

Correlation Between Human Tear Cytokine Levels and Cellular Corneal Changes in Patients With Bacterial Keratitis by In Vivo Confocal Microscopy

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PURPOSE. We investigated bilateral tear cytokine levels in patients with unilateral bacterial keratitis (BK) as associated with in vivo confocal microscopic (IVCM) alterations in corneal nerves and dendritiform immune cells (DCs).

METHODS. A total of 54 (13 BK, 13 contralateral, 28 healthy controls) tear samples was collected prospectively and analyzed by multiplex microbeads assay. The IVCM of the central cornea was performed on the same day, and assessed for corneal nerve and DC alterations.

RESULTS. Interleukin-1 β , IL-6, and IL-8 were significantly elevated only in affected eyes (66.6 ± 26.8 , 7174 ± 2430 , and 810 ± 315 pg/mL, respectively; $P = 0.04$, $P < 0.001$, and $P < 0.001$, respectively), compared to healthy controls (13.0 ± 4.0 , 171.8 ± 32.1 , and 56.5 ± 33.8 pg/mL). Levels of chemokine ligand 2 (CCL-2), IL-10, and IL-17a were elevated only in contralateral eyes (813 ± 478 , 86.7 ± 38.3 , and 3350 ± 881 pg/mL, respectively; $P = 0.02$, $P = 0.01$, and $P = 0.04$, respectively), compared to controls (73.7 ± 25.3 , 17.5 ± 4.9 , and 1350 ± 337 pg/mL). Triggering receptor expressed on myeloid cells (TREM)-1 was significantly elevated in affected (551 ± 231 pg/mL, $P = 0.02$) and contralateral unaffected (545 ± 298 pg/mL, $P = 0.03$) eyes compared to controls (31.3 ± 12.4 pg/mL). The density of DCs was significantly increased in affected (226.9 ± 37.3 cells/mm², $P < 0.001$) and unaffected (122.3 ± 23.7 cells/mm², $P < 0.001$) eyes compared to controls (22.7 ± 5.9 cells/mm²). Sub-basal nerve density significantly decreased in affected (3337 ± 1615 μ m/mm², $P < 0.001$) and contralateral ($13,230 \pm 1635$ μ m/mm², $P < 0.001$) eyes compared to controls ($21,200 \pm 545$ μ m/mm²). Levels of IL-1 β , IL-6, and IL-8 were significantly correlated with DC density ($R = 0.40$, $R = 0.55$, and $R = 0.31$, all $P < 0.02$) and nerve density ($R = -0.30$, $R = -0.53$, and $R = -0.39$, all $P < 0.01$).

CONCLUSIONS. Proinflammatory tear cytokines are elevated bilaterally in patients with unilateral BK, and are correlated strongly with alterations in DCs and nerve density as detected by IVCM.

Keywords: infectious keratitis, dendritic cells, corneal infection, tears, cytokines

Bacterial keratitis (BK) is a potentially blinding ocular condition of the cornea, which can result in severe loss of vision due to corneal scarring, corneal perforation, or endophthalmitis.^{1,2} Risk factors for BK include contact lens wear, ocular surface disease, trauma, chronic use of topical steroids, corneal surgery, diabetes, and neurotrophic keratopathy.³⁻⁵ During the past decade, in vivo confocal microscopy (IVCM) has been used increasingly to evaluate the structural and cellular changes in various corneal diseases.⁶⁻¹² It is being used in the diagnosis and management of corneal diseases, as well as in the assessment of disease severity.^{8,13} This technique is further being used to understand the pathophysiology of corneal diseases in patients, and has led to new insights into ocular and systemic diseases, such as keratoconus, herpetic keratitis, and diabetic neuropathy.^{6,14-17} Recently, we demon-

strated bilateral nerve alterations in patients with unilateral herpes simplex keratitis (HSK) and herpes zoster ophthalmicus (HZO) by IVCM.^{15,16} Moreover, we showed an inverse correlation between the nerve density and density of dendritiform immune cells (DCs) in patients with infectious keratitis, including bacterial, fungal, and *Acanthamoeba* keratitis.¹⁸ However, the mechanism and consequences of bilateral nerve alterations in unilateral infectious keratitis remain poorly understood.

Recent studies have suggested that proinflammatory cytokines in tears may have a key role in the pathogenesis of several corneal diseases, including dry eye disease,¹⁹ keratoconus,^{20,21} graft-versus-host disease (GVHD),²² conjunctivitis,²³ as well as in the development of corneal neovascularization.²⁴ However, currently, to our knowledge, there have been no reports on the

alterations of cytokine levels in patients with infectious keratitis. Furthermore, previous studies have assessed tear cytokine changes in either affected eyes^{21,24} or in bilateral diseases,²²⁻²⁵ but to our knowledge contralateral changes in tear cytokines in unilateral diseases have not been assessed before. Finally, although previous reports suggested that tear cytokine levels correlated with clinical findings, such as keratorefractive values in keratoconus²⁰ or with clinical severity score in dry eye patients,^{25,26} it is unclear if intracorneal cellular changes in DCs and corneal nerves as shown by IVCM correspond with changes in tear cytokines.

We hypothesized that alterations in corneal DCs and corneal nerves by IVCM would correlate with changes in proinflammatory tear cytokines, and that unilateral BK would alter bilateral tear cytokine levels. Thus, in the current study, we aimed to quantify bilateral tear cytokines levels, and correlate them with alterations in corneal nerves and DCs as detected by IVCM in unilateral BK.

PATIENTS AND METHODS

Patients

A prospective, single-center study was conducted in a masked fashion. Subjects with acute BK at presentation were recruited from the Cornea & Refractive Surgery Service at the Massachusetts Eye and Ear Infirmary (Boston, MA, USA), between 2011 and 2013. A total of 10 patients with a diagnosis of acute unilateral BK was included in this study and compared to 14 normal age- and sex-matched healthy control subjects. We collected 54 tear samples from the affected and contralateral clinically unaffected eyes of BK patients as well as from both eyes of healthy control subjects. In three BK patients, tear collection and IVCM were performed twice (Patients 8-10) to analyze changes over a 1- to 2-week time period after the initial visit. In all 3 patients, the infiltration and corneal epithelial defect improved after the treatment was started. All patients and healthy control subjects underwent slit-lamp biomicroscopy. Bacterial keratitis was diagnosed according to the patient's history, clinical examinations, and/or cultures. Patients with other causative organism, such as viral, fungal, and *Acanthamoeba* keratitis, were excluded. None of the healthy controls had trauma, surgery, contact lens use, or other ocular surface diseases, such as dry eye disease or conjunctivitis. None of the BK patients or healthy control subjects had any systemic immunological diseases or diabetes. The study protocol was approved by the Institutional review Board/Ethics Committee from the authors' institution, complied with the Health Insurance Portability and Accountability Act, and was conducted in accordance with the provision of the Declaration of Helsinki. All patients provided written informed consent after a detailed explanation of the nature of the study.

Tear Collection

Tear collection was conducted before the IVCM examination and instillation of any eye drops as described previously.²⁷ Briefly, 60 μ L of sterile saline solution were instilled into the inferior fornix by using a micropipette. Next, subjects were asked to look left, right, up, and down, four times without blinking, to mix the tear fluid content. Then, the diluted tears with sterile saline were collected from the inferior fornix using the micropipette and transferred to an Eppendorf tube. This process was repeated for the contralateral eye and for both eyes of control subjects, always using a new micropipette sterile tip for each eye. All tear samples were stored in individual Eppendorf tubes at -80°C .

In Vivo Confocal Microscopy

Laser IVCM (Heidelberg Retina Tomograph 3/Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany) was performed on the central cornea of BK patients and control subjects bilaterally after tear collection. This microscope uses a 670-nm wavelength diode laser source and is equipped with a $\times 63$ objective immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan). The microscope provides a $400 \times 400\text{-}\mu\text{m}$ section of the cornea with axial resolution of 1 to 4 μm . The technique used was the same as reported previously.¹⁸ Briefly, a disposable sterile polymethylmethacrylate (PMMA) cap (TOMO-cap; Heidelberg Engineering GmbH, Heidelberg, Germany) filled with hydroxypropyl methylcellulose 2.5% (GenTeal gel; Novartis Ophthalmics, East Hanover, NJ, USA) was mounted in front of the cornea module objective lens for each examination. After the instillation of topical anesthesia of 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX, USA) in both eyes, a drop of hydroxypropyl methylcellulose 2.5% (GenTeal gel) was applied to the inferior fornix. Further, hydroxypropyl gel was added to the outside tip of the TOMO-cap to improve optical coupling and the lens was advanced until the cap made contact with the surface of the cornea. Images were obtained from the epithelial layer to the endothelial layer using multiple scans in the sequence mode. A total of four to six sequence scans was recorded for each eye, at least 3 of which were sequence scans with particular focus on the subepithelial area, the sub-basal nerve plexus, and epithelial DCs, typically at a depth of 50 to 80 μm .

When a corneal ulcer was present with an epithelial defect or severe ulceration in eyes with BK, the ulcer and periphery of the ulcer were scanned. If the images of the ulcer were not suitable due to severe edema or opacity, the periphery of the ulcer (Fig. 1B, arrows) was chosen for the analysis to minimize the bias from the opacity or edematous ulceration. A minimum of 3 representative images of the sub-basal nerve plexus and epithelial DCs were selected for analysis for each eye. The images were selected from the layer immediately at or posterior to the basal epithelial layer and anterior to the Bowman's layer. The criteria to select the images were the best-focused and complete images, with the whole image in the same layer, without motion, without folds, and good contrast. Sub-basal nerve fibers and DC density and morphology were analyzed as described previously.¹⁸ Particularly, image analysis was performed using the ImageJ (developed by Wayne Rasband, National Institutes of Health [NIH], Bethesda, MD, USA; available in the public domain at <http://rsb.info.nih.gov/ij/>; accessed March 2014) and NeuronJ (available in the public domain at <http://www.imagescience.org/meijering/software/neuronj/>; accessed March 2014), a semiautomated tracing plugin for ImageJ, a free image analysis software distributed by the NIH. Three masked observers (TY, BMC, and YQ) evaluated the confocal images for the parameters as described below. Nerve density was assessed by measuring the total length of the nerve fibers in micrometers per frame of 0.16 mm^2 area and expressed as $\mu\text{m}/\text{mm}^2$. Number of nerves was defined as the number of total nerve fibers per frame of 0.16 mm^2 area. The DC parameters included DC density, DC area, numbers of dendrites per cell, and DC field. The DCs were defined morphologically as bright dendritiform, well-demarcated structures, predominantly found in the sub-basal area. The DC density was calculated by dividing the total number of DCs per each frame of 0.16 mm^2 , expressed as cells/mm^2 . The DC area was determined as the mean actual cell area of 5 representative DCs/frame from 3 representative frames (total 15 DCs) and was expressed as $\mu\text{m}^2/\text{cell}$. The number of dendrites is defined as the mean number of dendrites per cells of 5 representative

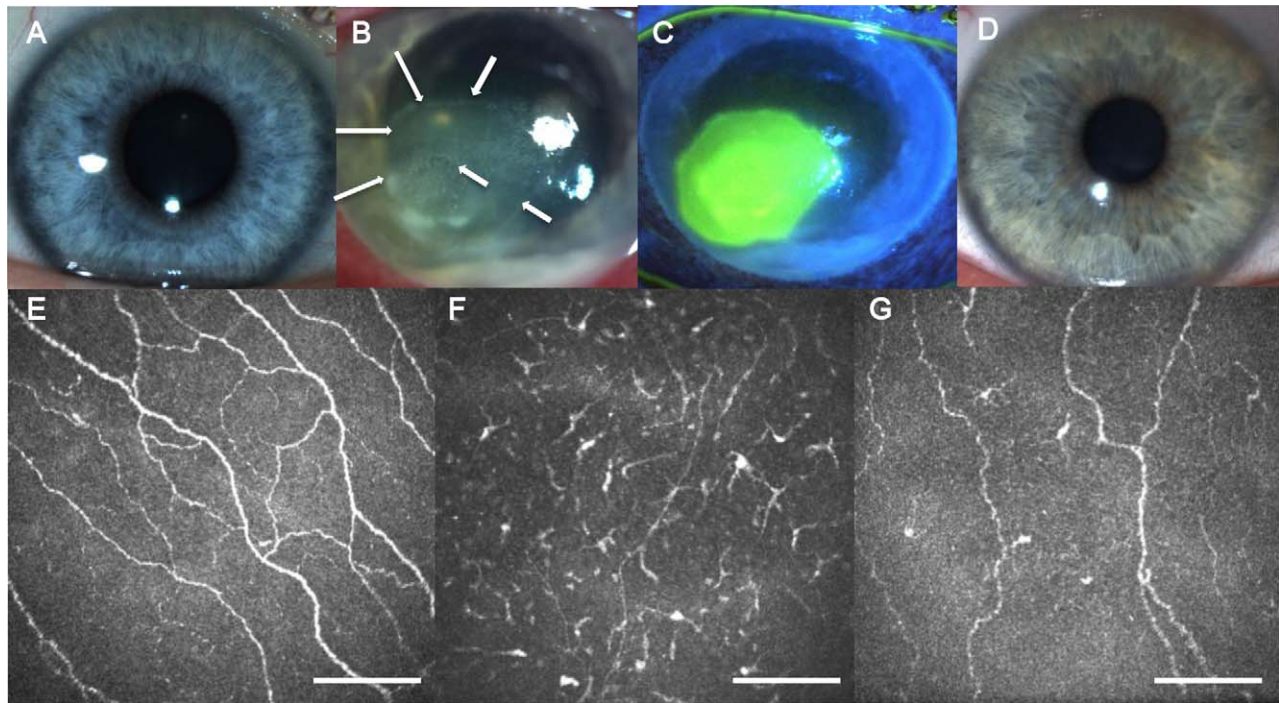


FIGURE 1. Slit-lamp photographs and in vivo confocal microscopy images. The representative slit-lamp photographs of a normal control eye (A), a clinically affected eye with unilateral BK (B, C), and its contralateral clinically unaffected eye (D). The IVCM images of a healthy control eye (E), a clinically affected eye with unilateral BK (F), and its contralateral clinically unaffected eye (G). Scale bars: 100 μ m.

DCs/frame from 3 representative frames. The DC field was defined as the span of each cell, which included the area of its mean cell body and dendrites of 5 representative DCs/frame from 3 representative frames and was expressed as $\mu\text{m}^2/\text{cell}$.

Tear Cytokine Protein Levels

The cytokine levels (IL-1Ra, IL-1 β , IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL-17a, fibroblast growth factor [FGF]-2, granulocyte/macrophage colony stimulating factor [GM-CSF], chemokine ligand [CCL]-2, and triggering receptor expressed on myeloid cells [TREM]-1) of tear samples were measured by Luminex (Luminex Corporation, San Antonio, TX, USA) beads-based multiplex immunoassay according to previous reports.²³ Briefly, 20 μ L of each tear sample were incubated with antibody-coated capture beads in an incubation buffer (PBS + 0.1% Tween-20 + 0.05% sodium azide) at room temperature. After 1 hour of incubation in the dark, the beads were washed with washing buffer (PBS + 0.1% Tween-20) by centrifugation, and biotinylated secondary antibodies were added for 30 minutes of incubation in the dark at room temperature. Plates were washed with washing buffer by centrifugation, and Phycoerythrin-labeled streptavidin was added for 15 minutes of

incubation in the dark at room temperature. After being washed twice in washing buffer by centrifugation, plates were resuspended in 50 μ L washing buffer, and assays were performed using a Luminex 200 instrument (Luminex Corporation). We used the STarStation v2.3 and STarCollate software program (Applied Cytometry Systems, Dinnington, UK) to determine sample cytokine levels by standard curve analysis using known concentrations of cytokines as standards.

Statistical Analysis

Data were analyzed using Prism for Windows version 6.04 (GraphPad Software, Inc., San Diego, CA, USA). To compare the sub-basal nerve density; DC density and morphology; and cytokine levels between control eyes, affected BK eyes, and contralateral unaffected eyes; 1-way ANOVA with Tukey's multiple comparisons post-test was performed. Spearman's correlation coefficient analysis was used to evaluate the correlation among sub-basal nerve density, DC parameters, and cytokine levels. For each test, differences were considered statistically significant at a *P* value of less than 0.05 and data were presented as mean \pm SE.

RESULTS

We collected 26 tear samples from 10 patients with BK and 28 tear samples from 14 healthy control subjects. Figure 1 shows the slit-lamp photographs and IVCM images of the representative cases of each group. Demographic data of BK and the healthy control group are summarized in Table 1. Clinical data of BK patients are presented in Table 2. The causative organisms were identified using culture examination in 5 of 10 patients. Five other patients were diagnosed with BK by their history and clinical findings, as well as their rapid response to the antibacterial therapy.

TABLE 1. Demographics of Patients and Control Subjects

	Healthy Controls	BK	Contralateral
Subjects, <i>n</i>	14	10	10
Tear samples, <i>n</i>	28	13	13
Mean age \pm SD, y	38.7 \pm 12.8*	40.1 \pm 18.9	40.1 \pm 18.9
Sex, M/F	8/6†	5/5	5/5

* *P* = 0.21, comparing age between healthy controls and BK group (*t*-test).

† *P* = 0.53, comparing sex between healthy controls and BK group (Fisher's exact test).

TABLE 2. Summary of Cases and Characteristics of Patients With BK

Case	Age	Sex	Cause of BK	Causative Organism	Initial CDVA	Topical Treatment	Tear Samples, n
1	78	M	Trauma	CNS	CF	VCM, TOB, BAC	1
2	40	F	CL-related	NA	CF	GFLX, TOB, Pred	1
3	24	F	CL-related	NA	HM	VCM, TOB	1
4	58	F	CL-related	NA	20/40	VCM, TOB	1
5	26	M	CL-related	NA	HM	VCM, MFX	1
6	66	F	CL-related	<i>P. aeruginosa</i>	20/400	VCM, TOB, CPFX	1
7	22	M	CL-related	<i>P. aeruginosa</i>	20/70	TOB, Pred	1
8	18	F	CL-related	<i>P. aeruginosa</i>	20/200	VCM, TOB	2
9	56	M	Long-term topical steroid use	<i>Streptococcus pneumoniae</i>	HM	VCM, TOB	2
10	45	F	Trauma	NA	20/25	MFX	2

M, male; F, female; NA, not available; CNS, coagulase negative streptococcus; *P.* Pseudomonas; TOB, tobramycin; VCM, vancomycin; BAC, bacitracin; MFX, moxifloxacin; GFLX, gatifloxacin; Pred, prednisolone; CPFX, ciprofloxacin.

Corneal Sub-Basal Nerve Plexus and DC Parameters by IVCM

Quantitative analysis of affected and unaffected eyes of BK patients and controls are shown in Figure 2. Patients with unilateral BK showed significant reduction in the sub-basal nerve plexus density and total number of nerve fibers in both eyes, when compared to controls. In particular, the mean nerve density was reduced to $3337 \pm 1615 \mu\text{m}/\text{mm}^2$ in affected BK eyes, and to $13,230 \pm 1635 \mu\text{m}/\text{mm}^2$ in contralateral eyes, compared to $21,200 \pm 545 \mu\text{m}/\text{mm}^2$ in controls (ANOVA, $P < 0.001$; Fig. 2A). The total number of nerve fibers was reduced to $2.0 \pm 0.9/\text{frame}$ in BK eyes and $7.6 \pm 1.1/\text{frame}$ in contralateral eyes, compared to $16.4 \pm 1.4/\text{frame}$ in controls (ANOVA, $P <$

0.001 ; Fig. 2B). The DC density was increased to 226.9 ± 37.3 cells/ mm^2 in BK eyes and 122.3 ± 23.7 cells/ mm^2 in contralateral eyes, compared to 22.7 ± 5.9 cells/ mm^2 in healthy controls (ANOVA, $P < 0.001$; Fig. 2C). The DC area was increased to $161.7 \pm 20.2 \mu\text{m}^2$ in BK eyes and $109.0 \pm 8.5 \mu\text{m}^2$ in contralateral eyes, compared to $80.7 \pm 5.8 \mu\text{m}^2$ in healthy controls (ANOVA, $P < 0.001$; Fig. 2D). There were no significant differences in number of dendrites and DC field among the groups (ANOVA, $P = 0.247$ and $P = 0.263$, respectively).

Tear Cytokine Concentrations

The detailed tear cytokine concentrations measured by the multiplex assay are shown in Figure 3. The concentrations of

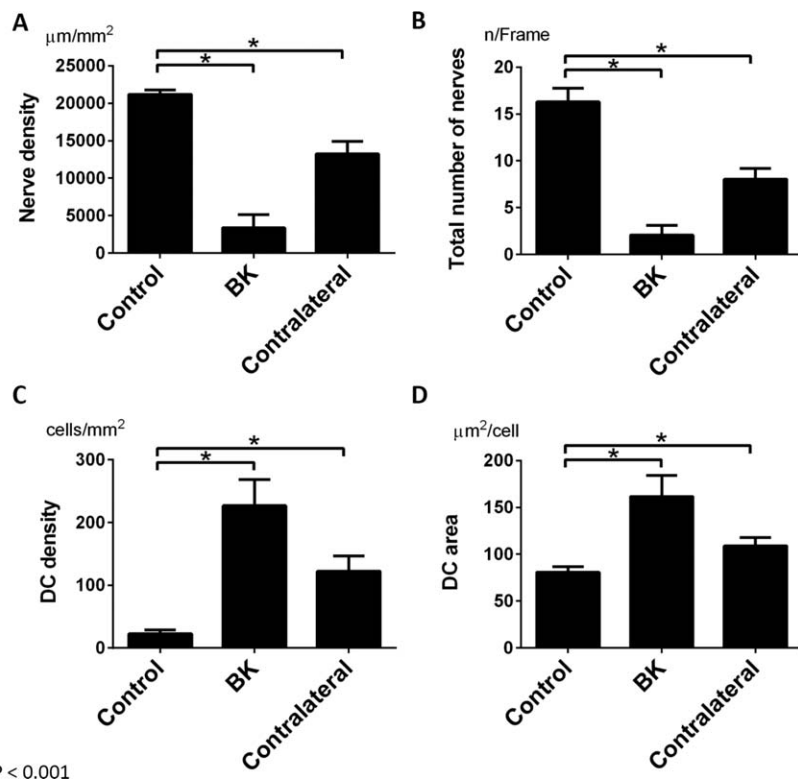


FIGURE 2. Corneal sub-basal nerves and dendritiform cells. The nerve density (A) and number of nerves (B) in eyes with unilateral BK and contralateral eyes were significantly decreased compared to normal eyes ($P < 0.001$, 1-way ANOVA). The DC density (C) and mean DC area (D) were significantly higher in eyes with unilateral BK and in contralateral unaffected eyes than in eyes of healthy controls ($P < 0.001$, 1-way ANOVA). A P value of less than 0.05 was considered statistically significant. Graph bars: represent average SE.

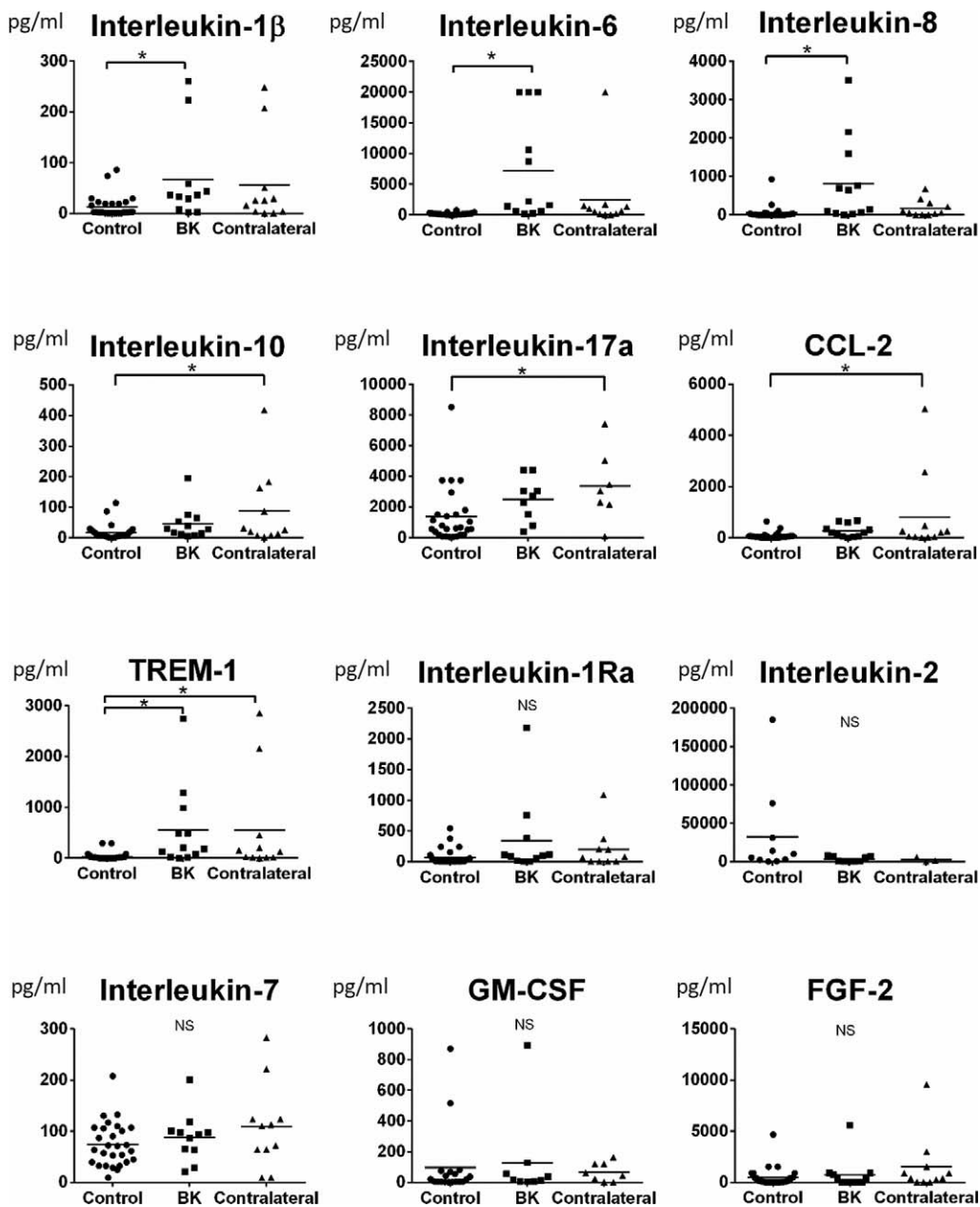


FIGURE 3. Tear cytokine levels. The IL-1 β , IL-6, and IL-8 levels in affected BK eyes ($P=0.04$, $P < 0.001$, and $P < 0.001$), but not contralateral eyes ($P > 0.05$), were significantly increased compared to controls. In contrast, IL-10, IL-17a, and CCL-2 were only significantly increased in contralateral unaffected eyes ($P = 0.01$, $P = 0.04$, and $P = 0.02$), but not in affected eyes ($P > 0.05$), compared to controls. The level of TREM-1 was significantly increased in affected eyes with BK ($P = 0.02$), as well as in contralateral unaffected eyes ($P = 0.03$), compared to normal eyes. There were no significant differences in levels of IL-1Ra, IL-2, IL-7, GM-CSF, and FGF-2 among the groups ($P > 0.05$). Error bars: represent the mean value for each group. A P value of less than 0.05 was considered statistically significant.

IL-1 β , IL-6, and IL-8 were significantly increased in affected BK eyes ($P=0.04$, $P < 0.001$, and $P < 0.001$), but not contralateral eyes ($P > 0.05$), compared to healthy controls. In contrast, the tear concentrations of IL-10, IL-17a, and CCL-2 were only significantly increased in contralateral unaffected eyes ($P = 0.01$, $P = 0.04$, and $P = 0.02$), but not affected eyes ($P > 0.05$), compared to healthy controls. The tear concentration of TREM-1 was significantly increased in affected eyes with BK ($P = 0.02$), as well as in contralateral unaffected eyes ($P = 0.03$), compared to controls. There were no significant differences in

levels of IL-1Ra, IL-2, IL-7, FGF-2, and GM-CSF among the groups ($P > 0.05$).

Correlation of Tear Cytokines With DC Parameters by IVCM

Tear concentrations of IL-1 β , IL-6, IL-8, and IL-17a were positively correlated with DC density ($R = 0.40$, $P = 0.001$; $R = 0.55$, $P < 0.001$; $R = 0.31$, $P = 0.01$; and $R = 0.34$, $P = 0.01$, respectively; Figs. 4A-D). Interleukin-1 β was correlated inversely with the number of dendrites ($R = -0.51$, $P = 0.004$;

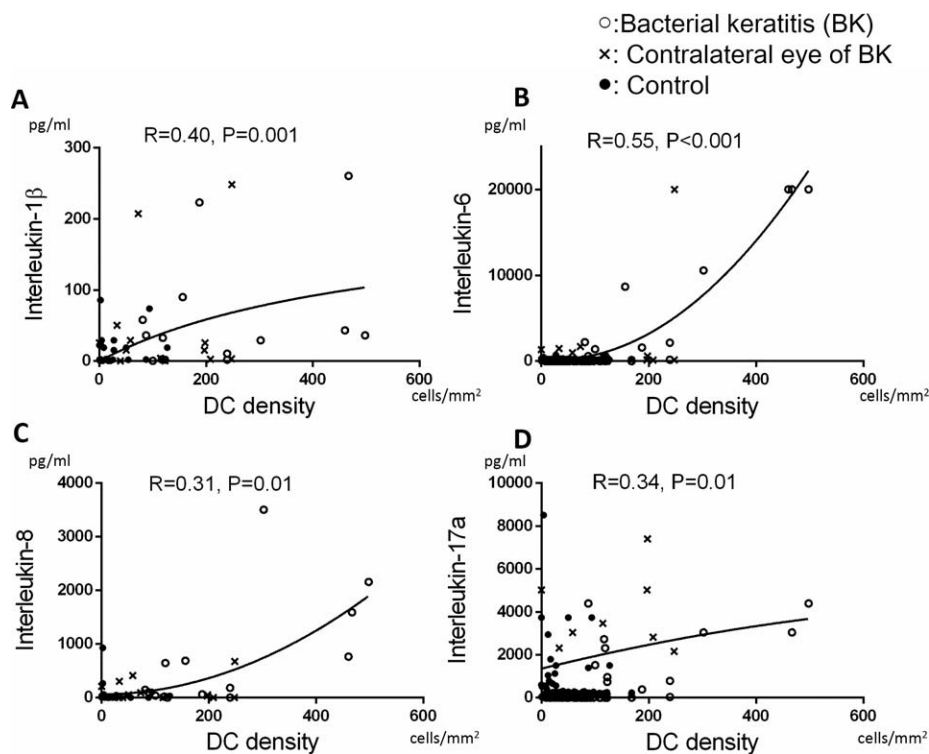


FIGURE 4. Correlation between tear cytokine levels and DC density. Levels of IL-1 β ($R=0.40$, $P=0.001$ [A]), IL-6 ($R=0.55$, $P<0.001$ [B]), IL-8 ($R=0.31$, $P=0.01$ [C]), and IL-17a ($R=0.34$, $P=0.01$ [D]) significantly correlated with DC density. A P value of less than 0.05 was considered statistically significant.

Fig. 5A), and IL-7 was inversely correlated with DC field ($R=-0.31$, $P=0.01$; Fig. 5B). However, other cytokines did not correlate with morphological parameters for DCs ($P>0.05$).

Correlation of Tear Cytokines and Sub-Basal Nerve Density

The tear concentrations of IL-1 β , IL-6, and IL-8 were inversely correlated with sub-basal nerve density ($R=-0.30$, $P=0.01$; $R=-0.53$, $P<0.001$; and $R=-0.39$, $P=0.001$, respectively; Figs. 6A-C). In addition, concentrations of TREM-1 were inversely correlated with sub-basal nerve density ($R=-0.35$, $P=0.005$; Fig. 6D). There were no significant correlations between all other cytokines and corneal nerve density ($P>0.05$).

Alterations of Cytokine Concentrations in Patients Over Time

In 3 cases (cases 8-10), the tear samples and IVCM images were obtained at 2 time points (Fig. 7A). In all these 3 patients, the BK improved clinically after the antibiotic treatment started. The average of DC density, IL-1 β , IL-6, and IL-17a decreased in the affected eyes as the BK was improving (Fig. 7B). However, surprisingly, the DC density, IL-1 β , and IL-6 increased in the contralateral eyes, although they remained clinically unaffected. The IL-17a level in the contralateral eyes were elevated at visit 1 and decreased as the BK improved.

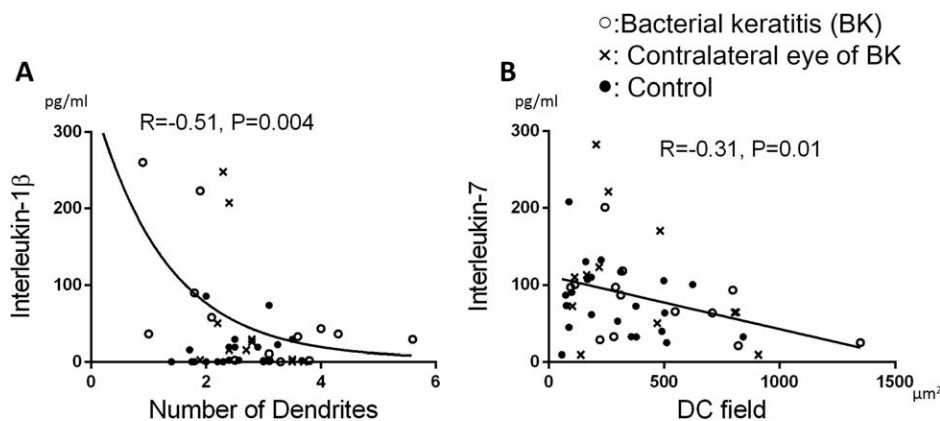


FIGURE 5. Correlation between cytokine levels and DC morphology. The number of dendrites was significantly correlated with IL-1 β ($R=-0.51$, $P=0.004$ [A]). The DC field was significantly correlated with IL-7 ($R=-0.31$, $P=0.01$ [B]). A P value of less than 0.05 was considered statistically significant.

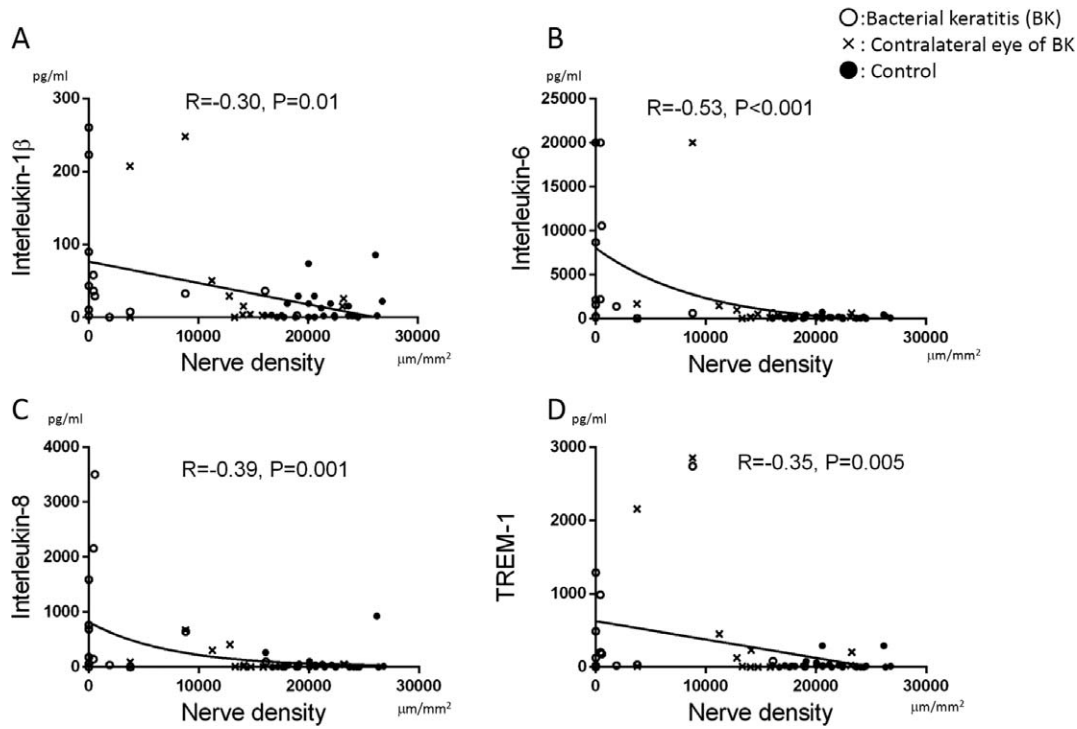


FIGURE 6. Correlation between nerve density and cytokine levels. Nerve density was significantly inversely correlated with IL-1 β ($R = -0.30$, $P = 0.01$ [A]), IL-6 ($R = -0.53$, $P < 0.001$ [B]), IL-8 ($R = -0.39$, $P = 0.001$ [C]), and TREM-1 ($R = -0.35$, $P = 0.005$ [D]). A P value of less than 0.05 was considered statistically significant.

DISCUSSION

The current study evaluates the tear cytokine concentrations and alterations in corneal DCs and nerves by IVCM in healthy control subjects and patients with unilateral BK. We demon-

strated correlation of proinflammatory tear cytokine levels with DC density and DC morphology as shown by IVCM. Recent advances in the technique of cytokine protein measurements from small volumes of fluid have enabled us to evaluate the tear cytokine concentrations in ocular diseases,

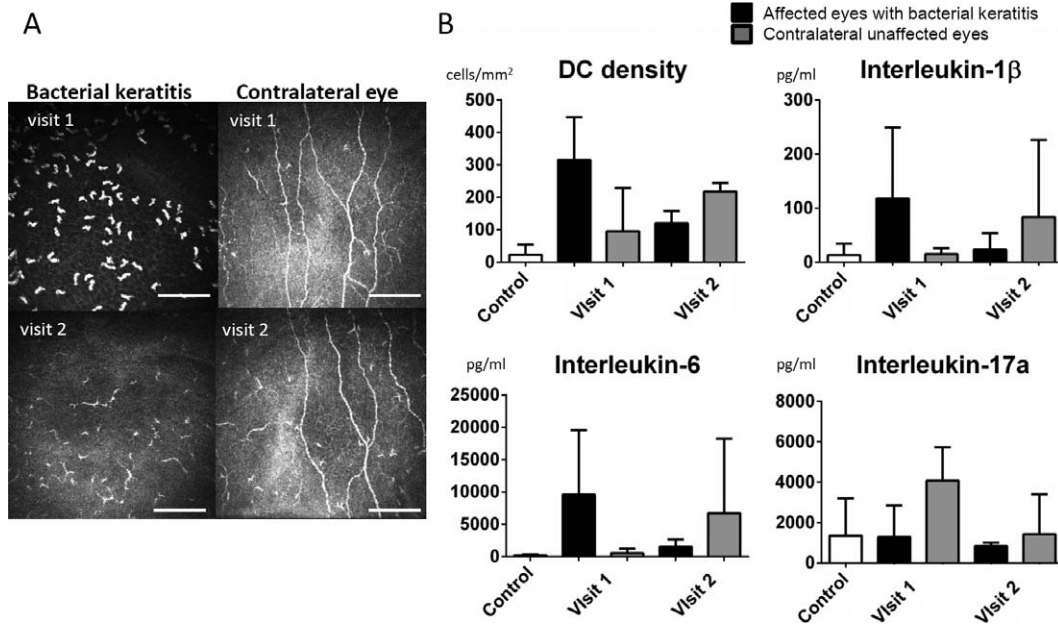


FIGURE 7. Alterations of DC density and tear cytokines over time. In 3 cases (case 8-10 in Table 2), tear samples and IVCM were obtained on the same day at visits 1 and 2. In all of these 3 patients, the BK improved clinically after the antibiotic treatment started. The representative IVCM images at visits 1 and 2 (A). Size bars: represent 100 μ m. The DC density, IL-1 β , IL-6, and IL-17a in the affected eyes decreased from visit 1 to visit 2. The DC density, IL-1 β , and IL-6 in the contralateral eyes gradually increased from visit 1 to visit 2, whereas IL-17a in the contralateral eyes increased at visit 1 and decreased at visit 2 (B).

such as dry eye disease, keratoconus, GVHD, and corneal neovascularization.^{19–25} Recently, Villani et al.²⁶ evaluated tear cytokine concentration and IVCM data in patients with rheumatoid arthritis and reported a decrease in tear IL-1 and IL-6 levels, as well as in DC density after systemic therapy. The current study assesses and correlates bilateral tear cytokine concentrations and microscopic cellular findings of corneal sub-basal nerves and DCs in affected and clinically unaffected contralateral eyes of patients with unilateral BK and compares them to normal control eyes. The bilateral changes in tear cytokines in unilateral BK are in line with our previous studies, demonstrating bilateral changes in corneal nerves in patients with unilateral HSK and HZO.^{15,16} To date, to our knowledge, the underlying mechanism of the bilateral alterations in IVCM finding in unilateral clinical corneal diseases have not been elucidated.

Interestingly, our study demonstrates significant correlations of proinflammatory cytokines to increased DC density and alteration in their morphology as shown by IVCM, as well as correlations of upregulated tear cytokine levels in BK with reductions in corneal sub-basal nerve density. Antigen-presenting cells, most prominently dendritic cells, have a crucial role in the defense against foreign organisms through the secretion of cytokines, presentation of foreign antigens, pathogen clearance, and wound healing. During acute infections, various cytokines have been shown to increase in response to the tissue damage and pathologic antigens in the corneal epithelium.^{28,29} However, to our knowledge the tear cytokines in eyes with BK have not been investigated to date.

In the current study, bilateral increase in the concentration of TREM-1 is shown in patients with unilateral BK. The TREM-1, a molecule increased bilaterally in BK patients, is expressed at high levels on monocytes and macrophages, and functions as an amplifier of inflammation in tissues infected by bacteria or fungi. The TREM-1 can stimulate the production of proinflammatory cytokines, and stimulates rapid neutrophil degranulation and oxidative burst.³⁰ In contrast, while TREM-1 is not upregulated in noninfectious diseases, TREM-2 regulates DCs, microglia and osteoclast during inflammation in the central nervous system and rheumatoid arthritis.³⁰ During corneal infections, TREM-1 has been shown to be upregulated by *Pseudomonas aeruginosa* or lipopolysaccharides (LPS) in an animal model.³¹ Although there were no correlations between TREM-1 levels and DC parameters in our study, TREM-1 concentration was inversely correlated with corneal nerve density. The bilateral increase in TREM-1 concentration in tear samples of patients with unilateral BK, to our knowledge, is the first report on TREM-1 elevation in patients with ocular diseases.

The tear concentrations of IL-1 β , IL-6, and IL-8 were elevated only in the affected eyes with BK, but not in contralateral clinically unaffected eyes. Interleukin-1 β , which is elevated in our study, has been shown to induce additional proinflammatory mediators, such as IL-6, IL-8, FGF-2, prostaglandin E₂, and cyclooxygenase-2.^{32–34} Moreover, IL-1 β enhances host defense against infections, by augmenting antimicrobial function of macrophages and initiating T helper (Th) 1 and Th17 adaptive immune responses.³⁵ Interleukin-6, which also was increased in tears of patients with unilateral BK, is a major proinflammatory molecule in corneal wound healing,^{36–39} infection,^{28,29} inflammation,^{28,40} and corneal transplantation models.^{41,42} During inflammation, IL-6 stimulates the maturation and trafficking of DCs, promotes the differentiation of B cells, and IL-2 production by T cells, and, thus, mediates adaptive immune responses against pathogens.^{43,44} Both IL-1 β and IL-6 have been established as important mediators of fever induced by LPS from gram-negative bacteria⁴⁵ and are produced by epithelial cells⁴⁶ and plasmacytoid dendritic cells

(pDCs).⁴⁷ Interleukin-8 is an important inflammatory mediator during viral and bacterial infections,⁴⁸ and increased IL-8 has been reported with LPS stimulation in corneal epithelial cells.⁴⁹ As such, elevated IL-8 concentration in tear samples of the affected eyes would be expected as shown in the current study.

While the changes in tear cytokines and corneal DC density can be rationalized by infection-induced inflammation in affected eyes, alteration of cytokine concentrations in the contralateral clinically unaffected eyes is intriguing. Interestingly, IL-17a was only significantly elevated in the contralateral eyes, but not the affected BK eyes compared to controls. Interleukin-17a regulates prophylactic host defense against infection via Th17 cell responses, which improves the mucocutaneous barrier function. Interleukin-17 also stimulates the release of antimicrobial peptides and chemokines for neutrophil recruitment.³⁵ Recently, in response to IL-6 stimulation, a population of neutrophils have been shown to produce IL-17a in an autocrine manner to activate fibroblasts and epithelial cells to produce chemokines and proinflammatory cytokines, which leads to enhancing reactive oxygen species and antifungal activity.⁵⁰ Interleukin-17 expression was reported to be elevated by 2000-fold in human corneal tissues with filamentous fungal keratitis⁵¹; however, to our knowledge, there have been no reports on contralateral alterations of IL-17a concentrations in human samples or animal experiments. Clinically, the incidence of bilateral corneal infection is 1% to 3% and is relatively rare.^{52,53} It is tempting to speculate that the elevation of IL-17a in contralateral eyes in this study may potentially be due to prophylactic defense mechanisms in these eyes to prevent infection in the fellow eye.

Specific mechanisms have to be substantiated by comprehensive studies in animals and human, to determine the sequence of events that take place after BK with regards to tear cytokine increase and alterations in DC density and morphology. While some of the increased cytokines may be pathogen-specific, others may be upregulated nonspecifically as a response to inflammation. In this study, IL-7 was negatively correlated with DC field. Interleukin-7 has been proposed to be the primary driver of homeostatic T cell proliferation and also regulates the expression of major histocompatibility complex (MHC)-II or human leukocyte antigen (HLA)-DR on DCs via IL-7 receptor α signaling.⁵⁴ While resident central corneal DCs demonstrate negative to low expression of MHC-II expression during steady state, DCs are capable of upregulating MHC-II during inflammation and migrate to draining lymph nodes.⁵⁵ Correlation of tear IL-7 concentration with morphological changes of DCs, suggestive of DC activation in patients with BK, may result in the use of IL-7 as a primer for DC activation in other inflammatory ocular surface diseases.

Our study has several limitations. These include the lack of serum cytokine level measurements, the limited number of cases and the methodology of tear collection after instillation of saline. In systemic diseases, serum as well as local tissue cytokine/chemokine concentrations are found to be elevated, such as in dry eye disease with systemic disease^{56,57} and allergic conjunctivitis.²³ However, in local eye disease, such as in keratoconus,²⁰ serum cytokine levels remain within normal limits, while tear cytokine levels are elevated. In the case of infectious keratitis, the inflammation generally is limited to the eye.⁵⁸ However, given that we did not obtain serum levels, we cannot comment on systemic cytokine alterations after BK. Further, our tear samples were collected after instillation of 60 μ L of saline.²⁷ Thus, the cytokine levels in the current study reflected the total amount of cytokines on the ocular surface.

In conclusion, our study demonstrated that increased proinflammatory tear cytokines correlated with increased corneal DC density and size. Moreover, we demonstrated that

unilateral BK can result in bilateral alterations in proinflammatory tear cytokines, which potentially could result in the development of chronic bilateral ocular surface disease in patients with unilateral BK.

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