Age-Related Conjunctival Disease in the C57BL/6.NOD-*Aec1Aec2* Mouse Model of Sjögren Syndrome Develops Independent of Lacrimal Dysfunction

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PURPOSE. To investigate parameters of ocular surface disease in C57BL/6.NOD-*Aec1Aec2* (Aec) mice with aging and their correlation with development of Sjögren syndrome (SS)-like lacrimal gland (LG) disease.

METHODS. Aec and C57BL/6 wild-type (B6) female mice were evaluated at 4, 12, and 20 weeks of age. Whole LG and eyes and adnexa were excised for histology and gene expression analysis and evaluated by flow cytometry and immunohistochemistry. Tear volume and goblet cell density was measured. Quantitative PCR evaluated T-cell-related cytokine expression in cornea and conjunctiva.

RESULTS. Both strains showed age-related conjunctival goblet cell loss that was more pronounced in the Aec strain and significantly greater than in B6 mice at 12 weeks. This was accompanied by CD4⁺ T-cell infiltration of the conjunctiva that was greater in Aec strain at 20 weeks. Aec mice had higher levels of IL-17A, IL-17R, IL-1 α , IL-1 β , and TNF- α in the conjunctiva, and they significantly increase with aging. Aec mice had greater lymphocytic infiltration of the LG and conjunctiva at 20 weeks that consisted of a mixture of CD4⁺ and CD8⁺ cells. Flow cytometry showed a significant increase in CD4⁺ T cells in Aec LG compared to B6 mice. Tear volume was significantly increased in both strains at 20 weeks.

CONCLUSIONS. Aec mice developed greater conjunctival goblet cell loss associated with lymphocytic infiltration of the LG and conjunctiva with aging. Increased expression of certain T helper or inflammatory cytokines in these tissues was observed in Aec mice. The conjunctival disease appeared to be due to inflammation and not a decrease in tear volume.

Keywords: C57BL/6.NOD-*Aec1Aec2*, inflammation, Sjögren syndrome (SS)-like disease

Sjögren syndrome (SS) is a chronic systemic autoimmune disease that targets primarily the salivary and lacrimal glands (LGs), resulting in severe dry mouth (stomatitis sicca) and dry eye (keratoconjunctivitis sicca), respectively.¹ Despite extensive efforts to define the genetic, environmental, and/or immunologic basis of human SS, the underlying etiology remains relatively unclear. This is due, in part, to the delay in diagnosis to an average 7 to 10 years after first symptoms, patients are seen in clinics only after onset of overt clinical disease, and comparative controls are unlikely to volunteer for exocrine gland biopsies. So, in an effort to identify the underlying pathogenesis of SS in humans, numerous mouse models in which various aspects of SS appear spontaneously or are experimentally induced are being intensively investigated.² Typically, these mouse models show lymphocyte infiltration of the lacrimal and salivary glands, increased expressions of proinflammatory and helper T helper (Th) cytokines, generation of autoantibodies (especially ANAs [antinuclear antibodies] and anti-M3R antibodies), and eventually decreased tear and saliva secretion.

In recent years, several mouse strains, including nonobese diabetic (NOD), MRL/*lpr*, CD25 knockout (KO) strains, and

C57BL/6.NOD-*Aec1Aec2* (Aec) mouse, have been used to study the immunopathogenic mechanisms of SS.²⁻⁹ The Aec mouse is a recently generated model in which two genetic intervals, the *Idd3* region (designated *Aec1*, *autoimmune exocrinopathy*) of chromosome 3 and the *Idd5* (designated *Aec2*) of chromosome 1, derived from the NOD mouse were bred into the SS-nonsusceptible C57BL/6 (B6) mouse.⁷ These two genetic intervals have been shown to be responsible for a SS-like disease manifestation to develop in the NOD. Because Aec mice have a B6 genetic background, these mice do not exhibit signs of autoimmune diabetes and abnormal development of LGs, and LG histology is normal prior to onset of SS-like disease.²

Aec mice exhibit several features found in human SS, including lymphocytic infiltration of salivary glands and LGs, reduced tear production, and the presence of serum autoantibodies.⁶⁻⁹ However, the disease process is not fully synchronized between individual mice,⁸ and the ocular surface disease has not been described. Therefore, in the present study, we compared LGs and ocular surface alterations between Aec and B6 strains at various time points.

METHODS

Animals

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

Breeding pairs of Aec mice in a B6 background were a kind gift from Ammon B. Peck (University of Florida, Gainesville, FL, USA) for establishing breeder colonies. They were housed in our vivarium in a pathogen-free environment that is managed by Baylor Center for Comparative Medicine (Houston, TX, USA). Mice were exposed to a 12-hour light cycle while in the vivarium with timers controlling the light/dark cycle.

Aec female mice were evaluated at 4, 12, and 20 weeks of age, and age-matched wild-type B6 female mice were used as controls. A minimum of 33 animals per time point (4, 12, and 20 weeks) per strain were used: 3 mice for histologic sections, 8 for evaluation of corneal permeability, 10 for tear collection, 6 for flow cytometry, and 6 for gene expression studies. An additional 20 mice were used for tear collection and flow cytometry at 4 and 20 weeks in both strains.

Histology, PAS Staining, and Immunohistochemistry

Eyes with ocular adnexa and extraorbital LGs were surgically excised, fixed in 10% formalin, and embedded in paraffin, and 8- μ m sections were cut. Sections were stained with hematoxylin and eosin for evaluation of morphology.

For immunohistochemistry, extraorbital LGs and the eyes and adnexa of mice from each age per strain ($n = 3$) were excised, embedded in optimal cutting temperature compound (OCT compound; VWR International, Suwanee, GA, USA) 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 .

Immunohistochemistry was performed in LGs and in the conjunctival epithelium to detect and count cells that stained positively for CD4 (clone H129.9, 10 $\mu\text{g}/\text{mL}$; BD Biosciences, San Diego, CA, USA), CD8 α (clone 53e6.7, 3.125 $\mu\text{g}/\text{mL}$; BD Biosciences), or CD19 (clone 6D5, 2 $\mu\text{g}/\text{mL}$; Abcam, Cambridge, MA, USA) antibodies as previously described.¹⁰ Positively stained cells were counted in the goblet cell-rich area of the palpebral conjunctiva using image analysis software (NIS Elements Software, version 3.0; Nikon, Inc., Melville, NY, USA). Results were expressed as the number of positive cells per 100 μm .

For PAS staining, the eyes with surrounding eyelids were fixed in 10% buffered formalin overnight and embedded in paraffin. Serial sections 5 μm thick were cut from each sample using a microtome (Microm HM 340E, ThermoScientific, Wilmington, DE, USA). The sections were deparaffinized and stained with 0.5% PAS for identification of goblet cells. The number of positively stained goblet cells in the superior and inferior conjunctiva was counted, and the length of the basement membrane between the first and last goblet cell was measured. The data are presented as the average number of goblet cells per millimeter per eyelid.

RNA Isolation and Real-Time PCR

Total RNA from conjunctiva, corneal epithelium, and LG (individually collected and individually pooled) from Aec and B6 mice at 4, 12, and 20 weeks was extracted by using RNeasy Plus Mini and RNeasy Plus Micro Kit (Qiagen's RNA isolation kit; Qiagen, Inc., Valencia, CA, USA) according to the

manufacturer's instructions, quantified by a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE, USA), and stored at -80°C . A minimum of six samples per strain and age were used, and one sample consisted of pooled samples of right and left eyes and LGs of the same animal. Samples were treated with DNase (Qiagen, Inc.) to prevent genomic DNA contamination, according to the manufacturer's instructions.

First-strand complementary DNA was synthesized from 1 μg of total RNA using random hexamers and Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Arlington Heights, NJ, USA), as previously described.⁵

Quantitative real-time PCR was performed with specific minor groove binder (MGB) probes (Taqman; Applied Biosystems, Foster City, CA, USA) and PCR master mix (Taqman Gene Expression Master Mix) in a commercial thermocycling system (StepOnePlus Real-Time PCR System; Applied Biosystems). Murine MGB probes were IFN- γ (Mm00801778_m1), IL-1 α (Mm00439620_m1), IL-1 β (Mm00434228_m1), IL-6 (Mm99999064_m1), IL-13 (Mm99999190_m1), IL-17A (Mm00439618_m1), TNF- α (Mm99999068_m1), matrix metalloproteinase (MMP)-3 (Mm00440295_m1), MMP-9 (Mm00442991_m1), small proline-rich protein 2a (SPRR2a; Mm00845122_m1), major histocompatibility complex (MHC) II (Mm00482914_m1), T-box transcription factor (T-BET; Mm00450960_m1), GATA-binding protein (GATA)-3 (Mm00484683_m1), RAR-related orphan receptor C (RORC; Mm00441139_m1), and interleukin-17 receptor (IL-17R; Mm00434295_m1). The hypoxanthine phosphoribosyltransferase 1 (*HPRT-1*) (Mm00446968_m1) gene was used as an endogenous reference for each reaction. The results of quantitative PCR were analyzed by the comparative C_t method in which the target of change = $2^{-\Delta\Delta C_t}$ was normalized by the C_t value of HPRT-1 and the mean C_t of relative mRNA level in the B6 group at 4 weeks of age was used as the calibrator.

Tear Volume Measurements

Phenol red thread tests were used to measure tear volume, as previously described.¹¹ Briefly, a phenol red-impregnated thread (ZONE-QUICK; Showa Yakuhin Kako Co., Tokyo, Japan) was held in the lateral canthus of each eye with forceps. A color change from yellow to red occurs in the thread when tears are absorbed. After 20 seconds, the thread was removed and the entire wet (red) portion was measured using imaging software (NIS Element-BR, version 3.0; Nikon, Inc.) after photographing with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon, Inc.). The distance was converted to volume according to a previously published standard.¹¹

Corneal Permeability

Corneal epithelial permeability to Oregon green dextran (OGD; 70,000 MWt; Invitrogen, Eugene, OR, USA) was assessed at each time point (4, 12, and 20 weeks) in Aec and B6 mice, as previously described.¹² Briefly, 0.5 μL of 50 mg/mL OGD was instilled onto the ocular surface 1 minute before euthanasia. The severity of corneal OGD staining was graded in digital images in the 2-mm central zone of each cornea by two masked observers, using NIS Elements Software (Nikon, Inc.). The mean fluorescent intensity measured by the software inside this central zone was transferred to a database and results averaged within each group. Results are presented as means $\pm$ SEM of gray levels.

Tear Washing and Epidermal Growth Factor (EGF) ELISA

Tear washings were collected from each age group from Aec and B6 mice, and tear EGF concentration was measured by a previously reported method.⁵ One sample consisted of tear washings pooled from both eyes of one mouse (2 μ L) in PBS plus 0.1% bovine serum albumin (8 μ L) and stored at -80°C until the assay was performed.

Tear EGF washings were collected from the same mice used for tear volume measurements on 2 consecutive days. Results are presented as means $\pm$ SEM (in picograms per milliliter).

Immunoglobulin Measurement in Tears

The levels of immunoglobulins (Ig; IgA, IgG1, IgG2b, and IgM) were measured in tears using an immunoglobulin in an isotyping magnetic bead panel (Milliplex Map Kit; EMD Millipore Corp., Temecula, CA, USA). One sample consisted of tear washings pooled from both eyes of 10 mice (20 μ L) in PBS plus 0.1% bovine serum albumin (5 μ L) and stored at -80°C until the assay was performed. Four samples from Aec and B6 mice at 4 and 20 weeks of age were used. Tear washings were collected for 3 consecutive days from mice and were later used for flow cytometry. A 25 μ L sample was used according to the manufacturer's protocol. Sera were collected from B6 mice at 4 weeks of age ($n = 3$) through cardiac puncture immediately after euthanasia, frozen at -80°C until ready to use, and then used as positive controls. Labeled immunoglobulins (kappa light chain) were read by Luminex (Luminex 100 analyser with xPONENT 3.1 software upgrade; Luminex Corp., Austin, TX, USA). At least 50 events per bead were read, and the data were analyzed using software (Milliplex Analyst; EMD Millipore Corp.). Biological replicates were averaged, and data are presented as picograms per milliliter.

Flow Cytometry Analysis of Infiltrating Cells

Single-cell suspensions of LGs from Aec and B6 mice at 4, 12, and 20 weeks were prepared as previously reported.⁵ In brief, right and left extraorbital LGs were excised, rinsed, and subjected to collagenase digestion for 1 hour at 37°C under constant agitation. Collagenase was neutralized by adding complete RPMI with fetal bovine serum (FBS); cells were filtered by using a 70- μ m cell strainer, centrifuged, and resuspended. Cells were stained with anti-CD16/32 (BD Biosciences), followed by cell-surface staining with FITC-conjugated anti-CD4 (GK 1.5; BD Biosciences), PE-anti-CD8 (clone 53-6.7; BD Biosciences), APC-B220 (clone RA3-6B2; BD Biosciences), APC-CXCR3 (clone 220803; R&D Systems, Minneapolis, MN, USA), PE-CCR6 (clone 140706; R&D Systems), or Pacific Blue-CD3 (clone 500A2; BD Biosciences). Single-cell preparations of splenocytes or cervical lymph nodes obtained from 4-week mice were stained with the same antibodies and served as positive controls. Propidium iodide exclusion was used to discriminate live cells.

For evaluation of regulatory T cells (Treg) in both strains at 4 and 20 weeks of age, cells were prepared as described above and incubated with a stain kit (LIVE/DEAD Fixable Blue Dead Cell Stain Kit [L-23105, Invitrogen-Molecular Probes, Eugene, OR, USA]), fixed and permeabilized (CytoFix/Cytoperm; BD Biosciences), followed by staining with PE-CD25 (clone 7D-4; BD Biosciences), APC-Foxp3 (clone FJK-16s; Ebioscience, San Diego, CA, USA), and Pacific Blue-CD45 (clone 30-F11; Biolegend, San Diego, CA, USA).

Cells were kept on ice until flow cytometry analysis was performed. A BD LSRII Benchtop cytometer (was used, and

data were analyzed with BD Diva software (version 2.1; BD Biosciences) and FlowJo software (version 10.1; Tree Star, Inc., Ashland, OR, USA).

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's post hoc testing was used for statistical comparisons parameters within strains. Two-way ANOVA with Tukey's post hoc testing was used for statistical comparison between strains. $P \leq 0.05$ was considered statistically significant. These tests were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Ocular Surface Disease

The uptake of OGD as a marker of corneal barrier function disruption was measured in Aec and B6 mice. Wild-type B6 mice had increased uptake of OGD with aging, significantly highest at 20 weeks. Aec mice had slightly, but not significantly greater OGD intensity compared with B6 mice at 4 and 12 weeks of age. However, there was no change in OGD staining with aging in Aec mice (Figs. 1A, 1B).

In order to determine if CD4^{+} T cells migrate to the ocular surface in Aec mice, the density of CD4^{+} T cells in the conjunctival epithelium was determined by immunohistochemical staining. We chose to count infiltrating CD4^{+} T cells within the conjunctival epithelium at the goblet cell-rich area because number of CD4^{+} T cells in this area was previously found to inversely correlated with number of goblet cells.¹³ As expected, we noted a significant increase in CD4^{+} T-cell infiltration in conjunctival epithelium in Aec mice compared to B6 mice at 20 weeks (2.07 ± 0.32 vs. 1.46 ± 0.41 cells, $P = 0.01$; Figs. 1C, 1D). There was a tendency toward increasing numbers of CD4^{+} T cells infiltrating the conjunctiva with aging in both Aec and B6 mice.

Filled goblet cells were identified in paraffin-embedded conjunctival sections stained with PAS. Consistent with disease progression, a significant decrease in the density of goblet cell in conjunctiva (cells per millimeter) was observed at 12 weeks and persisted at 20 weeks in both mouse strains (Figs. 1E, 1F). Goblet cell density was significantly lower in Aec mice compared to B6 mice at 12 weeks.

Cytokine Profile With Aging in Ocular Surface of Aec and B6 Mice

We examined expression of inflammatory cytokine and MMPs and T-cell-related cytokines at the ocular surface by real-time PCR. Our results are summarized in Figure 2.

We observed that Aec mice had significantly higher levels of IL-17A, IL-17R, IL-1 α , and IL-1 β mRNA transcripts (at 4 weeks), and significantly lower levels of IFN- γ , MHC II, IL-17R, and MMP-9 (at 12 and 20 weeks), and IL-17A and IL-1 β mRNA transcripts (at 20 weeks) in their cornea than did similarly aged B6 mice (Figs. 2A, 2B). No significant change was found in levels of IL-6, IL-13, and MMP-3 transcripts in the cornea in the two strains (data not shown). There was a significant age-related increase in expression of IL-1 α and TNF- α in Aec cornea with aging from 4 to 20 weeks.

Aec mice had significantly higher levels of IL-17A and IL-1 α (at 4, 12, and 20 weeks), IL-1 β and IL-17R (at 12 and 20 weeks), IL-6 (at 4 weeks), and TNF- α mRNA transcripts (at 12 weeks), and significantly lower levels of MHC II and SPRR2a mRNA transcripts (at 4, 12, and 20 weeks) in the conjunctiva than did

FIGURE 1. Ocular surface phenotype in Aec mice. **(A)** Corneal OGD fluorescence intensity score. *Bar graphs* show mean $\pm$ SEM of three independent experiments with four animals per experiment (8 eyes per experiment yielding a final sample of 24 eyes per age per strain). **(B)** Representative images of OGD staining of each strain. Original magnification: $\times 10$; *scale bar*: 100 μ m. **(C)** CD4⁺ T cells infiltrating the conjunctival epithelium. *Bar graphs* show mean $\pm$ SEM of one representative experiment with three animals per age and strain. **(D)** Representative images of conjunctiva sections immunostained for CD4 (in red). Original magnification: $\times 40$; *scale bar*: 25 μ m. **(E)** Number of PAS⁺ conjunctival goblet cells counted in paraffin-embedded sections expressed as number per millimeter. *Bar graphs* show mean $\pm$ SEM of one representative experiment with three animals per age and strain. **(F)** Representative images of conjunctival sections stained PAS used to generate the *bar graph* in **(E)**. Original magnification: $\times 40$; *scale bar*: 25 μ m. W indicates weeks. $^{**}P < 0.01$, $^{****}P < 0.0001$ for within-B6 strain comparison compared to age 4 weeks. $^{\wedge\wedge\wedge\wedge}P < 0.0001$ for within-Aec strain comparison compared to age 4 weeks. $^{\dagger}P < 0.05$ for 12 vs. 20 weeks intrastain comparison.

FIGURE 2. Quantitative gene analysis of mRNA transcripts in aged cornea and conjunctiva. (A, B) Relative fold of expression of T-cell-related cytokines and inflammatory cytokines in cornea of Aec and B6 mice at ages 4, 12, and 20 weeks. Bar graphs show mean $\pm$ SEM of one representative experiment with six to nine samples per strain per age. (Experiment was repeated twice with similar results.) One sample consisted of pooled right and left corneas of the same animal. (C, D) Relative fold of expression of T-cell-related cytokines and inflammatory cytokines in conjunctiva of Aec and B6 mice at ages 4, 12, and 20 weeks. Bar graphs show mean $\pm$ SEM of one representative experiment with six to nine samples per strain per age. (Experiment was repeated twice with similar results.) One sample consisted of pooled right and left conjunctivas of the same animal. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for within-B6 strain comparison compared to age 4 weeks. $\wedge P < 0.05$, $\wedge\wedge P < 0.01$, $\wedge\wedge\wedge P < 0.001$ for within-Aec strain comparison compared to age 4 weeks. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ for age 12 vs. 20 weeks intrastrain comparison.

B6 mice (Figs. 2C, 2D). There was a significant age-related increase in expression of IL-17A, IL-1 β , and TNF- α in Aec conjunctiva from 4 to 20 weeks. The IFN- γ significantly increased with aging in Aec conjunctiva.

Evaluation of LG Function

It has been shown previously that Aec mice exhibited a reduced salivary gland secretory response at 12 weeks of age.⁷ However, secretory function of LG in Aec mice has not been evaluated. To determine the secretory activity of LG, tear volume was measured by phenol red cotton-thread technique. We observed that tear volume in the Aec mice was significantly greater at 20 weeks than 4 and 12 weeks (Fig. 3A). The B6 mice also exhibited an increase in tear volume with age, reaching statistical significance by 20 weeks. No difference was found between strains at any of the three age groups.

Epidermal growth factor is a multifunctional cytokine that regulates proliferation, differentiation, and survival of epithelial cells.¹⁴ EGF in tears is generally accepted to be secreted from the LGs, and tear EGF concentration is significantly lower in murine models of Sjögren syndrome.¹⁰ We assayed EGF in tears of all mice of both strains at all three time points (Fig. 3B). We observed that Aec mice of 4 and 20 weeks had slightly lower EGF concentration in tear washings compared with same-age B6 mice. But, 12-week-old Aec mice had significantly higher EGF concentration compared with B6 mice of the same age.

Immunoglobulin A is commonly found in high concentration in normal human and rodent tears, while very low levels of IgG and IgM are normally found and are thought to be present secondary to transudation from plasma and/or inflammatory processes.^{15–17} Immunoglobulin A is produced locally within the LG, and IgA content in tears increases with age.¹⁸ Because both strains had increased tear volume at 20 weeks compared to 4 weeks, we assayed the tears from Aec and B6 at these time points to better evaluate the quality of tears using a multiplex assay. No difference at IgA, IgG1, and IgM levels were noted between Aec and B6 mice at 4 weeks of age (Figs. 3C–E). Significantly increased IgA levels were found in B6 compared to Aec (B6 > Aec; $P < 0.01$; Fig. 3C) and increased IgG and IgM levels were seen in Aec at 20 weeks (Aec > B6; $P < 0.05$ for both immunoglobulins; Figs. 3D, 3E). The ratio of IgA to IgG and IgA to IgM in B6 mice increased with aging from 4 to 20 weeks of age, indicating a greater production of IgA and low levels of IgG and IgM, while in Aec mice they did not change (Figs. 3F, 3G).

Taken together, these results indicate the composition of tears is altered in Aec at 20 weeks of age, and increased tear volume may be indicative of transudation of plasma from an inflamed conjunctiva.

Periductal lymphocytic infiltration has been noted in murine models of SS and the aged human LG.^{19–22} We compared the LG histology of Aec mice at 4, 12, and 20 weeks of age with age-matched B6 mice by hematoxylin and eosin-staining. The B6 mice LGs had minimal lymphocytic infiltration at all time points (Fig. 3H). Aec mice LGs showed more prominent periductal lymphocytic infiltration at all ages, and was greatest at 20 weeks of age (Fig. 3H). To better characterize the lymphocytic infiltration, LG sections from both strains were immunostained for CD4⁺, CD8⁺ T cells and B cells and also by flow cytometry. Aec mice showed increased CD4⁺ T-cell infiltration of the LG with aging that became especially intense at 20 weeks of age (Fig. 3I). Minimal infiltration with CD8⁺ T cells and B cells (data not shown) was seen in Aec and B6 LGs. Flow cytometric analysis of freshly isolated cells from LGs of all age groups and both strains was used to quantify the immune cell subsets that infiltrated the LG, including CD4, CD8, and B cells. We observed that CD4⁺ T

FIGURE 3. Lacrimal gland evaluation in Aec and B6 mice. (A, B) Tear volume (A) was measured by phenol red thread test, and tear EGF concentrations (B) were measured by ELISA. Tear washings from both right and left eyes from one mouse per age and strain were collected and pooled into a single tube yielding a final sample of 10 individual samples per group and age divided into two independent experiments with five samples per experiment. Bar graphs show mean $\pm$ SEM. (C-E) Tear immunoglobulin concentrations were measured by Milliplex map assay. One sample consisted of tear washings pooled from both eyes of 10 mice (20 μ L) in PBS plus 0.1% bovine serum albumin (5 μ L). Four samples from Aec

and B6 at 4 and 20 weeks of age were used. *Bar graphs* show mean $\pm$ SEM. (**F, G**) Tear IgA-to-IgG ratio and tear IgA-to-IgM ratio. (**H**) Representative images of hematoxylin and eosin-stained LG sections from mice aged 4 to 20 weeks. Normal LG morphology was noted in the B6 mice at all time points, in contrast to Aec LG, which showed lymphocytic infiltration (circumscribed by *black dotted lines*) at 20 weeks. Original magnification: $\times 10$; *scale bar*: 100 μ m. (**I**) Representative images of LG frozen sections immunostained for CD4 (in red, [**B**]). Original magnification: $\times 10$; *scale bar*: 100 μ m. (**J**) Flow cytometric analysis of CD4⁺ and CD8⁺ cells in LG of Aec and B6 mice at ages 4, 12, and 20 weeks. Right and left extraorbital LGs from one mouse per age and strain were excised and pooled into a single tube yielding a final sample of six individual LG samples per group and age divided into two dependent experiments with three samples per experiment. *Bar graphs* show mean $\pm$ SEM. (**K**) A representative dot plot of the percentage of CD4⁺ cells that are CCR6⁺ (for Th17) or CXCR3⁺ (for Th1) in the cervical lymph node (CLN) and LG. (**L**) A representative dot plot of the percentage of CD25⁺ cells that are *Foxp3*⁺ (for Treg) in the LG. * $P < 0.05$, ** $P < 0.01$ for within-B6 strain comparison compared to age 4 weeks. $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ for within-Aec strain comparison compared to age 4 weeks. $^{\dagger}P < 0.05$ for age 12 vs. 20 weeks intrastrain comparison.

cells significantly increased in Aec LG compared to B6 at 12 weeks ($16.08 \pm 4.0\%$ vs. $6.57 \pm 1.5\%$, $P < 0.01$) and 20 weeks ($18.13 \pm 3.3\%$ vs. $11.14 \pm 4.92\%$, $P < 0.01$) and was noted to increase with aging from ages 4 to 20 weeks in Aec mice (Fig. 3J). There was no significant difference in CD8⁺ T cells in Aec mice LG compared to B6 mice, but a progressive increase in CD8⁺ T cells with aging was noted in both strains, in roughly equal proportions with aging (Fig. 3J).

To better characterize the Th phenotype of infiltrating CD4⁺ T cells, expression of chemokine receptors and FOXP3 were evaluated by flow cytometry (Figs. 3K, 3L). CD4⁺CXCR3⁺ and CD4⁺CCR6⁺ cells identify Th1 and TH17 cells, while CD4⁺CD25⁺FOXP3⁺ cells are considered regulatory T cells.^{23,24} At 4 and 20 weeks of age, Aec mice had lower frequency of CD4⁺CXCR3⁺ cells than B6 of the same age, but the frequency of CD4⁺CCR6⁺ cells was higher. Aec mice had a 2.6-fold increase in the frequencies of both CD4⁺CXCR3⁺ and CD4⁺CCR6⁺, while B6 mice had a minimal increase in Th1 cells and approximately a 6-fold increase in TH17 cells with aging from 4 to 20 weeks (Fig. 3K). Analysis of CD4⁺CD25⁺FOXP3⁺ cells indicated that B6 mice have higher number of Tregs at 4 weeks (~ 4 -fold), and it remained elevated at 20 weeks of age, where the number was over 2-fold greater than Aec (Fig. 3L).

Taken together, these results show that Aec mice have increased LG lymphocytic infiltration with aging that consists mainly of CD4⁺ T cells with predominance of TH17 over Th1 cells. Although there is also an increase of Tregs within the LG, their frequency is still half that of wild-type B6 mice.

Inflammatory Cytokine Milieu in Aec LG

We examined expression of inflammatory and T-cell-related cytokines and T-cell transcription factors in the LG by qPCR (Figs. 4A, 4B). Aec mice had significantly higher levels of IFN- γ , IL-17A, IL-1 β , IL-6, T-BET and ROR γ T, but lower levels of IL-13 and GATA-3 mRNA transcripts in the LG than B6 mice at 4 weeks of age (Figs. 4A, 4B). Levels of IL-17A, IL-17R, IFN- γ , IL-1 β , IL-6, and TNF- α mRNA transcripts tended to increase in the LG with aging in B6 mice, with a greater increase in IL-17 (and IL-17R) than IFN- γ , mirroring the pattern of chemokine receptor expression seen by flow cytometry. Differential changes in some markers were observed with aging in Aec: IFN- γ , Tbet, ROR γ T, GATA-3, and IL-6 mRNA decreased, reaching statistical significance at 20 weeks for IFN- γ and IL-6 compared to levels at 4 weeks. A significant increase in the levels of IL-1 β , IL-17R, and TNF- α transcripts was observed in Aec mice at 12 weeks, with a subsequent decrease at 20 weeks. The levels of T-BET and GATA-3 decreased with aging in Aec mice. These results indicate that at a very early age, Aec mice have significantly increased IFN- γ , IL-1 β , IL-6, IL-17A, and very low levels of IL-13 and GATA-3 compared to B6 mice. While there is a decrease in IFN- γ with aging, the increase in IL-17A does not parallel that seen in B6 mice. The inflammatory

cytokine IL-1 β is significantly elevated at 12 weeks compared to B6 control mice ($P < 0.001$).

DISCUSSION

Aec mice, a congenic strain of the NOD model of autoimmune exocrinopathy, develop dry eye spontaneously with aging.² The course of disease and characteristics of dry eye in Aec mice is similar to those of human dry eye.^{2,7} A newly derived strain, known as C57BL/6.NOD-Aec1Aec2, has recombination in *Aec1* that results in shortening of this genetic region to 19.2 cm from 48.5 cm.⁹ This recombinant inbred strain has revealed that male mice maintain a full SS-like disease, whereas female mice exhibited no dacryoadenitis and no loss of LG

FIGURE 4. Quantitative gene analysis of mRNA transcripts in aged LG. (**A, B**) Relative fold of expression of T-cell-related cytokines and inflammatory cytokines in LG of Aec and B6 mice at ages 4, 12, and 20 weeks. *Bar graphs* show mean $\pm$ SEM expression of one representative experiment with six to nine samples per strain and age. One sample consisted of pooled right and left extraorbital LGs of the same animal. *** $P < 0.001$, **** $P < 0.0001$ for within-B6 strain comparison compared to age 4 weeks. $^{\wedge}$ $P < 0.05$, $^{\wedge\wedge}$ $P < 0.01$, $^{\wedge\wedge\wedge}$ $P < 0.001$, $^{\wedge\wedge\wedge\wedge}$ $P < 0.0001$ for within-Aec strain comparison compared to age 4 weeks. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$, †††† $P < 0.0001$ for age 12 vs. 20 weeks intrastrain comparison.

secretory function.⁹ In the present study, we have examined parameters of the ocular surface disease in Aec female mice with aging and their correlation with development of LG disease. Both humans and mice have been noted to develop dry eye disease with aging. The B6 strain has a predilection to a TH1 inflammatory response. Age-related autoimmunity of B6 mice contributes to development of dry eye disease with aging. McClellan et al.¹⁴ investigated the immunocytologic profile of age-related dry eye disease using three different ages of B6 mice: young (8 weeks), midlife (6–9 months), and elderly (24 months). Compared to B6, Aec mice exhibit several features found in human SS, including more severe conjunctival goblet cell loss and LG lymphocytic infiltration with aging. Age-related differences in proinflammatory and T-cell gene expression profile in the cornea and conjunctiva epithelium between the two strains were also observed.

We demonstrated that like SS, the ocular surface of Aec mice developed conjunctival goblet cell loss with aging, parallel with LG lymphocytic infiltration. We also observed age-related changes in proinflammatory and T-cell gene expression profile in the cornea and conjunctiva epithelium. These findings were equal to or greater than those observed in B6 mice, demonstrating that Aec mice are more prone to developing dacryoadenitis and conjunctival goblet cell loss.

Decreased density of mucin-filled goblet cells is a well-recognized feature of aqueous deficient dry eye.^{25,26} Goblet cell density has been found to correlate with severity of ocular surface disease in patients with aqueous tear deficiency.²⁷ Previously, we have found that the recipients of adoptively transferred CD4⁺ T cells from aged B6 donors developed conjunctival inflammation with CD4⁺ T-cell infiltration, increased IFN- γ expression, and decreased goblet cell density.¹⁴ In our study, older Aec and B6 mice showed an increased number of CD4⁺ T cells infiltrating the conjunctival epithelium, and this T-cell infiltration was accompanied by conjunctival goblet cell loss. The reduced goblet cell density may have been due to increased IFN- γ expression since both strains of mice showed increased expression of IFN- γ in the conjunctiva with aging, but this was significant only in the Aec strain.

The strong correlation between age and prevalence of dry eye disease has been well established.²⁸ In elderly people, changes in tear parameters, such as tear flow, tear meniscus height, and tear volume, are often found.^{29–33} In several mouse strains, changes in tear volume (increase, decrease, or no change) have been noted, depending on the genetic background.^{34–37} In particular, tear volume increased in several mouse models of dry eye, such as CD25KO, SPDEF KO, aging C57BL/6,^{5,34,35} In a recent report, Tan et al.³⁸ reported that Schirmer I test values of Aec mice showed a significant reduction in comparison with those of the B6 control mice at 16 and 20 weeks of age. However, in our study there was a discrepancy between inflammation of LG and tear production. Tear volume in Aec mice paradoxically increased with aging similar to that observed in B6 mice. Lymphocyte infiltration of LG was more prominent in older Aec mice. Tear volume is a composite of fluid secreted by the LG and corneal and conjunctival epithelium, inclusive of IgA, lysozyme, growth factors, and antimicrobial peptides. It is possible that the increase of tear volume in Aec and B6 mice reflects the normal increase in body size and weight of mice. We observed significant increase in IgA in tears in B6 mice with aging without any changes in Aec, despite increase in size of both strains. We also observed an increase in IgG and IgM in 20-week-old Aec mice, suggesting that quality of tears in aged Aec is altered compared to B6 mice. These results also suggest increased transudation of plasma proteins from inflamed tissues, as IgG and IgM are not very abundantly found in tears from normal subjects.^{17,18} Although decreases in tear volume

are normally associated with both dry eye disease and aging, increases in LG secretion have been reported to occur in response to ocular surface irritation.³⁹ It is possible that the observed increase in tear volume in the Aec strain could be due to reflex tearing or due to transudation from the conjunctiva. Moreover, our findings suggest that increases of inflammatory mediators with aging make a greater contribution to development of ocular surface disease, mainly conjunctiva, than inflammation in the LG.

Traditionally, keratoconjunctivitis sicca was attributed to tear deficiency from LG disease; however, it is not uncommon for SS patients to have clinically significant ocular surface disease in the presence of normal tear production and volume.⁴⁰ Our study provides additional evidence suggesting that LG and corneal/conjunctival inflammation are important factors in the pathogenesis of ocular surface epithelial disease and ocular discomfort in SS, even in the context of normal tear production or volume.

We had previously reported that neutralization of IL-17A ameliorates corneal barrier disruption in an experimental model of dry eye.⁴¹ IL-17A has been shown to cause barrier disruption in mice and increased expression of MMPs, which leads to increased desquamation and breakdown of tight junctions of the corneal epithelium.⁴¹ Recently, elevated levels of IL-17A protein were found in minor salivary glands of SS patients compared with normal control subjects.⁴² Interestingly, in our study, increased expression of IL-17A mRNA transcripts and increased OGD intensity score as markers of corneal barrier disruption were significantly higher in older B6 mice, whereas decreased expression of both IL-17A and MMP-9 mRNA transcripts and decreased OGD intensity score with aging was found in the cornea of Aec mice. In our study, there was no change in OGD staining with aging in Aec mice. In another mouse dry eye model study (*Spdef*^{−/−} mice), the increase in fluorescein staining with age in wild-type *Spdef*^{+/+} mice was not observed in *Spdef*^{−/−} mice.³⁴ A similar pattern was observed in the autoimmune CD25KO model where corneal barrier dysfunction was only observed at 12 weeks of age despite severe dacryoadenitis at 16 weeks.²²

The proinflammatory effects of IL-1 in the LG have been extensively studied by Zoukhri and colleagues.^{43–45} They showed that a single injection of IL-1 into the LG induced a mild decrease in LG secretion while inducing a robust, reversible inflammatory response that led to destruction of LG acinar epithelial cells.⁴⁴ Pellegrino et al.¹⁰ reported that the peak of IL-1 β (age 12 weeks) preceded the greatest increase in lymphocytic infiltration (age 16 weeks) of LG. Thus, IL-1 β may be critical in inducing inflammatory dysfunction in the LG. In our study, IL-1 β and TNF- α transcript levels peak in Aec LGs at age 12 weeks, while levels of IFN- γ , IL-1 β , and IL-6 were higher at 4 and 12 weeks of age in Aec compared to B6 mice.

Our results showed the LG in both strains is infiltrated by TH1 and TH17 cells; Aec mice show a greater increase in the percentage of Th1 cells, whereas B6 mice show a greater increase in Th17 cells with aging. This was accompanied by an increase of regulatory T cells in both strains, although the B6 mice had greater frequency of Tregs both at baseline and at 20 weeks. Nguyen et al.⁴⁶ identified the differential expression of transcription factors for CD4⁺ Th1, Th2, and Th17 cells in the submandibular glands of Aec mice. These included *Tbet* for Th1 cells, GATA-3 for Th2 cells, and ROR γ t for Th17 cells.⁴⁶ *Tbet* expression was up-regulated nearly 3.5-fold in glandular tissue from 4-week-old Aec mice, but returned to normal levels by age 8 weeks. In contrast, expression of both *Gata3* and ROR γ t did not increase until after age 12 weeks, peaked at age 16 weeks, and returned to normal by age 20 weeks.⁴⁶ These data are highly indicative of the disease profile described for Aec mice, in which IFN- γ expression is essential early in the

disease.⁴⁶ In our study, T-BET and RORC of LG were significantly increased in Aec mice compared with B6, while GATA-3 was decreased.

Although SPRR2 expression increased with age in Aec, the increase was less than that observed in B6 mice. The cause for the discrepancy between strains is not clear. In contrast, Aec mice developed significant conjunctival goblet cell loss with aging that paralleled the CD4⁺ T-cell infiltration in the conjunctiva and LG. Additionally, expression of the Th1 cytokine IFN- γ was increased in the conjunctiva and LG and was significantly higher in the Aec strain. Therefore, Aec appears to be a relevant model for the LG inflammation and conjunctival goblet cell loss that occurs in SS. Interestingly, ocular surface disease in the Aec mice was not accompanied by decreased tear volume but rather by altered composition, suggesting that it may be related to inflammation in those tissues.

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