser scanning immunofluorescent confocal microscopy of lacrimal gland sections stained for activated caspase-3 (in green, **A**) or processed for TUNEL assay (green, **B**) with propidium iodide nuclear counterstaining (in red) of C57BL/6 wild-type and CD25KO mice at 8, 12, and 16 weeks of age. Orange color in TUNEL indicates colocalization of green and red nuclear staining. The **white dotted outlines** circumscribe lymphocytic infiltration of LG of CD25KO mice at all ages. **C:** Mean $\pm$ SD of peroxidase content in tears. **Dotted lines** indicate the lowest value in the linear portion of the curve generated from the observed mean fluorescent intensities versus the observed concentrations of standards. **D:** mRNA transcript level of EGF expression in LG lysates of both strains at all time points. **E:** Merged images of laser scanning immunofluorescent confocal microscopy of lacrimal gland sections stained for EGF (in green) with propidium iodide nuclear counterstaining (in red) of C57BL/6 wild-type and CD25KO mice at eight weeks of age. *** $P < 0.001$ when compared with C57BL/6 at eight weeks of age.

Spontaneous Dacryoadenitis in CD25KO Mice Is Accompanied by Decreased LG Function

To determine whether the immunopathological changes detected over time in the lacrimal glands of CD25KO and C57BL/6 mice corresponded with decreased gland secretory function, we measured tear peroxidase levels and LG epidermal growth factor (EGF) expression in both strains.

Tear peroxidase levels were measured using a spectrofluorometric assay. This method measures levels of an acinar secreted protein, peroxidase, in comparison with measuring total protein output, where other proteins not contained in secretory granules, but released on stimulation, are measured along with secretory proteins such as peroxidase. As shown in Figure 3C, peroxidase activity in tears was highest at 8 weeks in wild-type mice. By contrast, no peroxidase secretion was detected in CD25KO mice at any age. The lack of any detectable peroxidase in the knockout model suggests an inflammatory mediated secretory dysfunction of the LG.

EGF, another lacrimal gland secretory product, has been previously shown to be decreased in patients with SS, as well as other forms of dry eye disease.⁴⁰ The expression of EGF was evaluated by real-time PCR in LG

lysates and by immunostaining in cryosections. EGF mRNA transcripts were significantly decreased in 8 week-CD25KO LG, as compared with C57BL/6 mice of the same age (Figure 3D). Time-related changes decreased EGF mRNA transcript levels at 12 weeks in C57BL/6 mice, while EGF transcript levels remained low in CD25KO mice. Laser confocal immunomicroscopy of LG at 8 weeks from both strains confirmed the low levels of expression of EGF in the CD25KO mice noted by real-time PCR. EGF immunoreactivity was noted diffusely in the acini of wild-type LG, but could not be detected in the CD25KO specimens (Figure 3E).

Taken together with the findings of the tear peroxidase assay, these results confirm the LG secretory impairment that takes place over time, and in the presence of autoimmune-mediated inflammation.

Spontaneous Shift in T-Helper-Associated Cytokine Profile Over Time in CD25KO

Before the discovery of the Th-17 subset, several studies in SS revealed a Th-1-predominant cytokine expression, highlighted by elevated levels of IFN- γ . Studies in NOD. IFN- $\gamma^{-/-}$ and NOD.IFN- γ R $^{-/-}$ mice fail to undergo apo-

ptosis and subsequently do not develop autoimmune disease of their salivary glands.⁴¹ Recent investigations focusing on the Th-17 pathway have detected an up-regulation of this family in salivary glands of SS patients and NOD mice.^{26,27} To characterize the T-helper phenotype associated with the dacryoadenitis observed in CD25KO mice levels of mRNA transcript encoding MMP-9, Th-1-, Th-2-, and Th-17-associated genes were evaluated using real-time PCR.

Young CD25KO LG were found to contain significantly higher Th-17 (TGF- β 1, IL-17A, IL-23R, IL-21, CCL20) and Th-1-associated cytokine transcripts (IFN- γ , IL-2, IL-12, IL-12RB1, IL-18R, T-bet,) than young wild-type mice (Figure 4, A and B). It is worth noting that CCL20, a potent chemoattractant molecule of Th-17-committed cells,¹⁴ displayed the greatest fold expression (400 fold) in CD25KO mice at 8 weeks of age, compared with wild-type mice. No significant changes were noted between strains in the expression of MMP-9, retinoid-related orphan receptor- γ , IL-17F, IL-17Ra, IL-22, IL-6, IL-23, STAT3, IL-15, or TGF- β 2 at any age (data not shown).

A significant time-related decrease in the Th-17-related cytokine (IL-17A, IL-23R, IL-21, CCL20) mRNA transcripts was observed in CD25KO LG (Figure 4A). This was countered by an initial decrease at 12 weeks, followed by an increase at 16 weeks in the expression of multiple Th-1 cytokines (IFN- γ , IL-2, IL-12, IL-12RB1, T-bet) in the CD25KO mice. In C57BL/6 wild-type LG, a significant time-related increase in mRNA levels of IL-2,

IL-18R, T-bet, TGF- β 1, IL-23R, and CCL20 was observed at 16 weeks, and in IL-17A at 12 weeks, with a subsequent decrease at 16 weeks (Figure 4B).

With respect to the Th-2 pathway, mRNA transcript levels of IL-4 and IL-13 were largely unchanged between both strains at all ages (data not shown). However, levels of GATA-3, the Th-2-specific transcription factor, were significantly higher in 8-week old CD25KO than C57BL/6 mice, and then proceeded to decrease at 12 and 16 weeks.

Taken together, these results suggest a time-dependent increase in the production of both Th-17 and Th-1 inflammatory mediators in C57BL/6 mice, whereas in CD25KO mice there is a switch from Th-17 to primarily Th-1 inflammation over time.

Discussion

CD25 (IL-2R α) knockout mice have been proposed as an animal model of SS, due to their propensity to develop a profound lymphocytic infiltrate of multiple organs, including the colon, lung, pancreas, and bone marrow, as well as both lacrimal and salivary exocrine glands.^{30,31} Our results in this present study showed that CD25KO mice displayed significant lacrimal inflammation as early as 8 weeks of age, highlighted by a CD8-predominant lymphocytic infiltrate and concomitant production of pro-inflammatory cytokines. This inflammatory response was paralleled by ductal epithelial apoptosis, an absence of tear peroxidase, and very low EGF mRNA transcripts at all ages, reflecting the severity of gland secretory dysfunction at even a young age in these mice. Additional findings of sparse EGF immunoreactivity at 8 weeks of age in CD25KO LG compared with wild-type mice are consistent with previous studies reporting decreased EGF expression in SS patients.⁴⁰ Meanwhile, our findings in C57BL/6 mice of time-related decreases in LG EGF expression suggests an impairment of LG protein secretion over time, a point supported by earlier reports.²⁻⁴

It is paramount to appreciate that the previously observed changes in LG structure and function are not the same between aging and autoimmune dry eye models, despite the fact aging itself is a risk factor for dry eye. Though this study aimed to evaluate both variables, our results must be considered independently. Because of the rapid onset and severity of autoimmunity CD25KO mice develop, early mortality (12 to 16 weeks) is often a consequence; therefore, time-related changes in the CD25KO mice could only be observed up to 16 weeks. Previous studies in the aging mouse model, which normally lives up to 36 months, have implicated increased oxidative stress (marked by lipofuscin deposition) secondary to hyperglycemia and insulin resistance, as well as mast cell infiltration as causative factors for early loss of gland function.²⁻⁴ Subsequently, a lymphocyte-driven inhibition of neural function is noted to occur in much older mice.²⁻⁴ In contrast, for autoimmune-mediated dry eye, an inflammatory cytokine-driven impairment of pre-synaptic and postsynaptic nerve function has been proposed to underlie glandular dysfunction early on in the

Figure 4. mRNA transcript levels of Th-17 (A) Th-1 and Th-2 (B) associated cytokines in the lacrimal glands of aged C57BL/6 and CD25KO mice (8, 12, and 16 weeks of age). Data are shown as the mean $\pm$ SEM ($n = 4$ /time point, bar indicates comparison between strains, asterisks indicate comparison within the same strain, versus 8 week, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; 8W = 8 weeks; 12W = 12 weeks; 16W = 16 weeks).

disease process.⁴² This hypothesis is further supported by our observations of a CD4⁺ T cell-rich lymphocytic infiltrate in young CD25KO mice.

Our findings in CD25KO LGs of peaked Ac-caspase-3 and TUNEL staining at 12 and 16 weeks, respectively, partially implicate epithelial apoptosis behind decreased gland activity. Ductal epithelial cells have been shown to be important in restructuring the electrolyte composition of tears and in secreting the water component. Thus, their destruction contributes to the dysfunctional tear film that develops in lacrimal keratoconjunctivitis. These results are in agreement with multiple human⁸⁻¹⁰ and animal^{35,37} studies of SS and SS-like disease, which demonstrated increased levels of apoptosis-related proteins in the exocrine tissue.

Also worth mention is the overall lack of lymphocytes undergoing apoptosis noted in the present study. Indeed, reports exist documenting the relative resistance of these cells to undergoing programmed death in SS, despite their own surface expression of Fas. It has been suggested their anti-apoptotic properties derive from an overexpression of Bcl-2.^{8,9} This expression profile gives them a propensity to replicate and survive in an otherwise destructive environment. This may also contribute to the increased risk patients with SS have of developing mucosal lymphomas.¹⁰

Both time-related changes and spontaneous autoimmune dacryoadenitis were found to be associated with increased expression of Th-1- and Th-17-related gene products, though CD25KO mice seemed to transition from a Th-17 to Th-1 bias at later time points, while the wild-type maintained a mixed composition. The T helper profile observed in CD25KO mice is comparable to previous findings in salivary gland biopsies from SS patients revealing both Th-1 and Th-17 committed cells.^{26,27} Given earlier reports of an IL-2-mediated Th-17 inhibition by transcriptionally suppressing its lineage factor, retinoid-related orphan receptor- γ ,³² an increase of Th-17 pathway cytokines would naturally be expected in the CD25KO mice, secondary to IL-2 inactivity. Specifically, mRNA levels of IL-17A, the signature cytokine of the Th-17 pathway, were found to be significantly higher in the LG of CD25KO mice than wild-type, with a marked time-related reduction. IL-17A expression has been identified previously in salivary gland tissue from SS patients and NOD mice.^{26,27} The contributions of IL-17A to autoimmunity and promoting inflammation are currently under intense investigation. Our group recently reported IL-17A-promoted MMP production on the ocular surface disrupts corneal epithelial barrier function in a murine model of dry eye, and *in vivo* neutralization of IL-17A significantly reduces corneal MMP expression, ameliorating dessicating stress-induced barrier dysfunction.²⁵ Additional studies in experimental murine dry eye have reported an IL-17A-mediated dysfunction of regulatory T cells, with a reduction of Th-17 cell expansion and restoration of Treg function after *in vivo* blockade of IL-17A.⁴³

An additional Th-17-associated cytokine, IL-21, was found to have increased mRNA expression in CD25KO compared with wild-type LG, with a significant time-related decrease. An emerging role for IL-21 in autoimmu-

nity is supported by observations of elevated levels of the cytokine and its receptor in the sera of patients with SS, rheumatoid arthritis, systemic sclerosis, and inflammatory bowel disease.⁴⁴ Additional studies in MRL/lpr mice modeling lupus revealed that IL-21 blockade with a soluble IL-21R-Fc fusion protein attenuated disease severity.⁴⁵ A natural product of activated CD4⁺ and natural killer T cells, IL-21 has been shown to stimulate the production of pro-inflammatory molecules by epithelial cells and fibroblasts, much like IL-17A.¹² However, IL-21 possesses an additional property of autocrine-mediated differentiation and amplification of mouse Th-17 cells in conjunction with TGF- β 1, and independent of IL-6.¹² In this study, levels of IL-6, the traditional Th-17 inducer, were not significantly elevated in the CD25KO LG, while IL-21 transcripts were >100-fold higher than wild-type at 8 weeks. Interestingly, the IL-21 gene is located within the *Id3* locus,⁴⁶ one of only two insulin-dependent diabetes (*Id*) susceptibility loci (out of over 19 identified) in the NOD mouse model implicated in the simultaneous exocrinopathy these mice develop.⁴⁷ Subsequently, a study of NOD spleen and pancreatic lymph nodes identified elevated expression of IL-21 and its receptor when compared with C57BL/6 controls.⁴⁶

Of particular interest was the near 400-fold increase in CCL20 mRNA transcripts in the LG of young CD25KO mice, compared with C57BL/6 mice. A potent chemoattractant of Th-17 cells, CCL20 production is known to be stimulated by IL-17A itself, in addition to the pro-inflammatory cytokines IL-1 and tumor necrosis factor- α .¹⁴ The significant increase in CCL20 observed in our study likely facilitated the influx of Th-17 CD4⁺ T cells into the LGs of CD25KO mice. Like IL-17A and IL-21, levels of CCL20 in the knockout model significantly declined with time. Taken together, this pattern of decline may be indicative of the chronic immune activation leading to a state of burnt-out inflammation and irreversible tissue injury. In accordance with this are the previously noted gross and histological findings of small, atrophic glands and massive fibrotic replacement of acinar tissue in the 16-week old mice as compared with the red, inflamed glands of the 8- and 12-week old CD25KO mice.

In addition to elevated Th-17 expression, we detected significantly increased levels of the Th-1 signature cytokine, IFN- γ , in the CD25KO mice, with peaks at 8 and 16 weeks. Increased production of IFN- γ in patients with SS has been well documented in the literature, with numerous studies detecting its overexpression in lacrimal and salivary glands.²⁷ Both NOD.IFN- γ ^{-/-} and NOD.IFN- γ R^{-/-} mice failed to undergo lymphocytic infiltration of the salivary gland, and retained normal secretory function.⁴¹ Increased levels of IFN- γ have also been found to up-regulate epithelial expression of Fas and caspase-8, thus potentiating the apoptotic pathway.⁸ Additionally, a recent study characterizing a transgenic mouse model of SS overexpressing IL-12, the chief stimulator of the Th-1 axis and subsequent IFN- γ production, detected focal lymphocytic infiltration of the salivary glands, LG, and the lungs, mimicking a SS phenotype.⁴⁸ Levels of IL-12, its

receptor, IL-RB1, and the Th-1 lineage specific transcription factor, T-bet, were all significantly increased in our animal model of SS when compared with C57BL/6 mice.

Another Th-1-related cytokine, IL-2, was found to be significantly higher in CD25KO than C57BL/6 mice. Though it has been shown to drive differentiation of naïve T cells toward Th-1 commitment, increased levels in this study could be due to a compensatory feedback mechanism from impaired IL-2 signaling at the receptor level. Normally, IL-2 signals through its high affinity receptor complex, IL-2R $\alpha\beta\gamma$, to mediate the development and maintenance of CD4⁺CD25⁺Foxp3⁺ regulatory T cells, which are crucial in preventing organ-specific autoimmunity.^{28,29} In humans, mutation of Foxp3 results in the lethal autoimmune disorder, immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome (IPEX), characterized by a congenital defect in Treg expression.⁴⁹ Similarly, Foxp3 mutant scurfy mice develop multi-organ autoimmunity and die at a young age.^{30,49} The importance of Tregs in suppressing ocular surface inflammation has been demonstrated by reconstituting T-cell-deficient nude mice with either pathogenic CD4⁺ T cells from donors exposed to dessicating stress alone, or in combination with *in vitro* expanded CD4⁺CD25⁺Foxp3⁺ regulatory T cells.⁵⁰ Nude mice reconstituted with both pathogenic CD4⁺ T cells and *in vitro* Tregs had significantly lower conjunctival cellular infiltration and tear levels of pro-inflammatory cytokines compared with the mice receiving only pathogenic effector T cells.⁵⁰ Other than dry eye, defective Treg function has been linked to the pathogenesis of several autoimmune diseases including multiple sclerosis and rheumatoid arthritis.

Altogether, it has become increasingly evident that a Th-1/Th-17 dichotomy is not so absolute, and there may be unique contributions of each pathway to the total makeup of autoimmunity. Notably, a murine model of experimental autoimmune encephalomyelitis where IFN- γ -producing Th-1 cells facilitated the entry of Th-17 cells into the central nervous system, illustrates the collaboration between the two pathways.⁵¹ Th-17 preparations that lacked IFN- γ -producing cells were unable to cause disease, leading the investigators to conclude that only Th-1 cells can access a noninflamed central nervous system.⁵¹ Though speculative, the possibility of Th-17 cells not being terminally differentiated and retaining the plasticity to develop into IFN- γ secreting Th-1 cells with age or under some unknown stimulus is intriguing, especially given recent reports of T cells known as “double producers” that simultaneously express IFN- γ and IL-17.⁵²

In summary, we demonstrated that the autoimmune exocrinopathy of the CD25KO LG is composed of a dynamic Th-1 and Th-17 response, with a Th-17 phenotype in young mice shifting to a Th-1-predominant expression at later time points. In addition, we provided evidence for a role of both ductal epithelial apoptosis, as well as defective effector T cell apoptosis, in the LG dysfunction accompanying the inflammatory infiltrate. The results of this study should yield further insight into the complex pathogenesis of Sjögren's syndrome.

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