Elevated Cytokine Levels in the Aqueous Humor of Eyes With Bullous Keratopathy and Low Endothelial Cell Density

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Purpose. To evaluate cytokine levels in the aqueous humor (AqH) of eyes with bullous keratopathy (BK) and low endothelial cell density (ECD).

Methods. A total of 145 AqH samples (60 BK, 16 low ECD, 35 corneal diseases with normal ECD, and 34 normal controls) were collected from consecutive patients who underwent corneal transplantation or cataract surgery. None of the patients had any clinically apparent inflammation at the time of AqH collection. The AqH levels of cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN-α, IFN-γ, monocyte chemotactic protein [MCP]-1, TNF-α, E-selectin, P-selectin, soluble intercellular adhesion molecule [sICAM]-1, granulocyte-macrophage colony-stimulating factor [GM-CSF], macrophage inflammatory protein [MIP] -1α and MIP-1β) were compared among the groups.

Results. The levels of IL-1α, IL-8, IL-17A, TNF-α, GM-CSF, MIP-1α, IFN-γ, and E-selectin in the AqH were significantly elevated in BK and low ECD eyes, compared with healthy controls (all $P < 0.03$). The levels of IL-4, IL-6, IL-10, IL-12p70, IL-13, MCP-1, MIP-1β, P-selectin, and sICAM-1 were significantly elevated only in BK eyes, compared with healthy control (all $P < 0.001$). There were no significant differences in AqH cytokine levels between corneal diseases with normal ECD and normal control eyes. Among BK eyes, the IL-6 and IFN-γ levels were elevated in eyes with pseudophakic BK (PBK), Fuchs' endothelial corneal dystrophy (FECD), postkeratoplasty, posttrabeculectomy, and postlaser iridotomy (LI) (all, $P < 0.04$), whereas IL-1α, IL-10, IL-17A, MIP-1β, and sICAM-1 levels were elevated only in PBK, postkeratoplasty, posttrabeculectomy, and post-LI eyes (all, $P < 0.05$).

Conclusions. Subclinical elevation of AqH cytokine levels may cause endothelial cell loss.

Keywords: bullous keratopathy, Fuchs' endothelial corneal dystrophy, aqueous humor, cytokine, endothelial cell density

Endothelial cell density (ECD) decreases with age,[1–3] and in various ocular conditions, including corneal endotheliitis, uveitis, pseudoexfoliation syndrome, and birth injury.[4–6] The reduction of ECD is exacerbated over time after intraocular surgery,[7–9] and is a serious complication after corneal transplantation because it may lead to endothelial decompensation and loss of vision.[10–12] Risk factors for postoperative endothelial cell loss after penetrating keratoplasty (PKP) include donor age, recipient age, graft diameter, lens status, glaucoma, graft rejection, and peripheral corneal diseases.[13–15] Recently, we reported that a decrease in ECD after Descemet stripping automated endothelial keratoplasty (DSAEK) was associated with severe preexisting iris damage. The ECD decrease was minimal in patients with a healthy iris, whereas it was rapid in patients with severe iris damage.[16] However, the reasons of the endothelial cell loss in eyes with iris damage are unknown.

Anatomically, the aqueous humor (AqH) must play an important role in reducing ECD. A combination of proinflammatory cytokines synergistically induces the apoptosis in corneal endothelial cells in vitro.[17] ECD is lower in eyes with a history of uveitis and is correlated with the flare in the anterior chamber, suggesting that environmental factors in the AqH can directly influence the survival of endothelial cells.[6] However, to the best of our knowledge, the association between reduced ECD and cytokine levels in the AqH is poorly understood. We hypothesized that elevation of proinflammatory cytokines in the AqH is associated with a decrease in ECD. We conducted a prospective study to determine cytokine levels in the AqH in eyes with various corneal disorders; bullous keratopathy (BK), low ECD, and corneal diseases with normal ECD.

Methods

This prospective consecutive study was performed in accordance with the Declaration of Helsinki. It was approved by the institutional ethics review board of Tokyo Dental College, Ichikawa General Hospital (I-15-51). Written informed consent was obtained from all participants.

Patients

A total of 145 consecutive patients who underwent corneal transplantation and cataract surgery at Tokyo Dental College

Table 1. Demographics of All Subjects

	BK	Low ECD	Corneal Diseases With Normal ECD	Control	*P* Value*
No. of eyes	60	16	35	34	
Age, y	75.0 ± 5.7	77.6 ± 6.5	68.3 ± 15.5	75.9 ± 7.4	0.074
Male/female	26/34	5/11	17/18	16/18	0.685
Axial length, mm	23.5 ± 2.1	23.2 ± 2.4‡	24.6 ± 2.0	23.7 ± 1.4	0.0117
ECD, cells/mm^2	NA	757 ± 331	2232 ± 644	2783 ± 334	<0.0001
CCT, μm	782 ± 150	532 ± 50	541 ± 136	534 ± 19	<0.0001

BK, bullos keratopathy; ECD, endothelial cell density; CCT, central corneal thickness; NA, not available.
* ANOVA.

from October 2015 to May 2016 were included. We did not perform corneal transplantation and cataract surgery in eyes with active inflammation in the cornea and the anterior chamber. Thus, this study did not include such eyes. In eyes with a history of uveitis (one eye with BK) and suspected endotheliitis (one eye with BK and two with low ECD), we confirmed that the anterior chamber did not contain cells, ciliary injections, and keratoprecipitates before surgery. Patients with severe ocular surface diseases (Stevens-Johnson syndrome, one eye; ocular cicatrical pemphigoid, one eye; thermal burn, one eye), or eyes in which we could not obtain more than 60 μL AqH due to a shallow anterior chamber (five eyes) were excluded. Low ECD was defined as ECD less than 1200 cells/mm^2.[18] Corneal diseases with normal ECD (defined as 1500 cells/mm^2 or more) included 18 eyes with old corneal scars, 11 eyes with hereditary corneal epithelial/stromal dystrophies (lattice corneal dystrophy 3a, 6 eyes; macular corneal dystrophy, 3 eyes; and granular corneal dystrophy 2, 2 eyes) and 6 eyes with keratoconus. None of the eyes with keratoconus had a history of acute hydrops. Healthy control subjects were defined as patients who underwent cataract surgery, without uveitis, corneal or intraocular surgeries, diabetes mellitus, or inflammatory systemic diseases such as ulcerative colitis or rheumatoid arthritis. All subjects in the healthy control group had an ECD more than 2000 cells/mm^2. Ultimately, 145 eyes of 145 patients were included.

Aqueous Humor Samples

The AqH was obtained under sterile conditions at the beginning of surgery after retrobulbar anesthesia in corneal transplantation or topical anesthesia in cataract surgery. First, paracentesis was placed at the clear cornea. Aqueous humor sample containing 70 to 300 μL was obtained using a 27-gauge needle taking care not to touch the iris, the lens, or corneal endothelium. The samples were centrifuged at 3000*g* for 5 minutes. The soluble factions were collected and stored at −80 degrees celsius until cytokine levels could be measured.

Protein Concentration Measurements

The protein concentrations of AqH samples were determined using the DC protein assay (Bio-Rad, Hercules, CA, USA). The reactions were based on the Lowry assay, and measured according to the manufacturer's instructions. In brief, BSA was used as a standard in the range of 0.23 to 1.37 mg/mL. Samples (5 μL) of BSA and AqH were added to 96-well microplates, followed by immediate addition of a mixture containing 25 μL reagent A+S and 200 μL reagent C. After 15 minutes of incubation at room temperature in the dark, the microplates were read at 690 nm and 405 nm using a microplate reader (Model 550; Bio-Rad). Concentrations were calculated by the subtraction method using the microplate manager system (Bio-Rad).

Cytokine Level Measurements

The cytokine levels (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN-α, IFN-γ, monocyte chemotactic protein [MCP]-1, TNF-α, E-selectin, P-selectin, soluble intercellular adhesion molecule [sICAM]-1, granulocyte-macrophage colony-stimulating factor [GM-CSF], and macrophage inflammatory protein [MIP]-1α and MIP-1β) in AqH samples were measured using Luminex (ProcaPlex kit; Luminex, San Antonio, TX, USA) beads-based multiplex immunoassay according to previous reports.[19] Briefly, 50 μL AqH samples were incubated with antibody-coated capture beads in an incubation buffer at room temperature. After 2 hours' incubation, the beads were washed three times using washing buffer, and phycoerythrin-labeled streptavidin was added for 30 minutes in the dark at room temperature. After having been washed three times with washing buffer, plates were resuspended in 150 μL reading buffer, and the assays were performed using a Luminex 200 (Luminex).

Statistical Analysis

The data were analyzed using Prism software (version 6.04 for Windows; GraphPad Software, Inc., San Diego, CA, USA). The D'Agostino and Pearson omnibus normality test was used to assess whether the data showed a normal distribution. To compare differences in cytokine levels among the groups, the Kruskal-Wallis test with Dunn's multiple comparisons test was used. Spearman's correlation analyses were used to evaluate the correlations between the AqH cytokine levels and the number of previous ocular surgeries; these included cataract surgery, trabeculectomy (TLE), vitrectomy, PKP, DSAEK, deep anterior lamellar keratoplasty (DALK), and laser iridotomy (LI). The data are expressed as the mean ± SD. A *P* value less than 0.05 was considered statistically significant.

Results

Patient Demographics

The mean age, sex, axial length, and ECD of the subjects are summarized in Table 1. The causes of BK included 16 eyes with pseudophakic BK (PBK), 13 eyes with Fuchs' endothelial corneal dystrophy (FECD), 12 eyes with postkeratoplasty (KP), 8 eyes with post-TLE, 8 eyes with post-LI, 1 eye with trauma, 1 eye with birth injury, and 1 eye with postendotheliitis (Table 2). In the PBK group, all of the eyes had posterior chamber IOLs and no eyes had an anterior chamber IOLs. Post-KP included eyes with endothelial decompensation after PKP (nine eyes), DSAEK (two eyes), and DALK (one eye). The causes of low ECD included seven eyes with post-LI, three with FECD, two with postendotheliitis, and four of unknown etiology. None of the subjects had active intraocular inflammation in the cornea and/or anterior chamber, such as cells in

Table 2. Demographics of Subjects With BK

	PBK	FECD	Post-KP	Post-TLE	Post-LI
No. of eyes	16	13	12	8	8
Age, y	74.4 ± 11.3	75.0 ± 5.7	67.6 ± 14.1	73.5 ± 13.2	75.6 ± 5.4
Male/female	8/8	6/7	7/5	4/4	0/8
Axial length, mm	24.1 ± 2.9	24.2 ± 2.3	23.7 ± 1.2	24.1 ± 1.2	22.2 ± 0.9
ECD, cells/mm^2	NA	NA	NA	NA	NA
CCT, μm	756 ± 144	697 ± 128	806 ± 182	801 ± 181	693 ± 17
No. of past surgeries	1.6 ± 1.1	0.5 ± 0.4	2.8 ± 1.5	2.5 ± 1.1	1.4 ± 0.7
Range	1–5	0–1	1–6	1–4	1–3
Median	1	0	3	2	1

BK, bullous keratopathy; PBK, pseudophakic bullous keratopathy; KP, keratoplasty; TLE, trabeculectomy; LI, laser iridotomy; NA, not available.

the anterior chamber, keratoprecipitates, corneal epithelial defects and infiltrates, and ciliary injections based on slit-lamp findings. There was a significant difference only in axial length between the groups (Table 1, $P = 0.0117$, ANOVA; $P = 0.0148$ between the low ECD and the corneal disease with normal ECD groups): presumably because the mean axial length was 26.8 ± 1.2 mm in eyes with keratoconus. The central corneal thickness (CCT) in the BK group was significantly larger than the low ECD, corneal disease with normal ECD, and control groups ($P < 0.0001$), whereas there were no significant differences in CCT among the low ECD, corneal disease with normal ECD, and control groups ($P > 0.99$).

Aqueous Humor Protein Level

The level of AqH protein was significantly higher in the BK group than in the low ECD, corneal disease with normal ECD, and control groups (Fig. 1) ($P = 0.0199$, $P < 0.0001$, and $P < 0.0001$, respectively), whereas there were no significant differences among the low ECD, corneal disease with normal ECD, and control groups (all, $P > 0.99$).

Cytokine Levels in AqH

The levels of IL-1α, IL-8, IL-17A, TNF-α, GM-CSF, MIP-1α, IFN-γ, and E-selectin in the AqH were significantly elevated in BK and low ECD eyes, compared with normal control eyes (Fig. 2, all $P < 0.03$). The levels of IL-4, IL-6, IL-10, IL-12p70, IL-13, MCP-1, MIP-1β, P-selectin, and sICAM-1 in the AqH were significantly elevated only in BK eyes (Fig. 3, all $P \leq 0.001$). There were no significant differences in cytokine levels between corneal diseases with normal ECD and healthy controls.

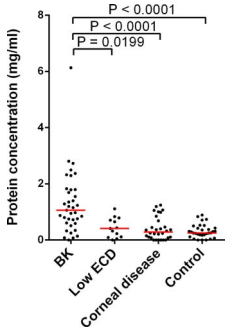

Figure 1. Protein level in AqH. The level of AqH protein was significantly higher in the BK group than the low ECD, corneal disease with normal ECD, and control groups ($P = 0.0199$, $P < 0.0001$, and $P < 0.0001$, respectively).

Cytokine Levels in the AqH of BK Subgroups

Among BK eyes, the IL-6 and IFN-γ levels in the AqH were significantly elevated in all PBK, FECD, post-KP, post-TLE, and post-LI eyes, whereas the levels of IL-1α, IL-4, IL-8, IL-10, IL-17A, MIP-1β, and sICAM-1 were elevated only in PBK, post-KP, post-TLE, and post-LI eyes, not in FECD eyes (Figs. 4A, 4B). Interleukin-12p70 and E-selectin levels were significantly elevated in PBK, post-TLE, and post-LI eyes (Fig. 4C), and IL-13 level was significantly elevated in PBK, post-KP, and post-LI eyes (Fig. 4C).

Correlations Between Cytokine Levels in AqH and the Number of Previous Ocular Surgeries

The cytokine levels in AqH are elevated after ocular surgeries, such as trabeculectomy and cataract surgery.[9,15,20–23] To assess the influence of previous ocular surgeries on the cytokine levels in AqH, we conducted a correlation analysis between the two (Table 3). The number of previous ocular surgeries was strongly positively correlated with the following cytokine levels: IL-1α ($r = 0.47$, $P < 0.0001$), IL-4 ($r = 0.46$, $P < 0.0001$), IL-6 ($r = 0.50$, $P < 0.0001$), IL-8 ($r = 0.50$, $P < 0.0001$), IL-10 ($r = 0.48$, $P < 0.0001$), IL-13 ($r = 0.38$, $P < 0.0001$), IL-17A ($r = 0.32$, $P < 0.0001$), MIP-1β ($r = 0.42$, $P < 0.0001$), sICAM-1 ($r = 0.42$, $P < 0.0001$), MCP-1 ($r = 0.26$, $P = 0.001$), P-selectin ($r = 0.26$, $P = 0.001$), and E-selectin ($r = 0.29$, $P = 0.0002$). The number of previous ocular surgeries was moderately positively correlated with the following cytokine levels: IL-1β ($r = 0.17$, $P = 0.049$), IL-12p70 ($r = 0.21$, $P = 0.009$), GM-CSF ($r = 0.26$, $P = 0.002$), TNF-α ($r = 0.20$, $P = 0.01$), and IFN-α ($r = 0.17$, $P = 0.03$). There were no significant correlations between the number of previous ocular surgeries and MIP-1α or IFN -γ levels (both, $P > 0.05$).

Discussion

The levels of cytokines, such as IL-1α, IL-8, IL-17A, TNF-α, and IFN-γ in AqH were significantly elevated in eyes with BK and low ECD, whereas the levels of cytokines, such as IL-6 and MCP-1, were elevated only in eyes with BK. Although there were individual differences in the cytokine levels among the etiologies of BK, IL-6 and IFN-γ levels were greater in all etiologies of BK, compared with healthy controls, whereas the levels of some cytokines, such as IL-1α, IL-8, IL-13, IL-17A, and MIP-1β increased in eyes with BK, except eyes with FECD.

Under normal conditions, the adult human cornea loses endothelial cells at a rate of 0.6% per year.[24] The annual rate of endothelial cell loss is 2.5% per year after cataract surgery,[21] and 2.6% to 7.8% per year after PKP with no postoperative complications.[11,25] Although some clinical factors, such as cataract surgery, anterior chamber IOL, glaucoma surgery,

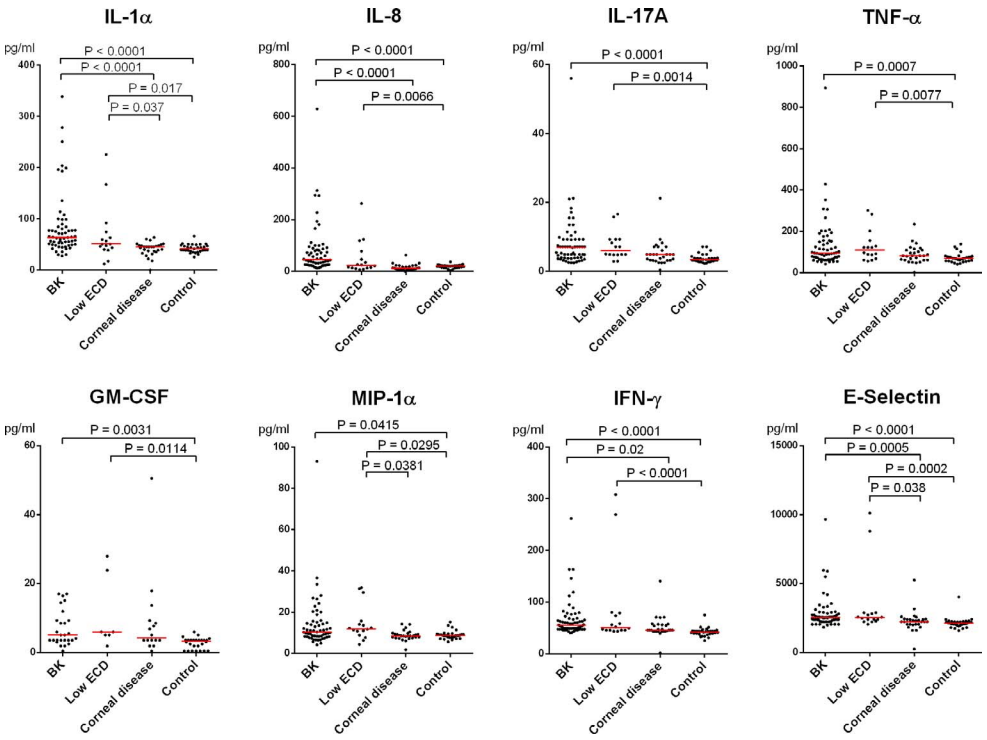

Figure 2. Elevated cytokine levels in eyes with BK, low ECD. The levels of IL-1α, IL-8, IL-17A, TNF-α, GM-CSF, MIP-1α, IFN-γ, and E-selectin in the AqH were significantly elevated in BK and low ECD eyes, compared with healthy controls (all $P \leq 0.03$). The *red lines* represent the median of each cytokine level.

uveitis, and iris damage score have been identified as the risk factors for reduced ECD,[6,9,13,16,25,26] the exact mechanism is unknown.

The corneal endothelium represents the innermost cellular layer of the human cornea, directly facing the AqH. In recent years, increased cytokine levels in AqH have been reported to be associated with pathogenesis and intraocular alteration in various ocular diseases; FECD,[22,27] graft rejection,[28,29] uveitis,[30–32] glaucoma,[19,32] AMD,[33,34] and retinal vein occlusion.[35–37] Maier et al.[28] evaluated IL-2, IL-4, IL-5, IL-10, and IFN-γ levels in 18 AqH samples that were collected at the time of PKP and reported that the cytokine levels in AqH were predictive of graft rejection. Kawai et al.[23] reported that AqH levels of MCP-1 and IL-8 were elevated 1 to 2 years after phacoemulsification because the proliferated lens epithelial cells produced MCP-1. However, little is known about the association between decrease in ECD and cytokine levels in AqH in vivo. Interestingly, Sagoo et al.[17] reported that the combined stimulation of IL-1α, IFN-γ, and TNF-α synergistically induced apoptosis in corneal endothelial cells in vitro. In the current study, we showed that the AqH levels of all of these cytokines were elevated in eyes with BK and low ECD. Although the exact mechanism of cytokine elevation needs to be confirmed in future studies, it is possible that AqH cytokines play an important role in reducing ECD. Because this was a cross-sectional study, to demonstrate the accelerated reduction of ECD by the specific cytokines, there is a need for a prospective longitudinal study on the effects of AqH cytokine levels on the long-term ECD decrease after corneal transplantation.

The levels of cytokines increased to different degrees in BK eyes. Only IL-6 and IFN-γ were significantly elevated in FECD eyes, compared with the control eyes; however, the increase was minimal, compared with the other BK eyes. Matthaei et al.[22] characterized the epithelial-mesenchymal transition (EMT)-related cytokines (TGF-β, MCP-1, and IL-1β) in the AqH and reported the simultaneous elevation of TGF-β and MCP-1 in pseudophakic FECD eyes, although there were no differences in EMT-related cytokines between phakic FECD eyes and healthy controls. Thus, they concluded that EMT-related aqueous cytokines did not have a primary role in in the pathogenesis of FECD.[22] In the current study, we did not find significant difference in MCP-1 levels between normal and FECD eyes (there were seven phakic and six pseudophakic eyes in the FECD group). Richardson et al.[27] conducted AqH proteome analyses in FECD eyes and found an elevation of complement C3, and a decrease in afamin, which is a protective protein against oxidative stress. Therefore, there might be some difference in the mechanism of ECD loss in FECD eyes compared with eyes with other etiologies of BK, such as PBK, post-KP, post-TLE, and post-LI. In contrast, IFN-γ was elevated in eyes with low ECD and BK in all etiologies, including FECD. Sugita et al.[38] reported that human corneal endothelial cells suppressed IFN-γ production by $CD\text{-}4^+$ T cells via programmed death-ligand 1 in vitro. In the current study, although we did not measure the IL-2 levels in AqH, the IL-4 levels were elevated in eyes with BK and low ECD. Previous in vitro studies have reported that endothelial cells inhibit IL-2 and IL-4 production by inhibiting T cells.[39,40]

It is probable that AqH cytokine levels are elevated in eyes with active inflammation, leading to ECD loss. Lapp et al.[41] reported that proinflammatory mediators from monocytes are sufficient to induce the cell death of a human corneal endothelial cell line in vitro and death of primary human corneal endothelial cells in corneal buttons ex vivo. Eom et al.[42] reported the elevation of IL-1 and IFN-γ levels after argon LI induced apoptosis of endothelial cells in an animal model. We postulate that these results[17,41,42] involved a rapid decrease in the ECD in response to proinflammatory stimuli. In the present study, it is noteworthy that AqH cytokine levels were elevated, although we collected all samples during a clinically

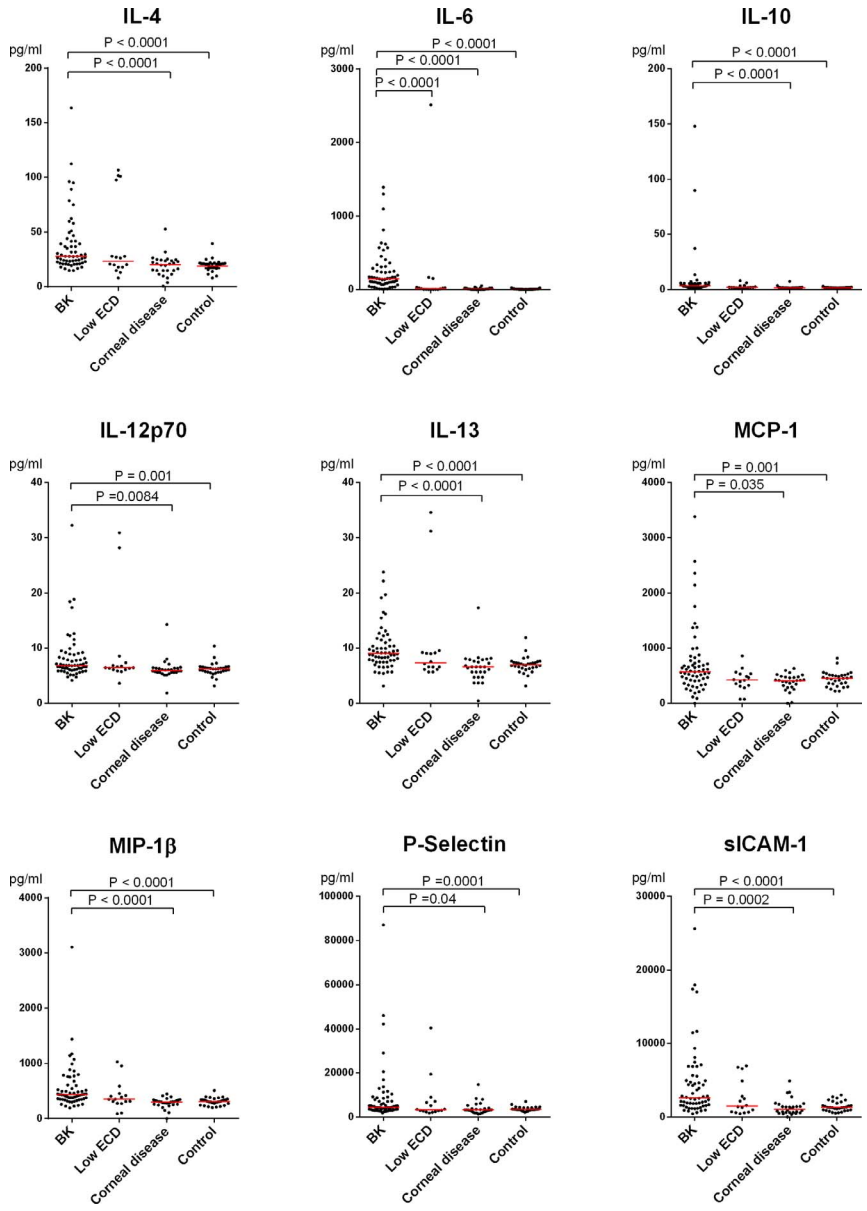

Figure 3. Elevated cytokine levels only in BK. The levels of IL-4, IL-6, IL-10, IL-12p70, IL-13, MCP-1, MIP-1β, P-selectin, and sICAM-1 in the AqH were significantly elevated only in BK eyes, compared with healthy controls (all $P \leq 0.001$). There were no significant differences in cytokines levels between corneal diseases with normal ECD and normal controls. The *red lines* represent the median of each cytokine level.

noninflammatory period. Reports on long-term ECD alteration have suggested that decrease in ECD may result from the breakdown of the blood-aqueous barrier (BAB).[7,21] Regarding chronic inflammation of the anterior chamber, a previous study that used laser flare photometry reported a correlation between ECD and flare in the anterior chamber of eyes with a history of uveitis.[6] Elevated flare can persist in people with a history of uveitis, even after the resolution of anterior chamber cells.[43] However, the laser flare photometer cannot identify specific proteins. In the present study, we found that cytokine levels in AqH were elevated in eyes with BK and low ECD. We postulate that chronic proinflammatory cytokines cause loss of endothelial cells as proposed by Armitage et al.,[11] who used biexponential models of rapid and slow components in ECD decrease.

We demonstrated that the aqueous levels of the adhesion molecules of E-selectin, P-selectin, and sICAM-1 increased in eyes with BK. These molecules play important parts in the other diseases, such as asthma,[44] atopic dermatitis,[45] and coronary artery disease.[46,47] Recently, Dohlman et al.[48] reported that E-selectin, not P-selectin, mediates the trafficking of immune cells in graft rejection after murine corneal transplantation. The current study is the first report on E-selectin, P-selectin, and sICAM-1 in human AqH. Future study on the incidence of graft rejection in eyes with elevated E-selectin may provide us a novel biomarker to predict the higher risk for graft rejection.

The presence of systemic inflammatory diseases and the administration of systemic or topical steroids can affect cytokine levels in AqH. However, none of our patients had systemic inflammatory diseases, such as rheumatoid arthritis, Sjögren's syndrome, or inflammatory bowel disease. In addition, although no patients were administered systemic steroid, topical steroids were used in 21 eyes in the BK group (10 eyes in post-KP, 8 eyes in PBK, and 3 eyes in post-TLE), when the AqH samples were collected. We compared protein

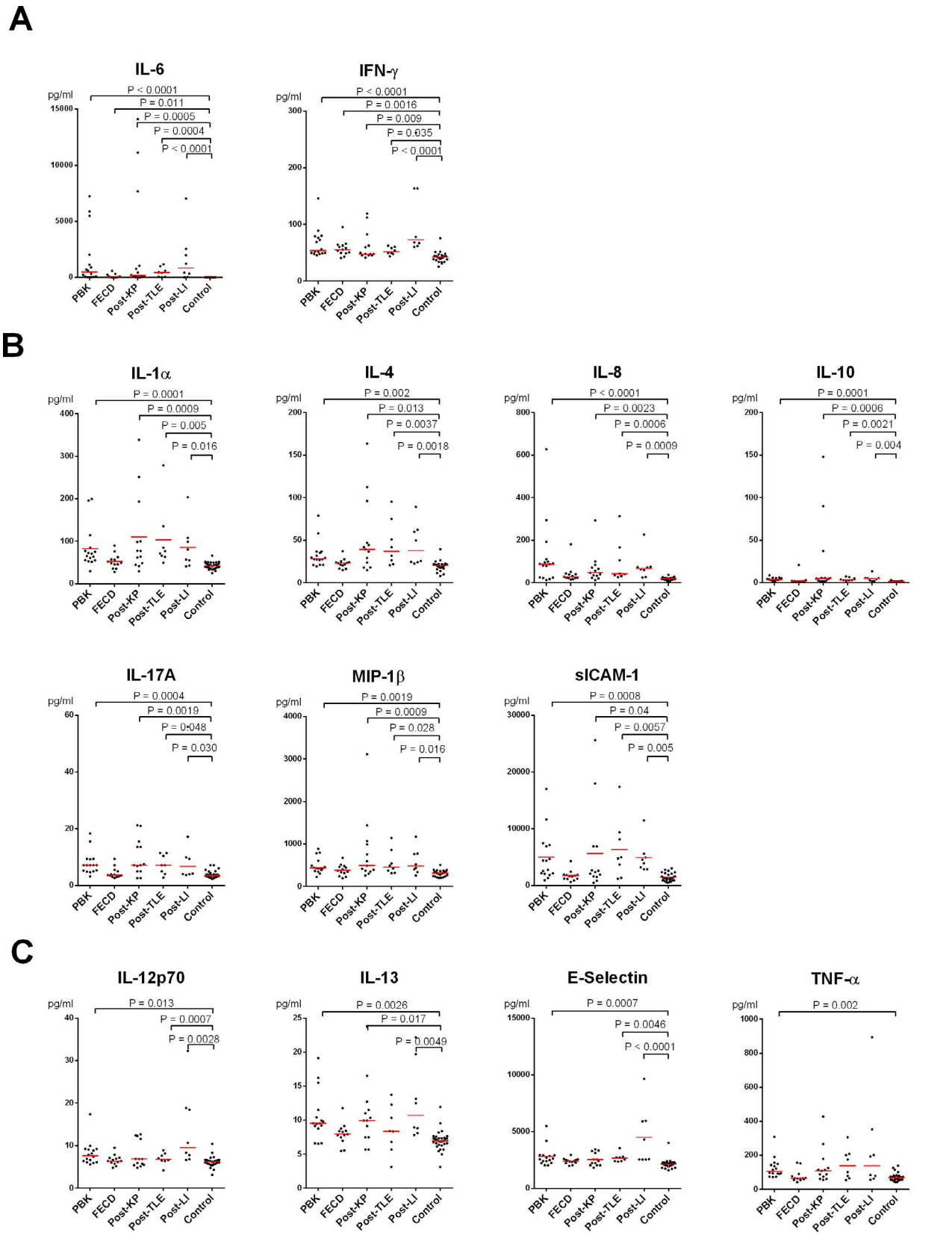

FIGURE 4. Cytokine levels in subgroups of eyes with BK. Among BK eyes, the IL-6 and IFN-γ levels in the aqueous humor significantly elevated in all subgroups including PBK, FECD, post- KP, post-TLE, and post-LI (**A**), whereas the levels of IL-1α, IL-4, IL-8, IL-10, IL-17A, MIP-1β, and sICAM-1 in the AqH were elevated only in PBK, post-KP, post-TLE, and post-LI, not in FECD eyes (**B**). The IL-12p70 and E-selectin levels were significantly increased in PBK, post-TLE, and post-LI eyes, and IL-13 level was significantly increased in PBK, post-KP, and post-LI eyes (**C**). The *red lines* represent the median of each cytokine level.

Investigative Ophthalmology & Visual Science

Table 3. Correlation Between Aqueous Cytokine Levels and the Number of Previous Ocular Surgeries

P Value	Cytokine
< 0.005*	IL-1α, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, MIP-1β, sICAM-1 MCP-1, E-selectin, P-selectin
< 0.05*	IL-1β, IL-12p70, GM-CSF, TNF-α, IFN-α
$0.05 \leqq$	MIP-1α, IFN -γ

Spearman's correlation analysis.
* All positive correlation.

and cytokine levels between eyes administered topical steroids (21 eyes) and those that did not receive them (39 eyes) in the BK group. The levels of protein and ICAM-1 were significantly higher in eyes given topical steroids ($P = 0.032$ and $P = 0.034$, respectively). However, there were no statistical differences in other cytokine levels ($P > 0.10$). Although there might have been bias due to the decision to use topical steroids in severe cases, we suggest that there must be some other detrimental factors causing the elevation of AqH cytokines.

Levels of specific cytokines in the AqH, such as IL-6, IL-10, MCP-1, P-selectin, and sICAM-1, were significantly elevated only in BK eyes, not in eyes with low ECD. Moreover, the increased levels of AqH protein, indicating breakdown of the BAB, were observed only in eyes with BK. We suggest that the differences in elevated cytokine levels between the BK and low ECD groups are important, because there would be expected to be no difference, if the elevated levels of these cytokines were the only cause for the reduction in ECD. BK may not only affect the condition of corneal edema due to endothelial pump dysfunction, but may also cause elevated AqH cytokines and breakdown of BAB after dysfunction in endothelial cell immunomodulatory properties.[38–40,49,50] Although inflammatory conditions might be a cause of endothelial cell loss in the current study, previous reports have shown that endothelial cells regulate inflammation by suppressing T-cell activation[38–40] and cytokine production[49] and promoting regulatory T cells.[50]

What is the reason for the subclinical inflammation in the anterior chamber? Under normal conditions, the anterior chamber is an immunosuppressive microenvironment. Streilein et al.[51] reported that ocular immune privilege is under neural control. The cornea is the most innervated tissue in the body, and corneal tissue, as well as the AqH, contain immunosuppressive neuropeptides.[51] The corneal endothelium transports the corneal fluid into the AqH via its pump function. It is tempting to suggest that the corneal endothelium transports immunosuppressive substances, such as neuropeptides, from the corneal tissue into the AqH, helping to maintain homeostasis in the anterior chamber and BAB. Moreover, recent clinical studies have shown that decreases in the corneal nerve are associated with ECD loss.[52,53] We previously reported that corneal denervation resulted in a decreased ECD, which could be rescued by the administration of neuropeptide in animal experiments. (Yamaguchi T, et al. *IOVS* 2014;55:ARVO E-Abstract 2077). Although the endothelial cells, iris, and ciliary body have immunosuppressive properties of their own,[54,55] future studies on interaction among the corneal nerve, ECD, BAB, and cytokines/neuropeptides in AqH will be invaluable in understanding the mechanisms of immune homeostasis in the anterior chamber.

This study had some limitations. First, the number of the previous ocular surgeries varied among the groups, which may have introduced bias. We confirmed the significant correlation between some cytokine levels in AqH and the number of previous surgeries, which is in line with previous studies.[20,22,23] However, although the number of the previous surgeries was small (0.5 ± 0.5, ranging from 0 to 1) in eyes with a low ECD, the aqueous levels of IL-1α, IL-8, IL-17A, TNF-α, GM-CSF, MIP-1α, IFN-γ, and E-selectin were significantly elevated in eyes with a low ECD, compared with healthy controls, suggesting that ocular surgeries were not the solitary factor responsible for the elevation of cytokine levels. Further studies using multivariate analyses are necessary to assess clinical factors associated with elevated levels of cytokines and decreases in ECD. Another limitation of this study is that we could not conclude that the elevation of proinflammatory cytokines directly caused the decrease in ECD. In the future, proteome analysis of AqH may enable the identification of some therapeutic targets to prevent ECD loss after ocular surgeries. Third, the effects of cataract surgery, trabeculectomy, and corneal transplantation on the ECD may be different. Although it is still controversial, trabeculectomy may have the greatest effect on ECD loss, based on previous studies and our clinical experience.[56–58] We plan to use multivariate analyses in future studies to compare the effects of surgeries on AqH cytokine levels and long-term ECD.

In conclusion, the levels of cytokines, such as IL-1α, IL-4, IFN-γ, and TNF-α are elevated in eyes with BK and low ECD. Among the etiologies of BK, the levels of IL-1α, IL-4, IL-8, IL-10, and sICAM were elevated except for eyes with FECD. The number of previous ocular surgeries was positively correlated with the levels of some cytokines, suggesting that the ocular surgery can alter the environmental conditions in the anterior chamber, leading to endothelial cell loss and BK.

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References

1. Carlson KH, Bourne WM, McLaren JW, Brubaker RF. Variations in human corneal endothelial cell morphology and permeability to fluorescein with age. *Exp Eye Res*. 1988;47:27–41.
2. Yee RW, Matsuda M, Schultz RO, Edelhauser HF. Changes in the normal corneal endothelial cellular pattern as a function of age. *Curr Eye Res*. 1985;4:671–678.
3. Moller-Pedersen T. A comparative study of human corneal keratocyte and endothelial cell density during aging. *Cornea*. 1997;16:333–338.
4. Pillai CT, Dua HS, Azuara-Blanco A, Sarhan AR. Evaluation of corneal endothelium and keratic precipitates by specular microscopy in anterior uveitis. *Br J Ophthalmol*. 2000;84:1367–1371.
5. Setala K. Corneal endothelial cell density in iridocyclitis. *Acta Ophthalmol (Copenh)*. 1979;57:277–286.
6. Alfawaz AM, Holland GN, Yu F, Margolis MS, Giaconi JA, Aldave AJ. Corneal endothelium in patients with anterior uveitis. *Ophthalmology*. 2016;123:1637–1645.
7. Ambrose VM, Walters RF, Batterbury M, Spalton DJ, McGill JI. Long-term endothelial cell loss and breakdown of the blood-aqueous barrier in cataract surgery. *J Cataract Refract Surg*. 1991;17:622–627.
8. Matsuda M, Miyake K, Inaba M. Long-term corneal endothelial changes after intraocular lens implantation. *Am J Ophthalmol*. 1988;105:248–252.

9. Numa A, Nakamura J, Takashima M, Kani K. Long-term corneal endothelial changes after intraocular lens implantation. Anterior vs posterior chamber lenses. *Jpn J Ophthalmol*. 1993;37:78–87.
10. Lass JH, Beck RW, Benetz BA, et al.; Cornea Donor Study Investigator Group. Baseline factors related to endothelial cell loss following penetrating keratoplasty. *Arch Ophthalmol*. 2011;129:1149–1154.
11. Armitage WJ, Dick AD, Bourne WM. Predicting endothelial cell loss and long-term corneal graft survival. *Invest Ophthalmol Vis Sci*. 2003;44:3326–3331.
12. Lass JH, Gal RL, Dontchev M, et al.; Cornea Donor Study Investigator Group. Donor age and corneal endothelial cell loss 5 years after successful corneal transplantation. Specular microscopy ancillary study results. *Ophthalmology*. 2008;115:627–632.e8.
13. Bertelmann E, Pleyer U, Rieck P. Risk factors for endothelial cell loss post-keratoplasty. *Acta Ophthalmol Scand*. 2006;84:766–770.
14. Lass JH, Benetz BA, Gal RL, et al.; Writing Committee for the Cornea Donor Study Research Group. Donor age and factors related to endothelial cell loss 10 years after penetrating keratoplasty: Specular Microscopy Ancillary Study. *Ophthalmology*. 2013;120:2428–2435.
15. Nishimura JK, Hodge DO, Bourne WM. Initial endothelial cell density and chronic endothelial cell loss rate in corneal transplants with late endothelial failure. *Ophthalmology*. 1999;106:1962–1965.
16. Ishii N, Yamaguchi T, Yazu H, Satake Y, Yoshida A, Shimazaki J. Factors associated with graft survival and endothelial cell density after Descemet's stripping automated endothelial keratoplasty. *Sci Rep*. 2016;6:25276.
17. Sagoo P, Chan G, Larkin DF, George AJ. Inflammatory cytokines induce apoptosis of corneal endothelium through nitric oxide. *Invest Ophthalmol Vis Sci*. 2004;45:3964–3973.
18. Yamazoe K, Yamaguchi T, Hotta K, et al. Outcomes of cataract surgery in eyes with a low corneal endothelial cell density. *J Cataract Refract Surg*. 2011;37:2130–2136.
19. Yamaguchi T, Calvacanti BM, Cruzat A, et al. Correlation between human tear cytokine levels and cellular corneal changes in patients with bacterial keratitis by in vivo confocal microscopy. *Invest Ophthalmol Vis Sci*. 2014;55:7457–7466.
20. Inoue T, Kawaji T, Inatani M, Kameda T, Yoshimura N, Tanihara H. Simultaneous increases in multiple proinflammatory cytokines in the aqueous humor in pseudophakic glaucomatous eyes. *J Cataract Refract Surg*. 2012;38:1389–1397.
21. Bourne WM, Nelson LR, Hodge DO. Continued endothelial cell loss ten years after lens implantation. *Ophthalmology*. 1994;101:1014–1022; discussion 1022–1013.
22. Matthaei M, Gillessen J, Muether PS, et al. Epithelial-mesenchymal transition (EMT)-related cytokines in the aqueous humor of phakic and pseudophakic Fuchs' dystrophy eyes. *Invest Ophthalmol Vis Sci*. 2015;56:2749–2754.
23. Kawai M, Inoue T, Inatani M, et al. Elevated levels of monocyte chemoattractant protein-1 in the aqueous humor after phacoemulsification. *Invest Ophthalmol Vis Sci*. 2012;53:7951–7960.
24. Bourne WM, Nelson LR, Hodge DO. Central corneal endothelial cell changes over a ten-year period. *Invest Ophthalmol Vis Sci*. 1997;38:779–782.
25. Ing JJ, Ing HH, Nelson LR, Hodge DO, Bourne WM. Ten-year postoperative results of penetrating keratoplasty. *Ophthalmology*. 1998;105:1855–1865.
26. Hollander DA, Giaconi JA, Holland GN, et al. Graft failure after penetrating keratoplasty in eyes with Ahmed valves. *Am J Ophthalmol*. 2010;150:169–178.
27. Richardson MR, Segu ZM, Price MO, et al. Alterations in the aqueous humor proteome in patients with Fuchs endothelial corneal dystrophy. *Mol Vis*. 2010;16:2376–2383.
28. Maier P, Heizmann U, Bohringer D, Kern Y, Reinhard T. Predicting the risk for corneal graft rejection by aqueous humor analysis. *Mol Vis*. 2011;17:1016–1023.
29. Maier P, Heizmann U, Bohringer D, Kern Y, Reinhard T. Distinct cytokine pattern in aqueous humor during immune reactions following penetrating keratoplasty. *Mol Vis*. 2010;16:53–60.
30. Kuiper JJ, Mutis T, de Jager W, de Groot-Mijnes JD, Rothova A. Intraocular interleukin-17 and proinflammatory cytokines in HLA-A29-associated birdshot chorioretinopathy. *Am J Ophthalmol*. 2011;152:177–182.e171.
31. El-Asrar AM, Struyf S, Kangave D, et al. Cytokine profiles in aqueous humor of patients with different clinical entities of endogenous uveitis. *Clin Immunol*. 2011;139:177–184.
32. Ohira S, Inoue T, Iwao K, Takahashi E, Tanihara H. Factors influencing aqueous proinflammatory cytokines and growth factors in uveitic glaucoma. *PLoS One*. 2016;11:e0147080.
33. Agawa T, Usui Y, Wakabayashi Y, et al. Profile of intraocular immune mediators in patients with age-related macular degeneration and the effect of intravitreal bevacizumab injection. *Retina*. 2014;34:1811–1818.
34. Roh MI, Kim HS, Song JH, Lim JB, Kwon OW. Effect of intravitreal bevacizumab injection on aqueous humor cytokine levels in clinically significant macular edema. *Ophthalmology*. 2009;116:80–86.
35. Okunuki Y, Usui Y, Katai N, et al. Relation of intraocular concentrations of inflammatory factors and improvement of macular edema after vitrectomy in branch retinal vein occlusion. *Am J Ophthalmol*. 2011;151:610–616.e611.
36. Kaneda S, Miyazaki D, Sasaki S, et al. Multivariate analyses of inflammatory cytokines in eyes with branch retinal vein occlusion: relationships to bevacizumab treatment. *Invest Ophthalmol Vis Sci*. 2011;52:2982–2988.
37. Jung SH, Kim KA, Sohn SW, Yang SJ. Association of aqueous humor cytokines with the development of retinal ischemia and recurrent macular edema in retinal vein occlusion. *Invest Ophthalmol Vis Sci*. 2014;55:2290–2296.
38. Sugita S, Usui Y, Horie S, et al. Human corneal endothelial cells expressing programmed death-ligand 1 (PD-L1) suppress PD-1+ T helper 1 cells by a contact-dependent mechanism. *Invest Ophthalmol Vis Sci*. 2009;50:263–272.
39. Kawashima H, Prasad SA, Gregerson DS. Corneal endothelial cells inhibit T cell proliferation by blocking IL-2 production. *J Immunol*. 1994;153:1982–1989.
40. Mi P, Gregerson DS, Kawashima H. Local regulation of immune responses: corneal endothelial cells alter T cell activation and cytokine production. *Cytokine*. 2000;12:253–264.
41. Lapp T, Zaher SS, Haas CT, et al. Identification of therapeutic targets of inflammatory monocyte recruitment to modulate the allogeneic injury to donor cornea. *Invest Ophthalmol Vis Sci*. 2015;56:7250–7259.
42. Eom Y, Kwon J, Heo JH, et al. The effects of proinflammatory cytokines on the apoptosis of corneal endothelial cells following argon laser iridotomy. *Exp Eye Res*. 2016;145:140–147.
43. Ladas JG, Wheeler NC, Morhun PJ, Rimmer SO, Holland GN. Laser flare-cell photometry: methodology and clinical applications. *Surv Ophthalmol*. 2005;50:27–47.
44. Bijanzadeh M, Ramachandra NB, Mahesh PA, et al. Soluble intercellular adhesion molecule-1 and E-selectin in patients with asthma exacerbation. Lung. 2009;187:315–320.
45. Hirai S, Kageshita T, Kimura T, et al. Soluble intercellular adhesion molecule-1 and soluble E-selectin levels in patients with atopic dermatitis. *Br J Dermatol*. 1996;134:657–661.
46. Khare A, Shetty S, Ghosh K, Mohanty D, Chatterjee S. Evaluation of markers of endothelial damage in cases of young myocardial infarction. *Atherosclerosis*. 2005;180:375–380.

Investigative Ophthalmology & Visual Science

47. Tzoulaki I, Murray GD, Lee AJ, Rumley A, Lowe GD, Fowkes FG. C-reactive protein, interleukin-6 and soluble adhesion molecules as predictors of progressive peripheral atherosclerosis in the general population: Edinburgh Artery Study. *Circulation*. 2005;112:976–983.
48. Dohlman TH, Di Zazzo A, Omoto M, et al. E-selectin mediates immune cell trafficking in corneal transplantation. *Transplantation*. 2016;100:772–780.
49. Sugita S, Kawazoe Y, Yamada Y, et al. Inhibitory effect of corneal endothelial cells IL-17-producing Th17 cells. *Br J Ophthalmol*. 2012;96:293–299.
50. Sugita S, Yamada Y, Horie S, et al. Induction of T regulatory cells by cytotoxic T-lymphocyte antigen-2a on corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2011;52:2598–2605.
51. Streilein JW, Okamoto S, Sano Y, Taylor AW. Neural control of ocular immune privilege. *Ann N Y Acad Sci*. 2000;917:297–306.
52. Muller RT, Pourmirzaie R, Paven-Langston D, et al. In vivo confocal microscopy demonstrates bilateral loss of endothelial cells in unilateral herpes simplex keratitis. *Invest Ophthalmol Vis Sci*. 2015;56:4899–4906.
53. Schrems-Hoesl LM, Schrems WA, Cruzat A, et al. Cellular and subbasal nerve alterations in early stage Fuchs' endothelial corneal dystrophy: an in vivo confocal microscopy study. *Eye (Lond)*. 2013;27:42–49.
54. Koh S-WM, Gloria D, Molloy J. Corneal endothelial autocrine VIP enhances its integrity in stored human donor corneoscleral explant. *Invest Ophthalmol Vis Sci*. 2011;52:5632–5640.
55. Streilein JW, Bradley D. Analysis of immunosuppressive properties of iris and ciliary body cells and their secretory products. *Invest Ophthalmol Vis Sci*. 1991;32:2700–2710.
56. Heindl LM, Koch KR, Bucher F, et al. Descemet membrane endothelial keratoplasty in eyes with glaucoma implants. *Optom Vis Sci*. 2013;90:e241–e244; discussion 1029.
57. Sugar A, Gal RL, Kollman C, et al.; Writing Committee for the Cornea Donor Study Research Group. Factors associated with corneal graft survival in the cornea donor study. *JAMA Ophthalmol*. 2015;133:246–254.
58. Sugar A, Tanner JP, Dontchev M, et al. Cornea Donor Study Investigator Group. Recipient risk factors for graft failure in the cornea donor study. *Ophthalmology*. 2009;116:1023–1028

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