Immunopathology and Infectious Diseases

Interleukin-1 as a Phenotypic Immunomodulator in Keratinizing Squamous Metaplasia of the Ocular Surface in Sjögren's Syndrome

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Chronic inflammation of the ocular surface in Sjögren's syndrome (SS) is associated with a vision-threatening, phenotypic change of the ocular surface, which converts from a nonkeratinized, stratified squamous epithelium to a nonsecretory, keratinized epithelium. This pathological process is known as squamous metaplasia. Based on a significant correlation between ocular surface interleukin (IL)-1 β expression and squamous metaplasia in patients with SS, we investigated the role of IL-1 in the pathogenesis of squamous metaplasia in an animal model that mimics the clinical characteristics of SS. Using autoimmune-regulator (aire)-deficient mice, we assessed lacrimal gland and ocular surface immunopathology by quantifying the infiltration of major histocompatibility complex class II⁺ (I-A^{d+}) dendritic cells and CD4⁺ T cells. We examined squamous metaplasia using a biomarker of keratinization, small proline-rich protein 1B. We used lissamine green staining as a readout for ocular surface epitheliopathy and Alcian blue/periodic acid-Schiff histochemical analysis to characterize goblet cell muco-glycoconjugates. Within 8 weeks, the eyes of aire-deficient mice were pathologically keratinized with significant epithelial damage and altered mucin glycosylation. Although knockdown of IL-1 receptor 1 did not attenuate lymphocytic infiltration of the lacrimal gland or eye, it significantly reduced ocular surface keratinization, epitheliopathy, and muco-glycoconjugate acidification. These data demonstrate a phenotypic modulation role for IL-1 in the pathogenesis of squamous metaplasia and suggest that IL-1 receptor 1-targeted therapies may be beneficial for treating ocular surface disease asso-

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Sjögren's syndrome (SS) is a chronic autoimmune disorder characterized by progressive, immune-mediated destruction of the salivary and lacrimal glands that results in dryness of the mouth and eye.¹ It is estimated to affect as many as 4 million people in the United States alone, making it the second most common autoimmune disease.² Among the clinical manifestations of SS, one of the most debilitating is the ocular surface epitheliopathy that results from aqueous tear deficiency, known clinically as keratoconjunctivitis sicca (KCS). Patients with SS-KCS experience persistent ocular irritation on a daily basis that is seldom relieved through the use of artificial tear supplements. Despite considerable progress in the field of dry eye research over the past decade, the pathogenesis of autoimmune-mediated KCS remains poorly understood and treatment options are largely palliative.

SS-KCS is often referred to as an "autoimmune epithelitis."^{3,4} The phenotypic architecture of the ocular surface consists of mucosal epithelia covered by a glycocalyx of membranous mucins and a preocular tear film stabilized by gel-forming mucins. In severe cases of SS-KCS, there is a pathological *trans*-differentiation of the ocular surface, from a nonkeratinized, stratified mucosal epithelium to a nonsecretory, keratinized epithelium. This process, known as keratinizing squamous metaplasia, is accompanied by loss or alteration of ocular surface mucins, increased epithelial stratification, cornification of the superficial epithelial cells and instability of the preocular tear film.^{5,6} Progression from the early stage of inflammatory epithelitis in SS-KCS to the later stage of chronic,

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pathological keratinization, causes considerable morbidity among patients with SS. Advanced epithelial keratinization in chronically inflamed eyes can couple with sub-epithelial fibrosis to cause corneal opacification and vision loss.

Although the immunopathology of the exocrine glands in SS has been intensely studied, the immunological events that drive phenotypic changes of the ocular surface have received relatively little attention.⁷ In the eyes of patients with SS, immunohomeostasis is disrupted by autoimmune-mediated inflammation. Acute inflammation of the ocular surface is initiated by proinflammatory mediators released from ocular mucosal epithelial cells under desiccating stress. Chronic inflammation ensues when local antigen-presenting cells are sensitized to autoantigens and the antigen-presenting cell-primed CD4⁺ effector T cells home to the ocular surface.⁸ The identity of effector cells and cytokine mediators responsible for modulating the acute and chronic stages of ocular surface inflammation in SS has been the focus of intense research. Nonetheless, the main determinant(s) that mediate(s) progression from inflammatory epitheliopathy of SS-dry eye disease to sight-threatening keratinization of squamous metaplasia remain(s) unknown.

Interleukin-1 (IL-1) is a proinflammatory cytokine produced by many cell types, including mucosal epithelial cells of the ocular surface and immune cells.^{9,10} Two biologically active forms of IL-1 (IL-1 α and IL-1 β) mediate immune responses via two receptors, functional IL-1 receptor type I (IL-1R1) and decoy type II receptor (IL-1R2).¹⁰ Increased levels of IL-1 α and IL-1 β have been identified in the tear fluid and conjunctival epithelium of patients with SS and non-SS-KCS.¹¹ Moreover, increased expression of IL-1 β has been demonstrated to have a functional role in the pathogenesis of dry eye in several different mouse models,^{12,13} and it is a potent inducer of squamous metaplasia in the chronically inflamed airway.¹⁴ Recently, we reported that IL-1 β induces squamous metaplasia in human corneal epithelial cells *in vitro*, through the activation of IL-1R1 with downstream p38 mitogen-activated protein kinase (MAPK) and transcription factor cAMP response element-binding protein (CREB).¹⁵ Although there is little doubt about the proinflammatory role of IL-1 in autoimmune-mediated ocular surface disease, a direct link between IL-1R1 and ocular squamous metaplasia in dry eye disease has never been established *in vivo*.

To study the immunopathogenesis of squamous metaplasia *in vivo*, we recently characterized a murine model of autoimmune-mediated keratinizing squamous metaplasia.^{16–18} Depletion of autoimmune regulator (aire), a transcription factor encoded by the *AIRE* gene (OMIM 240300), triggers a T-cell-mediated autoimmune disease that targets multiple organs, including the salivary and lacrimal glands. The exocrinopathy of aire-deficient mice mimics the histopathological characteristics of patients with SS. Using lissamine green staining (a clinical measure of epitheliopathy), small proline-rich protein 1B (SPRR1B) (a cornified envelope protein precursor that is highly expressed in keratinized epithelium) and periodic acid-Schiff staining (a histological stain for goblet cells),

we observed severe KCS, keratinizing squamous metaplasia, and decreased density of conjunctival goblet cells (GCs) in aire-deficient mice.¹⁶ These changes were accompanied by a substantial increase in ocular surface expression of IL-1 β .¹⁷ Although these studies demonstrated an association between IL-1 expression and squamous metaplasia, it was not clear whether IL-1 functioned to initiate inflammation and/or to perpetuate pathological alterations of the ocular phenotype.

In the current study we explored the role of IL-1 in autoimmune-mediated keratinization of the ocular surface. Surprisingly, abrogation of IL-1R1 signaling in aire-deficient mice did not attenuate the activation and mobilization of immune cells to the lacrimal gland or ocular surface. It did, however, significantly protect against ocular surface epitheliopathy, keratinizing squamous metaplasia and altered glycosylation of GC mucins. The implications of IL-1 as a local immunomodulator of the ocular mucosal phenotype in the setting of autoimmune exocrinopathy are further discussed.

Materials and Methods

All materials were purchased from Sigma-Aldrich (St. Louis, MO), except defined keratinocyte serum-free medium from Gibco-BRL (Grand Island, NY), Dispase II was from Roche (Indianapolis, IN), the periodic acid-Schiff (PAS) staining set was from American Master Tech Scientific, Inc. (Lodi, CA), diaminobenzidine (DAB) substrate was from Vector Laboratories (Burlingame, CA), hematoxylin was from Richard-Allan Scientific (Kalamazoo, MI), and 4',6'-diamino-2-phenylindole was from Molecular Probes (Eugene, OR). Lissamine green (1%) and fluorescein (0.5%) dyes were obtained from Leiter's Pharmacy and Compounding Center (San Jose, CA). Anti-CD4 and anti-I-A^d mouse monoclonal antibodies were from BD Pharmingen (San Diego, CA), anti-IL-1 β goat polyclonal antibody was from R&D Systems (Minneapolis, MN), and anti-Foxp3 rabbit polyclonal antibody was from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Human Subject Recruitment

Fifteen patients with SS were recruited from the University of California, San Francisco Oral Medicine clinic with approval of the University of California, San Francisco, Committee for Human Research. Patients who had KCS and i) a labial salivary gland biopsy showing focal lymphocytic sialadenitis (focus score >1) or ii) positive serological results for anti-SS-A/Ro or SS-B/La antibody were included in the study. Exclusion criteria included known diagnosis of hepatitis C, HIV infection, sarcoidosis, amyloidosis, graft-versus-host disease, systemic lupus erythema, rheumatic arthritis, or preexisting lymphoma. A quantitative ocular grading system for KCS, called the SICCA ocular staining score (OSS) was used to assess the severity of KCS epitheliopathy.¹⁹ The OSS ranges from 0 to 12. A score of "0" indicates no corneal or

conjunctival staining, whereas a score of 12 indicates severe staining of the cornea and conjunctiva with confluent fluorescein staining in the pupillary area and the presence of corneal filaments. An OSS ≥ 4 was considered positive for KCS. Impression cytology was performed on both eyes of each subject to harvest superficial conjunctival epithelial cells for RNA extraction. TaqMan-based ($n = 6$) or SYBR Green-based ($n = 9$) PCR chemistry was used to analyze IL-1 β and SPRR1B expression by quantitative RT-PCR.

Animal Model

Aire-deficient mice were generated by targeted disruption of the murine *Aire* gene (OMIM 240300) as described previously.²⁰ Aire-deficient mice were backcrossed into the nonobese diabetic (NOD) Lt/J background for more than 10 generations and then crossed with NOD mice deficient in IL-1 receptor type 1 (deletion of *IL-1R1* gene [OMIM 147810]) to create an aire/IL-1R1 double knockout (DKO). Together with age-matched heterozygous controls, four genotypes were studied: wild-type (aire^{+/+}IL-1R1^{+/+}), IL-1R1 knockout (KO) (aire^{+/+}IL-1R1^{-/-}), aire KO (aire^{-/-}IL-1R1^{+/+}), and aire/IL-1R1 DKO (aire^{-/-}IL-1R1^{-/-}). Genomic DNA isolated from tail clippings was genotyped for the *Aire* and *IL-1R1* mutation by PCR. Animals were studied at 8 weeks of age ($n =$ minimum of 6 group). Lissamine green dye, (1%, 6 μ l) was applied to the ocular surface to detect devitalized epithelial cells.

Immunostaining, Immunofluorescence, and Histology

Lacrimal glands and eyes were dissected from euthanized mice at 8 weeks of age and embedded in OCT (for immunostaining), or fixed in 10% phosphate-buffered formalin and embedded in paraffin (for histology). Immunohistochemical analysis for CD4 and I-A^d and immunofluorescence for IL-1 β , Foxp3, and SPRR1B expression in the lacrimal glands and ocular tissues were performed according to previously optimized protocols for aire KO mice established in our laboratory.¹⁶ Negative controls included the omission of the primary antibody or isotype control. Mucin contents of conjunctival GCs were identified in paraffin-embedded sections using Alcian blue (AB)-PAS.

Isolation of Murine Corneolimbic Epithelial Sheets

Four groups of 8-week-old mice were handled according to University of California, San Francisco animal welfare guidelines for animal care. Mouse corneolimbic sheets were isolated as reported previously.²¹ One eye per mouse was enucleated by forceps, washed in PBS, and digested by Dispase II (10 mg/ml) in keratinocyte serum-free medium at 4°C for 18 hours. Using jewelers' forceps, an intact corneolimbic epithelial sheet was

surgically peeled off each eye and used for RNA isolation. Three mice from each genotype were used for each experiment.

Transcriptional Profiling of HBE Cells Using TaqMan Gene Expression Assays

Total RNA was extracted from primary corneal epithelial cells using an RNeasy Mini RNA isolation kit (Qiagen, Hilden, Germany). Total RNA was eluted from mini columns with 30 μ l of RNase-free water. The amount of total RNA was quantified by optical density measurements at 260 nm (OD₂₆₀). Starting from 1 μ g of total RNA, 80 μ l of cDNA was synthesized by using TaqMan reverse transcription reagents, which contain oligo(dT)₁₆, random hexamer, RNase inhibitor, a deoxy-NTP mixture and MultiScribe reverse transcriptase. The reaction was performed for 10 minutes at 25°C, 15 minutes at 42°C, and 5 minutes at 95°C. cDNA was stored at -20°C until use. To compare the relative abundance of IL-1 β and IL-1RA transcripts expressed, a TaqMan Probe fluorogenic 5' nuclease chemistry-based gene expression assay with exon boundary-crossing primers (assay identification number: Mm00434228_m1 and Mm00434237_m1, respectively) was applied. Real-time PCR was performed using thermal cycling conditions at 95°C for 10 minutes as the initial step, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C for amplification in an ABI Prism 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). All assays were performed and compared in four technical replicates to housekeeping gene *GAPDH*. Data were obtained as threshold thermal cycle (C_t) values. Three biological replicates (three donors) were used for obtaining C_t data in each of the four groups of mice. After normalization with a housekeeping gene, the fold change derived from $\Delta\Delta C_t$ of KOs versus control was examined by analysis of variance with $P < 0.05$ being considered significant. Positive and negative quality controls for reproducibility, reverse transcription, and genomic DNA contamination were assessed and found to be acceptable.

Quantification of Lissamine Green Staining, SPRR1B Expression, Immune Cells, and Goblet Cells

Lissamine green-stained corneas were photographed using a Nikon COOLPIX5400 digital camera (Nikon Instruments, Inc., Melville, NY) fitted to an Olympus Zoom Stereo Microscope (Olympus America Inc., Center Valley, PA). Immunofluorescent samples were photographed with a Nikon Eclipse Ti-E epifluorescence microscope or Nikon C1Si laser scanning confocal microscope. ImageJ 1.40g software (<http://rsb.info.nih.gov/ij>, last accessed January 11, 2010) was used to quantify staining intensity in images of lissamine green, immunohistochemistry, and immunofluorescence.²² NIS Elements BR2.30 software (Nikon Instruments, Inc.) was used to quantify AB-PAS stained-GC images as reported previously.¹⁶ A masked, well trained

Figure 1. The squamous metaplasia biomarker, SPRR1B, as a predictor of IL-1 β and the OSS in human patients with Sjögren's syndrome. Superficial ocular epithelial cells from 15 patients with Sjögren's syndrome were harvested by impression cytology for RNA extraction. Ocular surface epitheliopathy was quantified using the SICCA OSS.¹⁹ To examine the consistency of real-time PCR data, both TaqMan- ($n = 6$, blue dots) and SYBR Green dye-based chemistry ($n = 9$, black dots) were used to examine mRNA expression of SPRR1B and IL-1 β . Data were normalized by *GAPDH* and $\Delta\Delta C_t$ values were analyzed by linear regression to test the null hypothesis that SPRR1B expression was independent of IL-1 β expression (A) and OSS (B). Each graph shows raw ΔC_t values as well as a regression line. Squared correlation coefficients (R^2) indicate the fraction of variance explained by the regression model. Correlations were considered statistically significant when computed P values were <0.01 .

observer quantified each cell type at $\times 200$ magnification in three sections per eye from at least six animals.

Statistics

Results are presented as means $\pm$ SD. We used analysis of variance to compare differences in CD4 $^+$ cell counts, I-A $^+$ cell counts, lissamine green staining, corneal SPRR1B, GC density, and percentage of AB $^+$ GCs of total GCs (sum of AB $^+$ PAS $^+$ and AB $^-$ PAS $^+$ cells) among the four groups of mice. We adjusted for multiple comparisons using the Bonferroni correction. We modeled the relationship between SPRR1B and markers of ocular surface damage and inflammation using linear regression. All analyses were done using the statistical software Stata 9.0 (StataCorp, College Station, TX) for Macintosh.

Results

Ocular Surface Epitheliopathy, Squamous Metaplasia, and IL-1 β Are Correlated in Human Patients with Sjögren's Syndrome

Using RNA isolated by impression cytology from 15 patients with SS-KCS, we analyzed SPRR1B and IL-1 β expression with TaqMan- and SYBR Green-based quanti-

Figure 2. Autoimmune-mediated inflammation in air-deficient mice mimics the lacrimal gland exocrinopathy and ocular surface keratinization of Sjögren's syndrome. **A:** Gross anatomy of lacrimal glands from air-deficient and air-sufficient mice, stained with anti-CD4 $^+$ T cell antibody. **B:** H&E staining of the lacrimal gland. **Inset** is the higher power view of the boxed area. **C:** External eye pictures of the ocular surface. **D:** Immunofluorescence staining of SPRR1B in the central cornea. **Dotted line** indicates the corneal endothelium. Scale bar = 50 μ m.

tative PCR. As shown in Figure 1A, ocular surface expressions of SPRR1B and IL-1 β were highly correlated in patients with SS-KCS. The significance of SPRR1B as a predictor of ocular surface pathology was further emphasized by its equally compelling correlation with clinical disease severity, as assessed using the SICCA OSS (Figure 1B). These data demonstrate the significance of SPRR1B as a biomarker of aberrant ocular surface keratinizing differentiation and link its expression to IL-1 β in patients with SS-KCS.

The Aire-Deficient Mouse as a Model of Sjögren Syndrome KCS with Squamous Metaplasia

In air-deficient mice we observed lymphocytic infiltration throughout the lacrimal gland that consisted predomi-

Figure 3. The IL-1 cytokine family in aire-mediated KCS is proinflammatory. **A:** Genomic DNA was isolated from tail clippings of heterozygous (+/-) and knockout (-/-) mice for each gene of interest (*Aire* and *IL-1R1*). Duplex PCR was used to detect the full-length target gene in wild-type mice or a shorter mutant form in knockout mice. As expected, the aire wild-type band was detected at 1000 bp, the aire knockout band at 600 bp, the IL-1R1 wild-type band at 350 bp, and the IL-1R1 knockout band at 172 bp. **B:** Transcriptional activity of proinflammatory IL-1 β and anti-inflammatory IL-1RA among four genotypic groups. Data were obtained from three individual mice in each group and are shown as relative quantitation (using wild-type mice *aire*^{+/+}*IL-1R1*^{+/+} as onefold in gene expression). **P* < 0.05, using analysis of variance. **C:** Confocal immunolocalization of IL-1 β -expressing cells in the cornea. Whereas wild-type and IL-1R1 KO mice showed minimal expression of IL-1 β in the corneal epithelium, both aire KO and aire/IL-1R1 DKO mice showed abundant expression of IL-1 β across the corneal epithelium. Scale bar = 70 μ m.

Figure 4. Dendritic cell activation and autoreactive CD4⁺ T-cell recruitment in aire-deficient mice with and without intact IL-1R1 signaling. **A:** Immunohistochemical analysis against MHC class II surface antigen I-A^d (DAB chromogen shown as brown) was used to identify activated antigen-presenting cells in the central corneas of wild-type (*aire*^{+/+}*IL-1R1*^{+/+}), IL-1R1 KO (*aire*^{+/+}*IL-1R1*^{-/-}), aire KO (*aire*^{-/-}*IL-1R1*^{+/+}), and aire/IL-1R1 DKO (*aire*^{-/-}*IL-1R1*^{-/-}). Aire-sufficient wild-type and aire-sufficient IL-1R1 KO mice served as controls. **Arrowheads** indicate subepithelial infiltrating APCs, whereas **arrows** indicate stromal infiltrating APCs. **B:** Central corneal I-A^d dendritic cells (brown) were quantified by DAB staining intensity among the four groups. Data are shown as means $\pm$ SD in arbitrary units. **C and D:** Immunohistochemical analysis against CD4⁺ T cells (brown) demonstrated dense infiltration of the lacrimal gland parenchyma and the limbal area of the ocular surface in both aire KO and aire/IL-1R1 DKO mice, with scarce cells noted in aire-sufficient controls. **Arrows** indicate intraepithelial infiltrating CD4⁺ T cells, while **arrowheads** indicate intrastromal infiltrating CD4⁺ T cells. **E:** DAB staining intensity of CD4⁺ effector T cells was analyzed by densitometry. Data are shown as means $\pm$ SD in arbitrary staining units. Analysis of variance was used to test for significant differences between groups with *P* < 0.05 considered statistically significant. **F:** Immunofluorescence to detect Foxp3⁺ T regulatory cells in the limbal tissues. Sporadic expression of Foxp3⁺ cells (**arrows**) in the spleen served as a positive control. Scale bar = 50 μ m.

Table 1. Distribution of Dendritic Cells and CD4⁺ T Cells in Three Anatomical Locations of the Ocular Surface

Cell type	Genotype	Central cornea	Limbus	Conjunctiva
CD4 ⁺	Aire ^{+/-} IL-1R1 ^{+/-}	6.65 ± 10.26	157.32 ± 263.89	197.88 ± 186.8
	Aire ^{+/-} IL-1R1 ^{-/-}	0.043 ± 0.106	6.49 ± 8.78	83.2 ± 93.21
	Aire ^{-/-} IL-1R1 ^{+/-}	114.16 ± 94.6*†	2084.43 ± 1301.57*†	390.69 ± 213.53†
	Aire ^{-/-} IL-1R1 ^{-/-}	73.53 ± 58.77	2084.13 ± 778.8*†	415.23 ± 210.18†
I-A ^{d+}	Aire ^{+/-} IL-1R1 ^{+/-}	0 ± 0	10.17 ± 14.93	118.09 ± 74.27
	Aire ^{+/-} IL-1R1 ^{-/-}	0 ± 0	18.66 ± 14.74	132.46 ± 42.01
	Aire ^{-/-} IL-1R1 ^{+/-}	1.0 ± 0.70*†	224.88 ± 155.75*	23.06 ± 15.96†
	Aire ^{-/-} IL-1R1 ^{-/-}	1.18 ± 0.55*†	284.18 ± 206.81*†	47.52 ± 38.22

Data are shown as means ± SD in arbitrary staining units. Analysis of variance was used to test for significant differences between groups with $P < 0.05$ considered statistically significant.

* $P < 0.05$ versus Aire^{+/-}IL-1R1^{+/-}.

† $P < 0.05$ versus Aire^{+/-}IL-1R1^{-/-}; no statistical difference was noted between Aire^{-/-}IL-1R1^{+/-} versus Aire^{-/-}IL-1R1^{-/-} either in CD4⁺ or I-A^{d+} cells in any anatomical location, analysis of variance.

nantly of CD4⁺ T cells (Figure 2A) and was completely absent in wild-type mice. Infiltrates were organized in a multifocal pattern that damaged the tear-secreting acinar cells and interlobular septa (Figure 2B). Lacrimal gland damage was accompanied by desiccation and keratinization of the ocular surface that progressed to complete loss of corneal transparency in the most severe cases (Figure 2C). SPRR1B expression was up-regulated in the cornified keratin layer that covered the superficial corneal epithelium, a phenotype consistent with epidermal-like metaplasia of the ocular mucosal epithelia (Figure 2D). The exocrinopathy and ocular keratinization were consistent with clinical characteristics of human disease, further substantiating the aire-deficient mouse as a model for studying autoimmune-mediated KCS with keratinizing squamous metaplasia.

IL-1 Cytokine Superfamily Is Proinflammatory in Aire-Mediated Keratoconjunctivitis Sicca

To better understand the role of IL-1 in autoimmune-mediated SS-KCS, we crossed aire KO and IL-1R1 KO mice to create an aire/IL-1R1 DKO. Gene mutations were verified by duplex PCR on genomic DNA isolated from tail clippings with specific primers provided by the manufacturer (Figure 3A). The IL-1 cytokine superfamily comprises proinflammatory cytokine members such as IL-1 α and IL-1 β , as well as an anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA). We sought to determine whether the equilibrium of IL-1/IL-1RA signaling shifted toward a proinflammatory state in our SS-KCS model. By quantitative PCR, we examined the transcriptional activity of IL-1 β and IL-1RA using mRNA isolated from the corneolimbic epithelium of wild-type, IL-1R1 KO, aire KO, and aire/IL-1R1 DKO mice ($n = 3$ mice in each group). Compared with wild-type and IL-1KO mice (negative controls), IL-1 β was significantly up-regulated in both aire KO and aire/IL-1R1 DKO mice ($P < 0.05$), whereas there was a corresponding decrease in IL-1RA (Figure 3B). Although decreased expression of IL-1RA in aire-deficient mice was not statistically significant, the ratio of proinflammatory cytokine IL-1 β to anti-inflammatory cytokine IL-1RA (a reported indicator of IL-1-mediated inflammation¹¹) was substantially increased in aire KO and aire/IL-1R1 DKO mice. Consistent with quantitative PCR

findings, immunofluorescence studies using confocal microscopy confirmed that IL-1 β was increased at the protein level and that IL-1 β -secreting cells were localized to the ocular surface epithelium (Figure 3C). Taken together, these results indicate the activation of proinflammatory IL-1 signaling in aire-mediated KCS and keratinizing squamous metaplasia.

IL-1R1 Is Not Essential for Aire-Mediated Exocrinopathy or Ocular Surface Infiltrates

To initiate a proinflammatory response on the ocular surface, IL-1 cytokine must bind its functional receptor, IL-1R1. Using aire-deficient mice with and without functional IL-1R1, we examined the infiltration of representative cells from the afferent and efferent arms of the adaptive immune response. Dendritic cells expressing major histocompatibility complex class II molecule, I-A^d, and T cells expressing CD4 were identified using immunohistochemical analysis.¹⁶ Although I-A^{d+} cells were nearly absent in the central corneas of aire-sufficient mice (wild-type and IL-1R1 KO), they were recruited throughout the full epithelial and stromal segment of corneas in aire-deficient mice (aire KO and aire/IL-1R1 DKO) (Figure 4A). Quantification revealed a significant increase in dendritic cell numbers in aire-deficient mice, compared with aire-sufficient controls; however, the difference between aire KO and aire/IL-1R1 DKO mice was insignificant (Figure 4B). CD4⁺ T cells, the main effector cells of SS-KCS, infiltrated the lacrimal gland (Figure 4C) and the ocular surface (Figure 4D) of aire-deficient mice but not the two aire-sufficient mouse lines. CD4⁺ T cell infiltration of the ocular surface in aire-deficient mice was essentially unaffected by the presence or absence of IL-1R1 (Table 1). Dense, multifocal infiltration of CD4⁺ T cells throughout the lacrimal parenchyma of aire KO mice caused significant disruption of acinar cells that mimicked the exocrinopathy of SS patients (Figure 4C). On the ocular surface, the distribution of CD4⁺ cells varied by anatomical locations (conjunctiva, limbus, and cornea) with infiltration most dense at the limbus, an anatomical junction between the cornea and bulbar conjunctiva (Figure 4, D and E). A quantitative summary of dendritic and CD4⁺ T cell distribution in each anatomical location of the ocular surface is provided in Table 1. To determine whether

Figure 5. Ocular surface epitheliopathy in aire-deficient mice with and without IL-1R1 signaling. **A:** Lissamine green staining of the ocular surface was used to reveal punctate epithelial cell damage (green). Compared with aire-deficient mice with IL-1R1 (aire KO), staining intensity was significantly reduced when IL-1R1 was simultaneously knocked-out (aire/IL-1R1 DKO). **B:** Lissamine green staining intensity was quantified by ImageJ with data reported as means $\pm$ SD. Analysis of variance was used to test for significant differences between groups with $P < 0.05$ considered statistically significant. Scale bar = 100 μ m.

CD4⁺ T regulatory (Treg) cells may exist within densely infiltrated areas and to explore their potential involvement in aire-mediated autoimmunity, we used an anti-Foxp3 antibody to look for the presence of Treg cells both in the eye and lacrimal gland. Interestingly, we did not find any Treg cells in either location, including those areas most densely infiltrated by CD4⁺ cells (Figure 4F). Taken together, these data indicated that aire-mediated CD4⁺ T cell infiltration of the lacrimal gland and ocular surface occurred independently of IL-1R1 signaling and that the infiltrating effector cell population did not contain activated Treg cells.

IL-1R1 Plays a Fundamental Role in the Pathogenesis of CD4⁺ T Cell-Mediated Ocular Surface Epitheliopathy and Squamous Metaplasia

To evaluate ocular surface damage, we used lissamine green dye to stain devitalized and/or mucin-deprived

Figure 6. Ocular surface keratinization in aire-deficient mice with and without IL-1R1 signaling. **A:** SPRR1B staining of the ocular surface revealed keratinization in aire KO mice. SPRR1B expression was highest in the superficial corneal epithelial cells with a decreasing gradient toward the basal layer in aire KO mice. SPRR1B staining in DKO mice was largely reduced and undetectable in wild-type and IL-1R1 KO mice. **Inset** is the high power view of the boxed area. The grey mode of SPRR1B immuno-signals was used to provide a better contrast. **B:** SPRR1B fluorescence intensity was quantified by ImageJ with data reported as means $\pm$ SD. Analysis of variance was used to test for significant differences between groups with $P < 0.05$ considered statistically significant. Scale bar = 100 μ m.

epithelial cells in all mice. Consistent with the clinical manifestation of severe SS-KCS, we observed numerous, punctate epithelial erosions throughout the corneal surface of aire KO mice, whereas aire-sufficient wild-type and IL-1R1 KO mice were free of lissamine green (Figure 5A). Interestingly, staining was dramatically reduced in aire/IL-1R1 DKO mice. Quantitative analysis confirmed that epithelial erosions in aire KO mice with intact IL-1R1 (aire^{-/-}IL-1R1^{+/-}) significantly outnumbered the other three groups, whereas staining in aire/IL-1R1 DKO mice was statistically indistinguishable from that in wild-type and IL-1R1 KO controls (Figure 5B). Likewise cornified envelope precursor SPRR1B was ectopically expressed in the corneas of aire KO mice but was undetectable in the two aire-sufficient mouse lines (Figure 6A). The distribution of SPRR1B was most abundant in the superficial layer, with a decreasing gradient toward the basal layer of aire KO corneal epithelium (Figure 6A, inset for aire^{-/-}IL-1R1^{+/-}). Densitometric analysis of immunofluorescent signal confirmed increased SPRR1B in aire KO mice compared with that in wild-type, IL-1R1 KO, and aire/IL-1R1 DKO (Figure 6B). Notably, aire/IL-1R1 DKO and aire-sufficient mice (wild-type and IL-1R1 KO) had similar levels of

Figure 7. Quantification and characterization of conjunctival goblet cells in aire-deficient mice with and without intact IL-1R1 signaling. **A:** PAS staining was used to identify the GC-rich zone in the mouse tarsal conjunctiva. **B:** The total number of PAS-positive (pink) GCs in a defined area of the GC-rich zone was determined, and data are expressed as mean GC number $\pm$ SD. **C:** AB-PAS staining was used to identify the presence of GCs containing acidic mucin glycoconjugates (blue, arrows) or neutral glycoconjugates (pink). Aire KO mice exhibited dramatic acidification of GC mucins that was significantly reduced in aire/IL-1R1 DKO mice and nearly absent in wild-type and IL-1R1 KO controls. **D:** Percentage of AB⁺ GCs to overall GC number (sum of AB⁺PAS⁺ and AB⁺PAS⁻ cells) was determined for each group. Data are reported as mean percentage $\pm$ SD. Analysis of variance was used to test for differences between groups with $P < 0.05$ considered statistically significant. Scale bar = 100 μ m.

SPRR1B expression. Collectively, these data demonstrate a central role for IL-1R1 in SS-like autoimmune-mediated ocular epitheliopathy and squamous metaplasia.

Aire Deficiency Causes Altered Conjunctival GC Phenotype in the Presence of Functional IL-1R1

A characteristic feature of squamous metaplasia is the alteration of conjunctival GCs. GCs are enriched in a defined zone of the mouse conjunctiva, known as the GC-rich zone (Figure 7A). Here, we examined the quantity and composition of GC mucins in aire-deficient mice

by PAS and AB staining. Although PAS is a histological method used to stain all forms of carbohydrate macromolecules in muco-glycoproteins, AB specifically stains acidic muco-glycoconjugates. Although there was no difference in the total number of GCs identified by PAS staining among the four groups of mice (Figure 7B), AB staining revealed significant acidification of glycosidic residues on GC mucins in aire KO mice (Figure 7C). Intriguingly, this unique biochemical shift of muco-glycoconjugate from neutral (PAS⁺AB⁻ pink cells) to acidic (PAS⁺AB⁺ blue/purple cells) was not observed in the two aire-sufficient lines (wild-type and IL-1R1 KO) and was

Figure 8. Epithelial damage, corneal keratinization, and acidification of GC mucins are highly correlated in Sjögren's syndrome-like autoimmune ocular surface disorder. **A–C:** Regression analysis was performed to examine the relationships between three phenotypic characteristics of the Sjögren's syndrome ocular pathology: lissamine green staining (epithelial damage), corneal expression of SPRR1B, and the percentage of AB⁺ conjunctival GCs. Squared correlation coefficients (R^2) were ~ 0.50 for each analysis, suggesting that each phenotypic characteristic predicted approximately 50% of the variability in the other two outcomes. Raw data are plotted with the best-fit regression line to demonstrate statistically significant correlations ($P < 0.05$) between SPRR1B and lissamine green staining (**A**), SPRR1B and percentage of AB⁺ GCs (**B**), and lissamine green staining and percentage of AB⁺ GCs (**C**). **D:** A hypothetical diagram presents a central role for IL-1 in the pathogenesis of KCS and squamous metaplasia of Sjögren's syndrome. In the progression of nonkeratinized KCS to keratinizing squamous metaplasia, IL-1 may initiate inflammatory epitheliopathy in the acute stages of disease and later act as an immunomodulator of epithelial phenotype in the setting of chronic inflammation.

significantly less pronounced in *aire*/IL-1R1 DKO mice. The ratio of AB⁺ GCs to total number of GCs (sum of PAS⁺AB⁻ and PAS⁺AB⁺ cells) indicated that approximately 55% of GCs contained acidic residues in *aire*-KO mice, which was strikingly higher than the 17% identified in *aire*/IL-1R1 DKO mice (Figure 7D). Approximately 4% of GCs in wild-type mice and 5% in IL-1R1 KO mice were AB⁺, making them statistically indistinguishable from those of *aire*/IL-1R1 DKO mice (Figure 7D).

Ocular Surface Epitheliopathy, Squamous Metaplasia, and GC Mucin Alteration Are Interrelated in Aire-KO Dry Eye Model

We used regression analysis to study the interrelationships between ocular epithelial damage, squamous metaplasia, and GC mucin acidification in *aire*-deficient mice. Using lissamine green staining (epitheliopathy), SPRR1B (squamous metaplasia), and percentage of AB⁺ goblet cells (mucin acidification) as our readouts, we found each of these pathological changes to be highly correlated to the others. SPRR1B expression was a significant predictor of both lissamine green staining ($R^2 = 0.48$, $P = 0.026$) and mucin acidification ($R^2 = 0.56$, $P = 0.010$) (Figure 8, A and B). Likewise lissamine green staining was highly correlated to mucin acidification ($R^2 = 0.53$, $P = 0.001$) (Figure 8C). Taken together, these data support a pathological link between compromised epithelial integrity, aberrant keratinization, and altered GC mucin content in the setting of SS-like autoimmune-mediated KCS. The potential role of IL-1R1 as an inflammatory mediator of ocular surface integrity and phenotypic modulator of ocular surface epithelium is summarized in Figure 8D.

Discussion

IL-1 is a versatile cytokine family that mediates inflammatory responses of immune cells and modulates growth and differentiation of epithelial cells.^{10,23,24} It represents an important mediator of inflammation and is reported to modulate pathological tissue remodeling in a number of autoimmune diseases, including rheumatoid arthritis and SS.^{1,25,26} IL-1R1 is the functional receptor that mediates cellular stimulation from IL-1 cytokine and is ubiquitously expressed in all tissues and organ systems of the body.²⁶ Notably, all of the key cells involved in SS exocrinopathy have been reported to express IL-1R1 (ie, dendritic cells, T lymphocytes, ocular epithelial cells, and lacrimal acinar cells).^{11,27–29} Whereas IL-1 is essential for maintaining ocular surface homeostasis, dysregulation of IL-1 has been implicated in eliciting ocular inflammation in patients with dry eye and in non-SS dry eye animal models.^{11,30} Our work demonstrates that IL-1 β expression on the ocular surface is a strong predictor of pathological keratinization in patients with SS (Figure 1). These clinical data suggest a potential role for IL-1 in mediating keratinizing squamous metaplasia in SS-KCS and emphasize the need for additional *in vivo* investigation of the inherent mechanism. Using a mouse model of autoimmune-mediated

KCS that mimics the glandular and ocular surface characteristics of SS, we further examined how IL-1 mediates the immunological events that lead to KCS and squamous metaplasia.

Although the events that link chronic inflammation to ocular surface keratinization are poorly understood, we previously demonstrated that CD4⁺ T cells are the main effectors driving ocular surface inflammation and squamous metaplasia in *aire* KO mice.¹⁶ Adoptive transfer of CD4⁺-enriched T cells from *aire* KO to non-disease-susceptible athymic nude mice conferred a SS-like disease phenotype that included pathological keratinization of the ocular surface.¹⁷ In the current study, we provided evidence that the ratio of the proinflammatory cytokine IL-1 β to anti-inflammatory IL-1RA is elevated in *aire*-mediated ocular surface disease (Figure 3B). This finding is in line with our clinical observations of increased IL-1 β on the ocular surface in patients with SS, mainly those who had significantly elevated expression of the keratinizing biomarker, SPRR1B (Figure 1A). These results are also consistent with a previous report showing an increased ratio of IL-1 β to IL-1RA in tears collected from patients with SS.¹¹ Taken together, our results demonstrate that IL-1 is a major mediator of epithelial damage and keratinization in response to autoreactive CD4⁺ T-cell-elicited ocular surface inflammation.

To further characterize the composition of CD4⁺ cells, we looked for the presence of Treg cells within those areas most densely infiltrated with lymphocytes. Interestingly, we did not detect any Treg cells in the ocular surface tissue or lacrimal glands (Figure 4F) of either *aire*-deficient or wild-type mice. An absence of Treg cells in the peripheral tissues suggested they were less likely to be actively involved in the pathogenesis of *aire*-mediated KCS. This assumption was consistent with previous reports demonstrating no apparent influence of *aire* on CD4⁺CD25⁺ Treg cell development in the exocrine gland or eye; rather, *aire* served to regulate the emergence of self-reactive effector cells.³¹ Another notable observation was the redistribution of tissue-resident dendritic cells and CD4⁺ cells from their limbal origin in the immune-privileged cornea to the central cornea in *aire* deficiency. This result implied the disruption of immune homeostasis in autoimmune-mediated KCS that occurred independent of IL-1 signaling.

Although the precise immune event(s) that caused ocular surface damage could not be determined in the current study, we clearly showed that ocular epitheliopathy and keratinization in response to CD4⁺ T cell-mediated inflammation was largely prevented in the absence of IL-1R1 signaling. In the eye, IL-1 cytokine is derived primarily from the resident epithelial cells.⁹ As a proinflammatory cytokine, IL-1 is known to facilitate antigen-presenting cell activation and costimulation of T cell function, together with an antigen or a mitogen.¹⁰ Locally, IL-1 can also function as an immunomodulator for epithelial differentiation through autocrine and/or paracrine signaling.^{23,32,33} Data in the current study suggest that, instead of costimulating antigen-presenting cells with autoantigen(s) or expanding/recruiting effector T cells to the ocular surface, IL-1 serves as a downstream

effector that bridges T-cell-elicited inflammation to the phenotypic transformation of ocular mucosa to epidermal-like epithelium.

In light of the connection between IL-1 and keratinization, we sought to evaluate ocular integrity and further characterize the mucosal phenotype in the presence and absence of IL-1R1. Ocular surface integrity is largely attributed to the barrier function constituted by tight junctions. IL-1 has been demonstrated to impair corneal barrier function by redistributing tight junction components ZO-1 and occludin in ocular inflammation.³⁴ Disruption of corneal barrier function in epitheliopathy has also been linked to up-regulation of keratinizing protein SPRR2 in an experimental mouse model of desiccating dry eye.³⁵ Using lissamine green staining as a readout for corneal epithelial damage, we observed a striking decrease in epitheliopathy in the absence of IL-1R1 (Figure 5, A and B). Moreover, lissamine green staining was highly correlated with SPRR1B expression in *aire*-deficient mice, supporting its use as a clinical correlate for squamous metaplasia that can be conveniently monitored in future explorations of therapeutic interventions.

Another key component that guards ocular integrity and maintains phenotypic homeostasis is the gel-forming mucin released from conjunctival GCs. Gel-forming mucins constitute the macromolecular scaffold of the aqueous tear film. The glycans decorating these mucin glycoproteins represent a rich ligand source for microbial binding, lubricate the ocular surface, and regulate physicochemical characteristics of the gel.³⁶ Several studies have demonstrated altered expression of mucins in dry eye syndrome.^{37,38} Although the carbohydrate content of ocular mucins has been reported to be altered in dry eye patients and in ocular keratinization caused by ocular cicatricial pemphigoid,^{37,39} our *aire*-KO mouse model is the first to directly link pathological glycosylation of ocular mucins to keratinizing squamous metaplasia in autoimmune-mediated dry eye disease. The high percentage of AB⁺ cells in the conjunctiva of *aire* KO mice indicated a dramatic acidification of GC muco-glycoconjugates (Figure 7C). Glycoconjugates confer a hydrophilic character to mucins that coat the epithelial surface and are essential to stabilize the preocular tear film and maintain epithelial barrier function.⁴⁰ Acidification of mucin glycans can occur due to increased sulfation or sialylation, both of which are observed in the pathological alteration of airway mucins in patients with cystic fibrosis.⁴¹ In the setting of autoimmune-mediated inflammation, we observed acidification of conjunctival GC mucins in eyes undergoing keratinizing squamous metaplasia, and these two events were highly correlated (Figure 8B). Moreover, the acidification of GC mucins was significantly attenuated by deletion of functional IL-1R1 signaling in the T-cell-infiltrated eye (Figure 7, C and D). To our knowledge, IL-1 β has only been shown to alter the expression of MUC5AC in airway GCs,⁴² making our report the first to demonstrate a role for IL-1 in aberrant glycosylation of gel-forming mucins in ocular inflammation. In the airway, IL-1 β regulates MUC5AC expression via a signaling pathway that involves p38 MAPK phosphorylation and downstream activation of transcription factor, CREB.⁴²

Interestingly, this same IL-1R1/p38 MAPK/CREB pathway led to SPRR1B up-regulation in corneal epithelial cells.¹⁵ The regulation of MAPK stress pathways in response to inflammation of the mucosal epithelia is undoubtedly complex, but the appearance of seemingly common pathways such as those described downstream of IL-1R1, suggest potential therapeutic targets in the management of ocular keratinization. Moreover, the strong interrelationships between ocular surface epitheliopathy, keratinization, and mucin acidification, indicated that KCS and squamous metaplasia are closely related disease entities that are mediated, at least in part, by IL-1/IL-1R1 signaling. Therefore, IL-1 might have a dual role in the pathology of SS-KCS, acting as an inflammatory mediator of acute epithelitis, as well as a phenotypic modulator that leads to aberrant keratinization (Figure 8D).

In summary, we report an important role for IL-1R1 in the pathogenesis of autoimmune-mediated KCS and squamous metaplasia. To our knowledge, this is the first *in vivo* study to establish a direct link between IL-1 and pathological keratinization of the ocular surface. Using a mouse model of KCS that mimics the human disease SS, we found that inhibition of IL-1R1 largely preserves the integrity and mucosal phenotype of the chronically inflamed ocular epithelium. Results of our study suggest that IL-1 receptor-targeted biological agents may represent a novel approach for managing patients with KCS who have keratinizing squamous metaplasia. Further study is necessary to determine the interplay of IL-1 with immune effector cells and the homeostasis of ocular mucosal epithelial phenotype in the setting of SS autoimmunity.

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