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Spontaneous T cell mediated keratoconjunctivitis in Aire-deficient mice

S Yeh, C S de Paiva, C S Hwang, K Trinca, A Lingappan, J K Rafati, W J Farley, D-Q Li, and S C Pflugfelder

Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, Houston, Texas, USA

Abstract

Background/aims—Patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) develop severe keratoconjunctivitis, corneal scarring and visual loss, but the precise pathogenesis is unknown. This study evaluated the ocular surface immune cell environment, conjunctival goblet cell density and response to desiccating environmental stress of the autoimmune regulatory (Aire) gene knockout murine model of APECED.

Methods—Aire-deficient and wild type (WT) mice were subjected to desiccating stress from a drafty, low-humidity environment and pharmacological inhibition of tear secretion for 5 days. Immune cell populations (CD4⁺, CD8⁺, CD11b⁺, CD45⁺) and goblet cell density were measured in ocular surface tissues and meibomian glands, and compared with baseline values.

Results—Greater CD4⁺ T cell populations were observed in the conjunctival epithelium of Aire-deficient mice ($p < 0.001$) compared with WT. Aire-deficient mice also had greater numbers of CD4⁺, CD8⁺, and CD11b⁺ cells in the peripheral cornea at baseline and following desiccating stress. The meibomian glands of Aire-deficient mice demonstrated greater CD4⁺, CD8⁺, CD45⁺ and CD11b⁺ cells at baseline ($p < 0.001$) and following desiccating stress. Conjunctival goblet cell density was lower at baseline and following desiccating stress in Aire-deficient compared with WT mice ($p < 0.001$).

Conclusion—Aire-deficiency leads to infiltration of CD4⁺ and CD8⁺ T cells on the ocular surface and meibomian glands, which is accompanied by goblet cell loss. Desiccating stress promotes this proinflammatory milieu. Immune-mediated mechanisms play a role in the severe blepharitis and keratoconjunctivitis in the murine model of APECED.

Autoimmune polyendocrinopathy (APECED) is an autosomal recessive condition caused by a mutation in the Autoimmune Regulatory (Aire) gene and consists of a constellation of autoimmune manifestations affecting endocrine glands, skin and the eye.¹² Systemic manifestations include adrenal insufficiency, hypoparathyroidism, chronic mucocutaneous candidiasis, hypothyroidism and autoimmune hepatitis.³ Ophthalmic features described in association with APECED include keratitis, conjunctivitis, blepharitis, cataract, uveitis and optic neuropathy.³⁴

Keratoconjunctivitis is the most common ophthalmic manifestation, occurring in 25–50% of APECED, and may develop prior to the onset of other systemic manifestations.³⁴ The ocular

Correspondence to: Professor S C Pflugfelder, Ocular Surface Center, Cullen Eye Institute, Baylor College of Medicine, 6565 Fannin Street, NC205, Houston, TX 77030, USA; stevenp@bcm.tmc.edu.

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surface inflammation that develops in APECED may lead to debilitating photophobia, chronic ocular pain and visual loss.⁵ Severe vision-threatening complications, including corneal scarring and spontaneous corneal perforation, have been reported,⁶ and corneal transplantation is associated with a high risk of rejection.⁷

The Aire gene product is thought to play a role in negative selection, the deletion of self-reactive T cells during thymic maturation.⁸ Specifically, the Aire gene product is thought to regulate the expression of ectopic (ie, non-thymic) proteins within the thymus to facilitate the deletion of auto-reactive T cells. Consistent with this hypothesis, DeVoss and associates showed that the failure to express a single retinal antigen, interphotoreceptor retinoid-binding protein (IRBP) in the thymus of Aire-deficient mouse resulted in ocular autoimmunity.⁹

The pathogenic mechanisms responsible for the severe ocular surface phenotype that develops in patients with APECED are incompletely understood. A study of the Aire-deficient murine model of APECED showed a dry eye phenotype manifesting as loss of ocular surface barrier function, decreased goblet cell density and increased epithelial stratification. An increased expression of proinflammatory cytokines by ocular surface cells was also seen.¹⁰ Other authors have proposed that limbal stem cell deficiency is central to the ocular surface findings APECED.^{11,12}

The potential role of environmental stressors in the pathogenesis of keratoconjunctivitis associated with APECED has not been explored either in patients or in Aire-deficient mice. We previously reported that in the non-obese diabetic (NOD) mouse, which is prone to developing ocular surface autoimmunity similar to Sjögren syndrome, desiccating environmental stress aggravates Sjögren syndrome-like lacrimal keratoconjunctivitis.¹³ The defective immunoregulation inherent in NOD-mice likely contributes to their inability to curb the ocular surface inflammation once it has been initiated.¹³

The purpose of this study was to further characterise the ocular surface immune environment in Aire-deficient mouse and to determine whether environmental stressors (ie, exposure to a desiccating environment) significantly alter the immune cell profile. Goblet cell loss, which is a hallmark of keratoconjunctivitis, was also evaluated before and after exposure to a desiccating environment.

METHODS

Animals

This research protocol was approved by the Baylor College of Medicine institutional animal care and use committee, and conformed to the standards in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

AIRE mice with a C57B6/J genetic background were purchased from Jackson Laboratories (Bar Harbor, Maine). Four-week-old C57B6/J mice served as controls. Mice were untreated or treated with subcutaneous injection of 0.5 mg/0.2 ml of the muscarinic receptor blocker scopolamine hydrobromide (Sigma-Aldrich, St Louis, Missouri) in alternating hindquarters four times per day to inhibit tear secretion and the desiccating stress of an air draft from a fan 16 h per day as previously reported.¹⁴ Mice were euthanised after 5 days of this treatment. Each experimental group consisted of three mice (six eyes) and all experiments were repeated.

Histology

Eyes and ocular adnexa were surgically excised, fixed in 10% formalin, paraffin embedded and 10 μm sections were cut. Sections were stained with periodic–Schiff (PAS) reagent for measuring goblet cell density and were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DMX 1200; Nikon, Garden City, New York). Goblet cell density in the superior and inferior conjunctiva was measured in three sections from each eye using image-analysis software (MetaVue 6.24r; Molecular Devices Metavue, Downingtown, Pennsylvania) and expressed as the number of goblet cells per 100 μm .

Immunohistochemistry

Immunohistochemistry was performed to detect and count cells in the conjunctival and corneal epithelium and stroma that stained positively for immune and inflammatory cell markers, including CD4, CD8, CD45 and CD11b. Cryosections from three mice per group were fixed in acetone at -20°C for 10 min. After fixation, endogenous peroxidases were quenched with 0.3% H_2O_2 in PBS for 10 min. The sections were then sequentially blocked with avidin/biotin blocking solution (Vector Laboratories, Burlingame, California) for 10 min. After blocking with 20% normal goat serum in PBS for 45 min, primary rat monoclonal antibodies against CD45 (clone 30-F11, 10 $\mu\text{g}/\text{ml}$), CD11b (clone M1/70, 6.25 $\mu\text{g}/\text{ml}$), CD4 (clone H129.9, 10 $\mu\text{g}/\text{ml}$) or CD8 (clone 53–6.7, 1.25 $\mu\text{g}/\text{ml}$), (all from BD Biosciences, San Jose, California) were applied and incubated for 1 h at room temperature. Sections were washed and incubated with biotinylated goat antirat antibody (BD Biosciences). The samples were incubated with NovaRed (Vector Laboratories) peroxidase substrate to give a red stain and counterstained with haematoxylin. Three sections from each animal were examined and digital images were obtained with an Eclipse E400 microscope equipped with a DMX 1200 camera (Nikon).

To determine the number of immune cells in conjunctival specimens, cells positively stained by each antibody were counted in the goblet rich areas of conjunctiva over a length of at least 500 μm of epithelium and to a stromal depth of 75 μm below the epithelium basement membrane using image-analysis software (MetaVue 6.24r; Molecular Device). Cell densities in the conjunctiva were expressed as the number of positive cells per 100 μm . To determine the number of immune cells within the cornea, cells were counted distances of 500 μm from the limbus. To determine the immune cell density in the region of the meibomian glands, a region of interest was circled and an area in μm^2 was calculated using image-analysis software (MetaVue 6.24r; Molecular Device). The number of cells within each region of interest was counted, and that number was multiplied by 10^6 to obtain an immune cell density with the units cells/ mm^2 .

Statistical analysis

Results are presented as mean (SD). A t test was used for between-group comparisons of inflammatory cell populations and goblet cell density, and two-way analysis of variance (ANOVA) with Tukey post hoc testing was used for statistical comparisons between multiple groups using GraphPad Prism 3.0 software (GraphPad Software; San Diego, California). A p value of <0.05 was considered statistically significant.

RESULTS

Aire-deficient mice develop keratoconjunctivitis

Keratoconjunctivitis is the most common eye manifestation in APECED. We compared the density of several immune cell populations within the ocular surface tissues of Aire-deficient mice with wild type (WT) control mice and evaluated the effects of desiccating

stress on these cell populations. CD4⁺ and CD8⁺ T cells have been observed qualitatively on histopathology sections of the ocular surface in Aire-deficient mice in one prior report.¹⁰ We quantitatively assessed and compared CD4⁺ and CD8⁺ T cell populations in the conjunctiva, cornea and meibomian glands of both Aire-deficient and WT control mice. The findings are summarised in table 1.

CD4⁺ T cell populations were found in significantly greater numbers in the conjunctival epithelium ($p<0.001$) of Aire-deficient mice compared with controls (table 1, fig 1).

The density of CD4⁺ was very low (<2 cells/500 μ m) in the peripheral and central corneal epithelium of WT mice. Both CD4⁺ and CD8⁺ T cell populations were significantly greater in the peripheral corneal epithelium and stroma, and the central stroma of Aire-deficient mice compared with WT.

Within the meibomian glands, significantly greater numbers of CD4⁺ T cells ($p<0.01$) were noted in the Aire-deficient mice, whereas the density of CD8⁺ T cells was significantly lower than WT.

CD45, a marker for bone marrow-derived cells, appeared to be fairly similar in the conjunctival epithelium and stroma, and in the corneal epithelium and stroma in Aire-deficient and WT mice. Aire-deficient mice appeared to have an elevated population of CD45⁺ cells within the meibomian glands ($p<0.001$).

CD11b, the beta2-integrin subunit of the Mac-1 (CD11b/CD18) complex, is one of the naturally occurring ligands for intercellular adhesion molecule-1 (ICAM-1). It is found on a number of leucocytes, including macrophages/monocytes, natural killer cells, dendritic antigen-presenting cells (APCs) and a subset of resting T cells. Prior studies have identified CD11b⁺ cells in normal human conjunctival epithelium.¹⁵ The number of CD11b⁺ cells within the conjunctival epithelium and stroma, and within the peripheral corneal epithelium was similar between Aire-deficient and WT control mice (table 1). The number of CD11b⁺ cells in the peripheral corneal stroma was higher ($p<0.05$) in Aire-deficient compared with WT controls. The density of CD11b⁺ cells in the central corneal epithelium and stroma was similar in both groups.

CD11b⁺ cell populations were greater in the meibomian glands of Aire-deficient mice than WT mice at baseline ($p<0.01$), but this was not observed following desiccating stress.

Effects of desiccating stress on the ocular surface of aire-deficient mice

We have previously reported that exposure of the ocular surface of C57BL/6 and NOD mice promoted migration of CD4⁺ T cells into the conjunctival epithelium and decreased conjunctival goblet cell density.¹³ Based on these findings, we evaluated the effects of desiccating stress on the ocular surface of Aire-deficient mice in the current study. The results of these studies are presented in table 1.

In unstressed mice, the number of CD4⁺ T cells in the conjunctival epithelium was greater in Aire-deficient mice compared with WT controls ($p<0.001$), whereas the density of CD4⁺ T cells in the conjunctival stroma was comparable ($p>0.05$). There was no change in the density of these cells in the conjunctival epithelium or stroma following 5 days of desiccating stress in Aire-deficient mice. In contrast, CD4⁺ T cells increased following desiccating stress in WT control mice. Aire-deficient mice exhibited similar numbers of CD8⁺ T cells in the conjunctival epithelium and stroma at baseline as controls, and desiccating stress did not appear to alter CD8⁺ cell populations in either Aire-deficient or WT mice.

The numbers of CD4⁺ and CD8⁺ T cells infiltrating the peripheral corneal epithelium and stroma in Aire-deficient mice were significantly greater than in WT mice both at baseline and following 5 days of desiccating stress ($p < 0.001$ for CD4⁺ T cells in peripheral corneal epithelium and stroma; $p < 0.001$ for CD8⁺ T cells in the peripheral corneal epithelium and stroma). Following desiccating stress, there was no significant change in the density of CD4⁺ and CD8⁺ T cells in the peripheral corneal epithelium and stroma of Aire-deficient mice.

Interestingly, the number of CD45⁺ leucocytes decreased in the conjunctival epithelium of control mice following desiccating stress ($p < 0.01$) but remained relatively unchanged in Aire-deficient mice. CD11b⁺ cells were unchanged in both Aire-deficient and WT mice before and after desiccating stress in ocular surface epithelia (conjunctiva and cornea). Within the meibomian glands, CD11b⁺ cells decreased in the Aire-deficient mice following desiccating stress ($p < 0.01$).

Aire-deficient mice have fewer goblet cells than C57BL/6 mice

Conjunctival goblet cell loss is recognised as a hallmark of a number of ocular surface inflammatory disorders (ie, Sjögren syndrome, Stevens–Johnson syndrome, ocular cicatricial pemphigoid).¹⁶¹⁷ Consequently, we evaluated conjunctival goblet cell densities in both Aire-deficient and control mice. Goblet cell densities were significantly lower in Aire-deficient mice versus WT mice at baseline ($p < 0.001$) and following desiccating stress ($p < 0.001$). In WT mice, the number of goblet cells decreased significantly ($p < 0.001$) following induction of keratoconjunctivitis; however, this phenomenon was not observed in Aire-deficient mice (table 2). The goblet cell density in Aire-deficient remained significantly lower than WT mice following desiccating stress ($p < 0.001$).

DISCUSSION

To gain a better understanding about the pathogenesis of the chronic ocular surface inflammatory disease that develops in APECED, we characterised the immune cellular environment of the ocular surface and meibomian glands, and evaluated the effects of desiccating stress on these cell populations in Aire-deficient mice and WT control mice. We also measured the density of conjunctival goblet cells before and after desiccating environmental stress.

Compared with WT mice, Aire-deficient mice demonstrated significantly greater numbers of CD4⁺ T cells in the superior and inferior conjunctiva. These areas of conjunctival epithelia are typically rich in goblet cells, mucin-producing cells that are critical to tear film and ocular surface homeostasis. The decreased density of conjunctival goblet cells in Aire-deficient mice described herein is consistent with prior qualitative observations,¹⁰ and if Aire gene defects in humans have similar consequences, goblet cell loss could play a role in the severe ocular surface phenotype observed in patients with APECED. Following desiccating stress, the goblet cell density of the Aire-deficient mice remained lower than in WT mice, potentially reflective of their severe ocular surface phenotype at baseline.

We also observed a CD4⁺ cellular infiltrate in the meibomian glands of Aire-deficient mice at baseline. Following desiccating stress, CD4⁺ T cell populations in the meibomian glands of WT mice more closely resembled those found in Aire-deficient mice. The meibomian glands typically function in the production of the hydrophobic lipid layer of the tear film, and disruption of meibomian gland function may contribute to the chronic ocular surface disease state of APECED patients. Posterior blepharitis has been described in APECED,¹² and these findings support an inflammatory basis of meibomian gland disease in APECED, leading to the loss of lipid secretions and subsequent tear-film instability.

Relatively few T cells were observed in the peripheral cornea of WT mice both at baseline and following exposure to desiccating stress. In contrast, Aire-deficient mice demonstrated significantly greater CD4⁺ and CD8⁺ T cell populations in both the peripheral corneal epithelium and stroma. This finding is compelling, as patients with keratoconjunctivitis associated with APECED may develop persistent peripheral corneal inflammatory cell infiltrates, superficial corneal neovascularisation with lipid deposition and subsequent anterior stromal scarring, which may progress to severe visual loss.³⁻⁷ The limbus is considered to be the most immunologically active portion of the cornea, and multiple diseases that feature inflammation and neovascularisation in this region have been described (eg, Mooren ulcer, peripheral ulcerative keratitis, phlyctenulosis and staphylococcal marginal keratitis).

We also observed that CD11b⁺ and CD45⁺ cells were significantly greater in the meibomian glands of Aire-deficient compared with WT mice. Immune cells that express CD11b include a number of antigen-presenting cells, and altered CD11b expression patterns have been previously observed in Sjögren syndrome patients.¹⁸ CD45 identifies a conserved transmembrane glycoprotein expressed exclusively on bone-marrow-derived cells, and various CD45 isoforms exist, some of which interact with T lymphocytes and their signal transduction pathways. The presence of large numbers of these immune cells, which may include other leucocytes and APCs, is supportive of the immunologically active environment in the eyelids.

Potential limitations of this study include the small numbers of mice evaluated and our use of Aire-deficient mice of only one genetic background. One prior study evaluated the ocular surface T cell infiltrates of Aire-deficient mice with BALB/c and NOD Lt/J backgrounds,¹⁰ and it would be useful to fully characterise the immune cellular response in these mice as well.

Despite these limitations, the findings described herein were repeated, and the baseline ocular surface inflammation appeared severe in the majority of Aire-deficient mice evaluated. A potential extension study could evaluate the effect of desiccating stress on ocular surface immune cells prior to the onset of CD4⁺ and CD8⁺ T cell infiltration. Interestingly, in patients with APECED, keratoconjunctivitis may be the first manifestation of widespread systemic autoimmunity, but the role of environmental triggers is unknown. Elucidation of potential early mechanisms of ocular surface disease in the murine model could provide an insight into the human situation.

The pathogenic mechanisms underlying the severe ocular surface disease observed in patients with APECED are incompletely understood. Our findings of CD4⁺ T cell infiltrates in goblet-rich areas of conjunctiva and CD8⁺ T cell infiltrates in the peripheral corneal epithelium and stroma are supportive of immune-based ocular surface inflammation. Whether a corneal autoantigen targeted by T cells is involved or whether specific soluble factors mediate T cell homing to the peripheral corneal epithelium and stroma remains to be determined. The loss of thymic expression of IRBP in Aire-deficient mice was recently found to lead to retinal autoimmunity;⁹ however, a similar corneal or ocular surface antigen has yet to be identified.

The loss of goblet cells of Aire-deficient mice and CD4⁺ T cell infiltrates within the meibomian glands suggests that inflammatory-mediated disruption of the mucin- and lipid-components of the tear film may be additional contributing factors. Interestingly, both at baseline and following desiccating environmental stress, Aire-deficient mice exhibited greater ocular surface immune cell populations than WT mice; it is possible that the severity of the inflammation in Aire-deficient mice made it difficult to appreciate subtle worsening

following exposure to environmental stressors. Further characterisation of the ocular surface immune environment of Aire-deficient mice may provide additional insight into pathogenesis of keratoconjunctivitis in APECED, as well as other visually debilitating ocular surface inflammatory diseases.

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Figure 1.

Representative immunohistochemical and PAS staining showing immune cell populations and goblet cells in Aire-deficient and control mice prior to desiccating stress. Greater numbers of CD4⁺ T cells are found within the conjunctival epithelium of Aire-deficient mice (A) compared with wild type mice (B). A significantly greater number of CD4⁺ T cells were found in the central corneal stroma of Aire-deficient mice (C, stroma highlighted by inset) compared with wild type mice (D). Within the meibomian glands, there were a greater number of CD4⁺ (pictured), CD8⁺, CD45⁺ and CD11b⁺ cells in Aire-deficient mice (E, region of interest outlined by red line) than wild type control mice (F, region of interest outlined by red line). PAS staining showed significantly fewer goblet cells in the inferior and superior conjunctiva of Aire-deficient mice (G) compared with wild type mice (H).

Table 1
Summary of immune cell populations in Aire-deficient and wild type (WT) mice before (UT) and after (5D) desiccating stress

		CD4+		CD8+		CD45+		CD11b+	
		WT	Aire	WT	Aire	WT	Aire	WT	Aire
Goblet-cell-rich area of conjunctiva (cells/100 μ m)									
Conjunctival epithelium	UT	1.32 (0.40)	2.48 (1.04) ***	1.12 (0.38)	1.72 (1.50)	4.54 (0.98)	4.80 (2.87)	1.61 (0.28)	1.79 (0.30)
	5D	2.06 (0.32) ††	2.08 (1.6)	0.54 (0.36)	1.11 (1.07) **	2.70 (1.37) ††	3.38 (0.65)	1.49 (0.83)	1.19 (0.35)
Conjunctival stroma	UT	3.08 (1.2)	3.38 (1.72)	0.64 (0.85)	0.61 (1.02)	6.06 (1.97)	8.4 (1.84)	7.23 (0.57)	9.77 (4.13)
	5D	1.94 (0.65)	3.14 (1.96) *	0.14 (0.20)	0.59 (0.98)	6.15 (1.55)	7.68 (3.40)	7.13 (2.17)	6.42 (2.81)
Cornea (cells/500 μ m)									
Peripheral cornea epithelium	UT	1.06 (1.4)	6.75 (2.6) ***	0.38 (0.65)	2.83 (3.37) *	1.4 (2.6)	16.7 (14.8)	1.1 (1.9)	7.7 (10.8)
	5D	0.65 (1.22)	5.17 (3.92) ***	0.16 (0.37)	2.67 (1.63) ***	1.0 (0.82)	3.8 (1.6)	0.4 (1.1)	1.3 (1.5)
Peripheral cornea stroma	UT	7.4 (4.2)	105.6 (80.67) ***	3.2 (3.9)	16.0 (9.01) ***	3.6 (3.8)	33.0 (33.9)	2.8 (3.2)	25.3 (26.8) *
	5D	3.2 (2.3)	98.0 (40.81) ***	0.34 (0.94)	19.2 (12.3) ***	3.5 (3.9)	23.3 (21.6)	1.5 (2.7)	18.3 (11.0) *
Central cornea stroma	UT	0.64 (0.79)	0.46 (0.63)	0.27 (0.63)	1.17 (1.3) *	0 (0)	2.3 (1.5) **	0 (0)	0.67 (1.2)
	5D	0.35 (0.75)	0.77 (1.03)	1.67 (2.61)	2.00 (2.95)	0 (0)	1.33 (1.0)	0 (0)	0.17 (0.41)
Meibomian gland (cells/mm ²)	UT	1.9 (1.47)	4.83 (5.5) *	0.43 (0.71)	2.0 (3.63)	0 (0)	9.3 (11.2)	0.38 (0.74)	12.0 (12.5) *
	5D	1.05 (2.06)	5.5 (6.3)	0.44 (0.72)	3.0 (7.3)	0.75 (0.96)	6.7 (7.8)	0.29 (0.76)	3.8 (3.3) *
Meibomian gland	UT	350.22 (106.46)	587.30 (247.5) **	406.45 (178.51)	74.78 (112) ***	362.0 (57.12)	1135.7 (207.6) ***	190.60 (207.2)	974.40 (469.7) **
	5D	470.58 (203.76)	542.46 (214)	222.95 (66.82) †	39.31 (60.84) ***	457.6 (151.7)	1066.0 (428.2) *	415.34 (147.3)	675.3 (256.7) ††

5D, Aire-deficient or WT mice that were exposed to desiccating environment for 5 days; UT, untreated mice, that is Aire-deficient or WT mice that were not treated with a desiccating environment.

* p<0.05;

** p<0.01;

*** p<0.001 (WT vs Aire, eg, central corneal epithelium, WT UT vs Aire-deficient UT);

† p<0.05;

^{††} p<0.01 (5D vs UT, e.g. central corneal epithelium, WT UT vs WT 5D).

All values are expressed as mean (SD).

Table 2

Goblet cell density

Cells/100 μm	C57	Aire
Goblet cells UT	6.81 (1.05)	4.03 (1.01) ***
Goblet cells 5D	4.99 (0.40) ^{†††}	3.76 (1.25) ***

All values are expressed as mean (SD).

p<0.001 (C57 vs Aire, eg, central corneal epithelium, C57 UT vs Aire-deficient UT);

^{††}
p<0.001 (5D vs UT, eg, central corneal epithelium, C57 UT vs C57 5D).

5D, Aire-deficient or WT mice that were exposed to desiccating environment for 5 days; UT, untreated mice, that is Aire-deficient or WT mice that were not treated with desiccating environment.