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Andrea Cruzat,[1,2] Wolfgang A. Schrems,[1] Laura M. Schrems-Hoesl,[1] Bernardo M. Cavalcanti,[1] Neda Baniasadi,[1] Deborah Witkin,[1] Deborah Pavan-Langston,[1] Reza Dana,[1] and Pedram Hamrah[1,3]

[1]Ocular Surface Imaging Center and Cornea & Refractive Surgery Service, Massachusetts Eye & Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, United States
[2]Department of Ophthalmology, Pontificia Universidad Católica de Chile, Santiago, Chile
[3]Boston Image Reading Center and Cornea Service, New England Eye Center, Department of Ophthalmology, Tufts Medical Center, Tufts University School of Medicine, Boston, Massachusetts, United States

Correspondence: Pedram Hamrah, Department of Ophthalmology, Tufts Medical Center, Boston, MA 02111, USA;
phamrah@tuftsmedicalcenter.org,
p_hamrah@yahoo.com.

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Purpose. To analyze the contralateral unaffected eyes of patients with microbial keratitis (MK) for any immune cell or nerve changes by laser in vivo confocal microscopy (IVCM).

Methods. A prospective study was performed on 28 patients with MK, including acute bacterial, fungal, and *Acanthamoeba* keratitis, as well as on their contralateral clinically unaffected eyes and on control groups, which consisted of 28 age-matched normal controls and 15 control contact lens (CL) wearers. Laser IVCM with the Heidelberg Retinal Tomograph 3/Rostock Cornea Module and Cochet-Bonnet esthesiometry of the central cornea were performed. Two masked observers assessed central corneal dendritiform cell density and subbasal corneal nerve parameters.

Results. The contralateral clinically unaffected eyes of patients with MK demonstrated significant diminishment in nerve density (15,603.8 ± 1265.2 vs. 24,102.1 ± 735.6 μm/mm^2), total number of nerves (11.9 ± 1.0 vs. 24.9 ± 1.2/frame), number of branches (1.7 ± 0.2 vs. 19.9 ± 1.3/frame), and branch nerve length (5775.2 ± 757.1 vs. 12,715.4 ± 648.4 μm/mm^2) ($P < 0.001$ for all parameters) compared to normal controls and CL wearers. Further, dendritiform cell density in the contralateral unaffected eyes was significantly increased as compared to that in controls (117.5 ± 19.9 vs. 24.2 ± 3.5 cells/mm^2, $P < 0.001$).

Conclusions. We demonstrate a subclinical involvement in the contralateral clinically unaffected eyes in patients with unilateral acute MK. In vivo confocal microscopy reveals not only a diminishment of the subbasal corneal nerves and sensation, but also an increase in dendritiform cell density in the contralateral unaffected eyes of MK patients. These findings show bilateral immune alterations in a clinically unilateral disease.

Keywords: microbial keratitis, in vivo confocal microscopy, dendritic cells, corneal nerves, contralateral

The in vivo study and assessment of corneal inflammation and innervation is important due to its clinical applicability in the management and treatment of ocular surface diseases.[1] Dendritic cells are the most potent antigen-presenting cells (APCs) of the body and mediators of both innate and adaptive immune responses. Given their strategic location in the epithelium and anterior stroma, they are ready to respond to invading pathogens in the cornea and ocular surface.[2,3] Corneal inflammation results in increased dendritic cell density, and their upregulation of major histocompatibility complex class (MHC)-II expression, as dendritic cells mature and become activated to generate immune responses.[3,4]

Until recently, the assessment of corneal inflammation has been possible only through slit-lamp biomicroscopic examination. However, the current availability of clinical in vivo confocal microscopy (IVCM) provides an opportunity to study corneal changes after microbial keratitis (MK) at a cellular level.[5–7] The use of this noninvasive imaging technique provides a resolution of images comparable to that with histochemical methods. In vivo confocal microscopy allows systematic studies of corneal epithelial, stromal, and endothelial cells and nerve morphology and density, as well as the study of immune cells such as corneal epithelial dendritiform cells in patients.[8–11] Increased corneal dendritiform cell density has been observed by IVCM in patients with dry eye,[9] with infectious keratitis,[5] in immune-mediated inflammatory diseases,[10] and in patients with pterygia.[11]

The role of corneal innervation is of great interest to clinicians and scientists due to its protective function on the ocular surface through regulation of corneal sensation and the blinking reflex, epithelial integrity, proliferation, and wound healing with the precise release of trophic factors.[12] Several recent studies on humans and animals have shown an association between corneal innervation and both apoptosis

and corneal stem cell homeostasis.[13] Specific changes in corneal nerve function and morphology after infection, corneal surgery, and other ocular or systemic pathologies (e.g., diabetes, dry eye disease, contact lens [CL] use, keratoconus, corneal dystrophies) have received increased attention in recent times.[1] Indeed, recently our group has shown that patients with unilateral herpes simplex keratitis (HSK)[6] and herpes zoster ophthalmicus (HZO)[7] have a significant bilateral loss of the corneal nerve plexus as compared to controls, demonstrating bilateral changes in clinically unilateral diseases. These findings were confirmed in an experimental study of trigeminal axotomy using a new mouse model for neurotrophic keratopathy, which demonstrated the loss of subbasal nerves in both the axotomized and contralateral eyes as soon as 24 hours after unilateral axotomy.[14] More recently, we demonstrated that patients with MK have a significant reduction in the subbasal corneal nerve plexus and a concurrent increase in dendritiform cell density.[5] Therefore, given our previously observed contralateral changes in corneal nerves in patients with HSK and HZO, we hypothesized that in nonherpetic MK, corneal nerve alterations in contralateral clinically unaffected eyes would be present and would be associated with concurrent increase in inflammatory cells, such as dendritiform cells.

Thus, the aim of the current study was to analyze the contralateral clinically unaffected eyes of patients with MK for corneal immune cell alterations and their correlation to subbasal corneal nerve changes by laser IVCM.

Patients and Methods

We performed a prospective, cross-sectional, controlled, single-blinded study. Twenty-eight patients with diagnosis of MK were included in the study. Both the affected and the contralateral clinically unaffected eyes were studied. Twenty-eight eyes of 28 normal volunteers constituted the control group after being screened and ruled out for dry eye disease or any other corneal pathology. Due to the high frequency of CL wear in the infectious keratitis patients, we also enrolled an additional age-matched control group of 15 bilateral soft CL wearers from the community. Patients were recruited from the Cornea & Refractive Surgery Service of the Massachusetts Eye & Ear Infirmary, Boston, Massachusetts, who presented with MK between 2009 and 2010. The study was Health Insurance Portability and Accountability Act compliant, conducted in accordance with the requirements of the Declaration of Helsinki, and approved by the Institutional Review Board/Ethics Committee from the institution. Participants signed a written informed consent. Detailed history was taken from the patients. The study excluded subjects with any other comorbidities such as history of previous infectious keratitis, ocular inflammatory disease, ocular trauma, ocular surgery, or diabetes. Patients receiving systemic corticosteroid therapy at the time of the examination were also excluded.

Slit-lamp biomicroscopy was performed on all subjects. Diagnosis of acute infectious keratitis was made according to the clinical history, clinical examination, and positive corneal cultures and/or positive confocal findings (in the case of fungal or *Acanthamoeba* keratitis). Duration of the infection was expressed from the time when patients presented with clinical signs and symptoms of infectious keratitis. Patients were followed at the Cornea Service, and none of them showed any clinical signs of infection in the contralateral eye during their follow-up.

Laser scanning IVCM with the Heidelberg Retina Tomograph 3/Rostock Cornea Module (HRT3/RCM) (Heidelberg Engineering GmbH, Heidelberg, Germany) was performed on the central cornea of both eyes of all MK patients and in one randomly selected eye of normal controls as previously described.[5] The HRT3/RCM uses as a diode laser with a wavelength of 670 nm, providing images that represent a corneal section of 400 × 400 μm. Adjacent images are separated by approximately 1 to 4 μm and have a lateral resolution of 1 μm/pixel. Acquired images are 384 × 384 pixels in size and are stored at a speed of 30 frames per second. The distance from the cornea to the microscope is kept stable by a single-use disposable sterile polymethylmethacrylate cap (Tomo-Cap; Heidelberg Engineering GmbH), filled with a layer of hydroxypropyl methylcellulose 2.5% (GenTeal gel; Novartis Ophthalmics, Basel, Switzerland). Both eyes are instilled with topical anesthesia 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX, USA) followed by a drop of the lubricant hydroxypropyl methylcellulose 2.5% (GenTeal gel; Novartis Ophthalmics). Another drop of the lubricant is applied on the outside tip of the Tomo-Cap to improve and facilitate the optical coupling to the examined eye; then the microscope is manually advanced until the gel has contacted the central surface of the cornea. A total of four to eight scans in sequence and automatic volume scan mode were obtained in the center of each cornea with special emphasis on epithelial dendritiform cells at the level of basal epithelial cells, on the subbasal nerve plexus, Bowman's layer, and anterior stroma at depths from 20 to 100 μm. When a corneal ulcer was present with an epithelial defect in the central cornea, both the ulcer and the surrounding area were scanned and analyzed. The scans yielded at least 100 to 300 images of the subbasal layer.

Central corneal sensitivity was evaluated with the Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres, France) on all patients and controls as previously described.[6] In brief, this test mechanically stimulates corneal nerves by pressing a retractable 6-cm-long monofilament nylon thread of 0.12-mm diameter against the anterior corneal surface, shortening in steps of 1.0 cm if a positive response is not obtained. If a positive response is obtained, the thread is advanced by 0.5 cm. The longest filament length resulting in a positive response was considered the corneal sensitivity threshold, which was verified twice.

Image Analysis

An experienced masked observer selected three representative images for corneal subbasal nerves and three for dendritiform cell analysis from the central cornea of each eye from at least 50 good-quality images from each cornea that satisfied the criteria for selection. The best-focused complete images, with the whole image in the same layer and good contrast, were chosen from the basal epithelial layer and anterior to the Bowman's layer. Epithelial dendritiform cell density and the corneal subbasal nerve plexus were quantified by two masked observers with the National Institutes of Health free software, ImageJ (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; available at http://rsb.info.nih.gov/ij/ [in the public domain]) and the plug-in NeuronJ (http://www.imagescience.org/meijering/software/neuronj/ [in the public domain]).[15]

Main nerve trunks were quantified by measuring their length and the total number of main trunks in one image (Fig. 1E). Nerve branches were calculated by measuring total length and the number of branches emerging from the main trunks in one image. Total nerve density was assessed by measuring the total length of all the nerve fibers in one frame in micrometers per millimeter squared (μm/mm^2), and the number of total nerves was defined as the absolute count of all nerves, including main nerve trunks and branches in one image (Fig.

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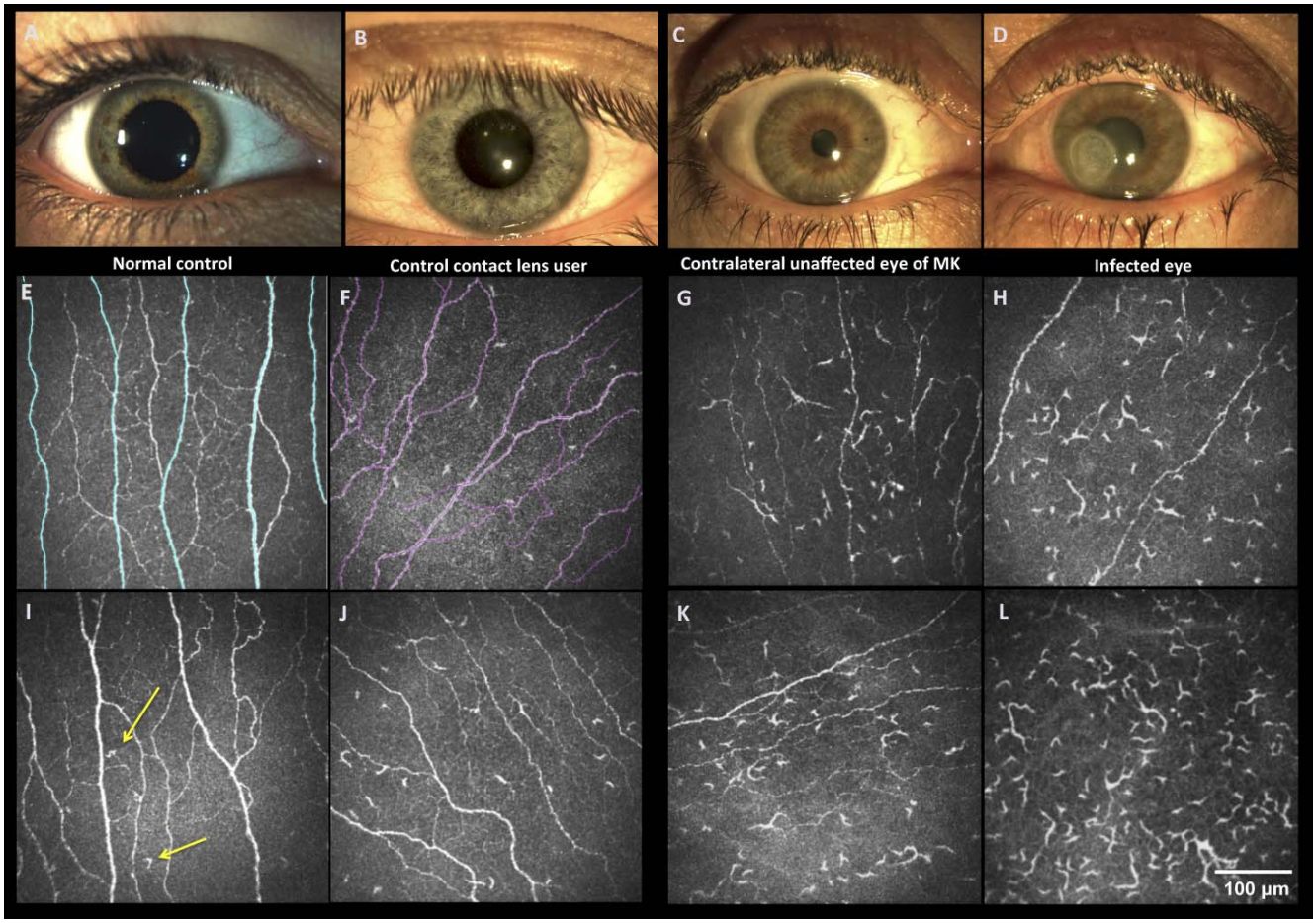

FIGURE 1. Corneal subbasal nerve plexus and dendritiform cells. (**A**) Slit-lamp photo of normal control. (**B**) Slit-lamp photo of control contact lens user. (**C**) Slit-lamp photo of contralateral clinically unaffected eye. (**D**) Slit-lamp photo of fungal keratitis. (**E–H**) In vivo confocal microscopy (IVCM) images of subbasal corneal nerve plexus in each group. (**E**) IVCM image from normal control. NeuronJ tracings of main nerve trunks of the subbasal nerve plexus. (**F**) NeuronJ tracings of total nerves, including main nerve trunks and branches of the subbasal nerve plexus in a control contact lens user. (**I–L**) Representative IVCM images of epithelial dendritiform cells (shown with *arrows*) in each group. (**L**) IVCM images from eye with fungal infection. Reduction of the corneal subbasal nerve plexus with increased density of the dendritiform cells is observed in the infected and the contralateral clinically unaffected eye of patients with microbial keratitis.

1F). Dendritiform cell quantification (cells/mm^2) was performed using Cell Count software (Heidelberg Engineering GmbH) in the manual mode by identifying bright individual dendritiform structures with cell bodies in each image at the level of basal epithelium or subbasal nerve plexus. For cells that overlapped with the edge of the frame, the observers assessed two sides of the image from which to include cells.

Statistical Analysis

We analyzed results with Student's *t*-test, analysis of variance (ANOVA), Pearson correlation coefficient, and linear regression analysis. We used Bonferroni for adjustment for multiple comparisons. Differences were considered statistically significant for P values less than 0.05. Means and standard error of mean are reported for the parameters analyzed. Analyses were performed with STATA software (version 11, 2009; StataCorp LP, College Station, TX, USA).

A priori power analysis was conducted using data from a previous study in MK[5] to estimate a sufficient sample size to achieve a power of 80% and alpha error of 0.05. Post hoc power analysis was conducted after the study was completed to determine the power of the study, confirming the appropriate sample size for the study with a power of more than 98% for statistically significant variables.

RESULTS

We analyzed the contralateral clinically unaffected eyes and the infected eyes of 28 patients with MK, including bacterial (n = 15), fungal (n = 8), and *Acanthamoeba* (n = 5) keratitis. Results were compared to a control group of 28 normal eyes and to a second control group of 15 eyes of 15 CL users. Demographic data of patients and controls are presented in Table 1. There was no significant difference in age between the groups (patients versus controls P = 0.17; patients versus control CL users P = 0.24).

Dendritiform Cells by IVCM

Quantitative analysis of dendritiform cell density for all groups is shown in Table 2. In the contralateral clinically unaffected eyes of patients with MK, the increase in epithelial dendritiform cell density was significantly higher as compared to both normal controls and control CL users (117.5 ± 19.9 vs. 24.2 ± 3.5 vs. 33.0 ± 7.8 cells/mm^2; $P < 0.001$) (Figs. 1, 2). The mean epithelial dendritiform cell density was also significantly higher

Table 1. Demographic Data of Controls and Patients With Microbial Keratitis

	Controls	Control CL Users	MK Affected Eye	Contralateral Eye
Sample size, *n*	28	15	28	28
Age, y	44.1 ± 2.7	32.5 ± 2.1	38.4 ± 3.2	38.4 ± 3.2
Sex, male/female	7/21	5/10	16/12	16/12
Central corneal sensation, 0–6 cm	6.0 ± 0.0	6.0 ± 0.0	3.8 ± 0.4*	5.4 ± 0.2
MK etiology, bacterial/fungal/*Acanthamoeba*	n/a	n/a	15/8/5	n/a
MK onset, d	n/a	n/a	20.7 ± 5.3	n/a

Statistical analysis performed by ANOVA with Bonferroni correction for multiple comparisons. Values reported as mean ± standard error. *P* value for age of patients versus controls, $P = 0.17$; and versus controls CL users, $P = 0.24$. n/a, not applicable.

* Statistically significant $P < 0.001$.

in the affected eye of patients with MK as compared to both normal controls and control CL users (384.4 ± 55.8 cells/mm^2; $P < 0.001$).

When patients are grouped by different infectious etiologies (bacterial versus fungal versus *Acanthamoeba*), no significant differences in dendritiform cell density were observed in the contralateral clinically unaffected eyes between groups ($P = 0.91$). The difference in mean dendritiform cell density between normal controls and control CL users was not significant ($P = 0.32$). The intraclass correlation coefficient for the two masked observers for the dendritiform cell density parameters was 0.99.

Corneal Subbasal Nerve Plexus by IVCM

A summary of corneal nerve parameters for the eyes with MK, their contralateral clinically unaffected eyes, and the normal control and the control CL users is reported in Table 2. Contralateral clinically unaffected eyes of patients with MK showed a significant reduction in the corneal subbasal nerve plexus parameters when compared to controls and to control CL users, albeit less in magnitude than the ipsilateral nerve changes (Fig. 1).

Contralateral clinically unaffected eyes of MK patients demonstrated a significant diminishment of corneal nerves as compared to both control groups, including total nerve length ($P < 0.001$) and total nerve number ($P < 0.001$) and, predominantly, the length of nerve branches ($P < 0.001$) and number of branches ($P < 0.001$) (Fig. 3). Nerve branches in the contralateral eyes were as severely affected as in the infected eyes, with no significant difference between these two groups ($P = 0.5$). We did not find a significant decrease in main nerve trunks in the contralateral clinically unaffected eye as compared to both control groups ($P = 0.3$).

As expected, the ipsilateral nerve changes were even more dramatic. We found a significant diminishment of the corneal nerves in the infected eyes as compared to both normal controls and control CL users, including decreased nerve density expressed as total nerve length and total number ($P < 0.001$), main nerve trunk length and number ($P < 0.001$), and branch length and number ($P < 0.001$), respectively (Fig. 3). The comparison of patients with different infectious etiology (bacterial versus fungal versus *Acanthamoeba* keratitis) did not show significant differences in subbasal nerve density in the contralateral clinically unaffected eye ($P = 0.59$).

Corneal sensation of the contralateral clinically unaffected eyes, as measured by the Cochet-Bonnet esthesiometer, was decreased (5.4 ± 0.2 cm) as compared to that in both normal controls (6.0 ± 0.0 cm) and control CL users (6.0 ± 0.0), although this was not as noteworthy ($P = 0.64$) as the reduction of corneal sensitivity in the affected eyes (3.8 ± 0.4 cm; $P < 0.001$) (Fig. 4).

The intraclass correlation coefficient for the two masked observers for the nerve parameters ranged between 0.75 and 0.96.

Correlation and Regression Analysis

Pairwise correlations and regression analysis were used to correlate the corneal morphologic features observed by IVCM with clinical or functional parameters. We observed a significant linear correlation between the increase in dendritiform cell density and the reduction in the total nerve length of the subbasal nerve plexus ($R = –0.63$; $P < 0.001$) (Fig. 5). Further, both the increase in dendritiform cell density and the reduction in subbasal corneal nerves were significantly correlated to the duration of the infection ($R = 0.53$; $P < 0.001$; $R = –0.41$, $P < 0.001$, respectively). We did not observe a significant correlation between dendritiform cell density and age ($P = 0.087$). As expected, we found a significant correlation between corneal nerve reduction and the corneal sensitivity decrease in patients with MK ($R = 0.65$, $P = 0.001$).

Discussion

In the current study we demonstrate, through laser IVCM, that patients with MK not only have a significant increase in immune dendritiform cell density in the infected eyes, but also demonstrate these changes in the contralateral clinically unaffected eyes when compared to controls. To our knowledge, this is the first study showing immune cell alterations in the contralateral clinically unaffected eyes in MK.

Nerve damage in infected eyes could be related to the level of inflammation or directly related to the infectious pathogens, where fungi, *Acanthamoeba*, or bacteria could destroy nerves. Previous studies have shown that *Acanthamoeba* trophozoites are able to destroy nerves in vitro both by cytolysis and by ingestion.[16] In addition, the pharmacotherapy for MK is usually prolonged and toxic.[17–19] Berry et al.[18] have shown that exposure to antibiotics, antifungals, or their combinations results in increased drug-dependent toxicity. Thus, it is conceivable that the nerve damage observed in the affected eye could be due to the use of numerous combinations of antimicrobial eye drops by patients with MK. However, the underlying mechanisms that result in decreased nerve density and increased immune cell density in contralateral clinically unaffected eyes remain elusive.

Tissues exposed to the external environment, such as epithelial surfaces of the skin, respiratory, urinary, digestive tracts and the cornea, are densely innervated by nociceptors, transducing the noxious stimuli instantaneously and several orders of magnitude faster than the innate immune system, to be the first responder in host defense.[20] The axonal reflex, that is, the antidromic action potentials that are transmitted back down to the periphery, result in a local release of neural

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Table 2. Dendritiform Cell Density and Corneal Subbasal Nerve Plexus Parameters in Control Groups, Contralateral Unaffected Eyes, and Affected Eyes With Infectious Keratitis

	Dendritiform Cell Density, Cells/mm²	Total Nerve Length, µm/mm² [µm/Frame]	Main Trunk Length, µm/mm² [µm/Frame]	Branch Length, µm/mm² [µm/Frame]	No. of Total Nerves, *n*/Frame	No. of Main Trunks, *n*/Frame	No. of Branches, *n*/Frame
Normal control	24.2 ± 3.5	24,102.1 ± 735.6 [3,856.3 ± 117.7]	11,386.7 ± 330 [1,821.9 ± 52.8]	12,715.4 ± 648.4 [2,034.5 ± 103.7]	24.9 ± 1.2	5.0 ± 0.2	19.9 ± 1.3
Control CL users	33.0 ± 7.8	23,716.6 ± 754.6 [3,794.6 ± 120.7]	11,798.3 ± 468.5 [1,887.7 ± 75]	11,918.3 ± 700 [1,906.9 ± 112]	23.5 ± 1.3	5.0 ± 0.2	18.6 ± 1.3
Contralateral unaffected eye	117.5 ± 19.9*†‡	15,603.8 ± 1,265.2*†‡ [2,496.6 ± 202.4]	9,828 ± 745.5‡ [1,572.5 ± 119.3]	5,775.2 ± 757.1*†‡ [924.0 ± 121.1]	11.9 ± 1.0*†‡	4.8 ± 0.3‡	1.7 ± 0.2*†
Contralateral bacterial	125.8 ± 25.8‡	14,520.3 ± 1,664.8*†‡ [2,323.2 ± 266.4]	9,542.3 ± 993.3‡ [1,526.8 ± 158.9]	4,978 ± 840*‡ [796.5 ± 134.4]	11.7 ± 1.5*†‡	4.7 ± 0.4‡	1.6 ± 0.3*†
Contralateral *Acanthamoeba*	111.9 ± 23‡	18,047.2 ± 3,870.2‡ [2,887.6 ± 619.2]	9,994.1 ± 2,028‡ [1,599.1 ± 324.5]	8,053.1 ± 3,100.3‡ [1,288.5 ± 496]	12.8 ± 1.8*†‡	5.3 ± 0.8‡	2.1 ± 0.5*†
Contralateral fungal	105.4 ± 51‡	16,108.1 ± 2,232.2*†‡ [2,577.3 ± 357.2]	10,262.1 ± 1,510.7‡ [1,641.9 ± 241.7]	5,846.1 ± 1,012.6*† [935.4 ± 162]	11.8 ± 1.8*†‡	4.6 ± 0.7‡	1.7 ± 0.4*†
MK affected eye	384.4 ± 55.8*	5,939.3 ± 1,248.1* [950.3 ± 199.7]	4,029.1 ± 814.4* [644.7 ± 130.3]	1,910.2 ± 504.5* [305.6 ± 80.7]	4.1 ± 0.9*	2.2 ± 0.5*	1.4 ± 0.4*

Statistical analysis performed by ANOVA with Bonferroni correction for multiple comparisons. Values reported as mean ± standard error.

* $P < 0.008$ in comparison to normal controls.

† $P < 0.03$ in comparison to control CL users.

‡ $P < 0.02$ in comparison to affected eye.

Figure 2. Dendritiform cell density in contralateral clinically unaffected eyes in microbial keratitis (MK) patients, their infected eye, normal controls, and control contact lens (CL) users. Contralateral unaffected eyes of patients with MK had significantly higher epithelial dendritiform cell density compared to normal controls and to control contact lens users. *Error bars* represent standard error from the mean. *$P < 0.0001$ compared to control group. ₸$P < 0.001$ compared to control group contact lens user. +$P < 0.001$ compared to infected eye. Statistical analysis performed by ANOVA with Bonferroni correction for multiple comparisons.

mediators. This "neurogenic inflammation" corresponds to the inflammatory symptoms that result from the release of bioactive substances from primary sensory nerve terminals that have been activated. The release of sensory neuropeptides, particularly substance P (SP) and vasoactive intestinal peptide (VIP), and the neuroimmune interactions can play a major role in the pathophysiology of allergic, inflammatory, and autoimmune diseases by amplifying pathologic immune responses.[21,22] The release of neuropeptides such as SP and calcitonin gene-related peptide (CGRP) is a major initiator of neurogenic inflammation by acting on target cells in the periphery, such as vascular endothelial cells, smooth muscle cells, mast cells, and immune cells, releasing additional neuropeptides, nitric oxide, and cytokines,[20] leading to inflammation, which is characterized by vasodilation, swelling, and hypersensitivity.[23] Several findings support that sensory neuropeptides in the periphery not only act in the vasculature but also can directly attract and activate humoral components of inflammation, innate, and adaptive immune cells.[23–25] Anatomical studies have shown a direct apposition of nerve terminals to dendritic cells. Release of neuropeptides such as CGRP and VIP can induce degranulation or cytokine production,[25] altering dendritic cell function toward certain T helper cell responses by promoting the production of cytokines.[24]

Although it may be surprising to find increased dendritiform cells and decreased nerves in the contralateral unaffected eye, there is previous evidence of bilateral nerve changes in unilateral diseases at the eye level, but not of immune cell changes as found in the current study. Our group has previously reported bilateral changes in corneal nerves after unilateral HSK[6] and HZO.[7] In the latter, we discuss that bilateral nerve alterations in unilateral HZO cases could be explained by central nervous system (CNS)-mediated contralateral effects as has been previously shown in contralateral undamaged areas after peripheral nerve lesions.[26–28] In an extensive review, Koltzenburg et al.[26] discuss several non-ophthalmic studies, which have demonstrated that various unilateral diseases or peripheral unilateral nerve lesions can

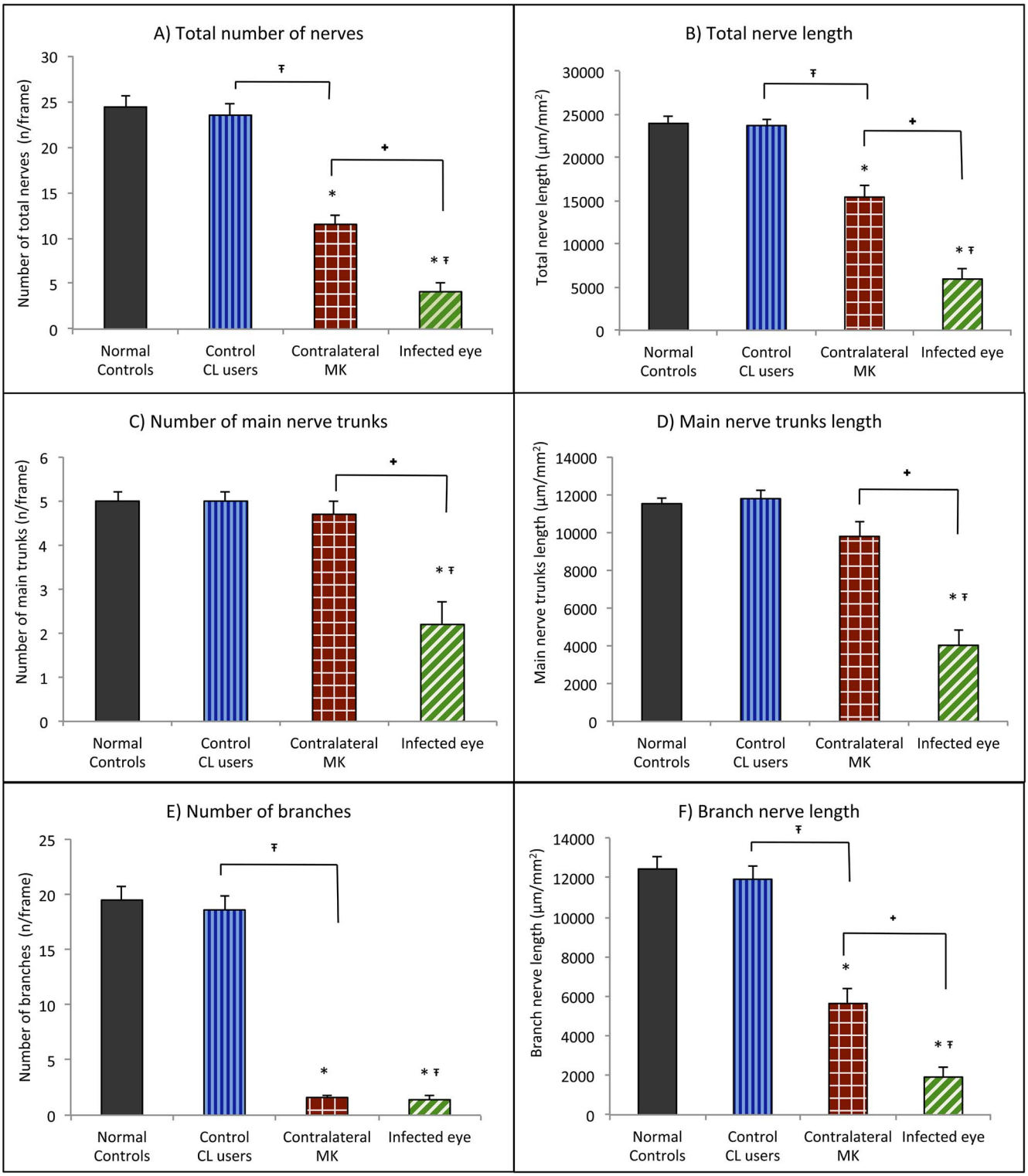

Figure 3. Comparison of subbasal corneal nerve alterations in contralateral clinically unaffected eyes in microbial keratitis (MK) patients, the infected eyes, normal controls, and control contact lens (CL) users. Contralateral unaffected eyes of patients with MK showed statistically significant diminishment of most of the corneal nerve parameters compared to normal controls and control contact lens users. Affected eyes, as expected, showed a bigger decrease in nerve parameters as compared to controls. (**A**) Total number of nerves. (**B**) Total nerve length. (**C**) Number of main nerve trunks. (**D**) Main nerve trunk length. (**E**) Number of branches. (**F**) Branch nerve length. *Error bars* represent standard error of mean. $^{*}P < 0.001$ compared to normal control group. $\mp P < 0.001$ compared to control group contact lens (CL) user. $+P < 0.001$ compared to affected eye. Statistical analysis performed by ANOVA with Bonferroni correction for multiple comparisons.

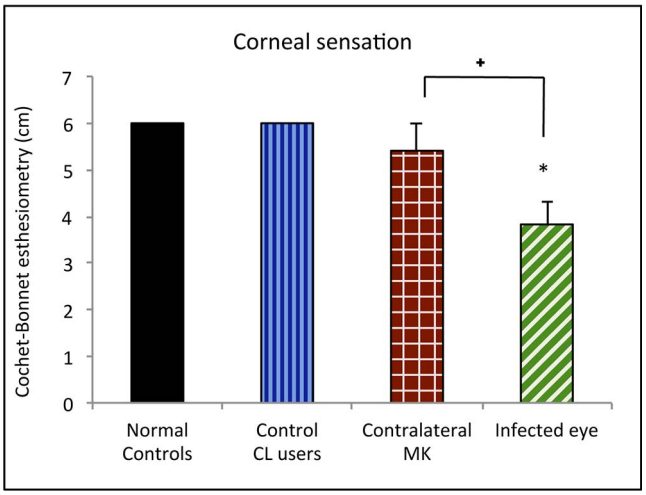

Figure 4. Corneal sensation by Cochet-Bonnet esthesiometry in contralateral clinically unaffected eyes in microbial keratitis (MK) patients, infected eyes, normal controls, and control contact lens (CL) users. Contralateral unaffected eyes of patients with MK had decreased corneal sensation compared to normal controls and to control contact lens (CL) users ($P = 0.64$), but this was not as noteworthy as in the infected eye. *Error bars* represent standard error of mean. $^{*}P < 0.001$ compared to normal control group and contact lens (CL) user. $+P < 0.001$ compared to infected eye. Statistical analysis performed by ANOVA with Bonferroni correction for multiple comparisons.

cause bilateral, topographically precise responses. The evidence of contralateral mirror responses is seen not only with neuronal injuries, but also in inflammatory lesions and responses in both animal models and in early reports in humans.[27] In addition, Streilein et al.[29] demonstrated that after intraocular inoculation of live virus in mice, the virus was found in both sides of the brainstem, as well as in the contralateral noninoculated eye of the mice. In line with these data, studies have shown that unilateral herpes infection can cause dryness in both eyes.[30] Finally our group recently demonstrated bilateral nerve alterations in an experimental unilateral neurotrophic keratopathy model by trigeminal axotomy in mice as soon as 24 hours after unilateral axotomy.[14]

Our study, to our knowledge, is the first to demonstrate a contralateral immune or inflammatory response following a unilateral corneal insult in patients. Laser IVCM reveals the presence of corneal epithelial dendritiform cells in both the infected and the contralateral eye. We cannot, however, categorically define dendritiform cells as dendritic cells. Our identification is based on morphologic features of these cells, as we are unable to use phenotypic markers due to the in vivo nature of this study. While other cells, such as macrophages, can have a dendritic morphology as well, previous studies have shown that bone marrow–derived cells present in the corneal epithelium are predominantly dendritic cells. Previous studies in mice[3,31,32] and human corneas[8,33,34] have confirmed this. Moreover, according to a recent paper by Knickelbein et al.,[35] the immune cells situated in the basal corneal epithelium are exclusively $CD11c^{+}$ dendritic cells and $CD207^{+}$ Langerhans cells. Moreover, Guthoff et al.[36] described the different types of inflammatory cells based on morphology with the laser confocal microscope and parallel studies with immunohistochemical staining, showing that cell morphology, diameter of the cell body, and location of the cell aid the clinician in the correct interpretation of the confocal data.[36]

Contralateral responses in unilateral inflammatory lesions, showing symmetrical inflammatory changes, have previously been shown in rheumatologic diseases.[27] Animal models have shown that inflammatory mediators can induce contralateral pain in symmetric areas after sciatic nerve damage.[28] Kidd et al.[37] induced a monoarthritis using latex spheres injected into the knee of male rats and showed that contralateral knee cellular infiltrates, mainly monocytes, were significantly elevated when compared with control animals and joints elsewhere in the affected rat. In addition, Bileviciute et al.[38] demonstrated neuropeptide release from the contralateral joint

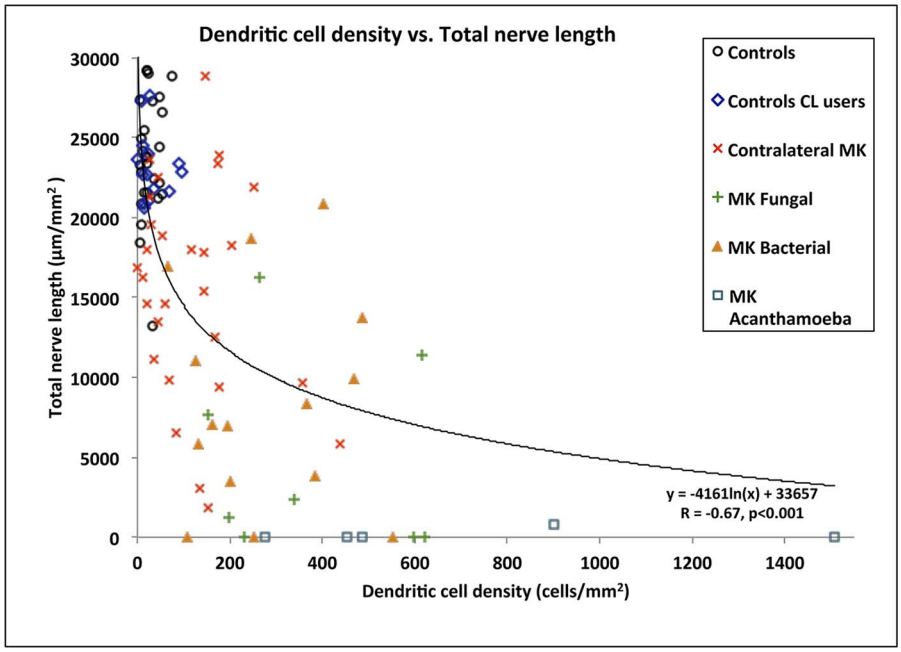

Figure 5. Correlation between dendritiform cell density increase and corneal nerve diminishment per group. A linear model between total nerve length and the log of dendritiform cell density was assumed. R^2 and P values are shown, indicating a significant correlation. Statistical analysis performed by Pearson correlation and linear regression analysis.

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of rats following a monoarthritis with no signs of systemic inflammation. These contralateral responses were reduced in magnitude compared with the ipsilateral side, similar to what was seen in the current study.[38] Nevertheless, although all contralateral eyes were clinically unaffected, one cannot exclude the possibility of subclinical infections in the contralateral eyes of acute MK patients, which could theoretically result in subbasal corneal nerve diminishment and increased dendritiform cell density. However, this possibility is remote as no signs of fungal and *Acanthamoeba* elements were detected in any patients by IVCM, and the corneas remained completely clear by clinical examination.

Theoretically, it is possible that some of the increase in dendritiform cell density and corneal nerve alterations may be due to CL use alone and not due to the neurogenic inflammation triggered by infectious keratitis, particularly in the contralateral eyes. Previous studies have shown that long-term CL wear does not appear to affect corneal nerve density, distribution, or morphology.[39,40] However, Zhivov et al.[41] demonstrated a minimal increase in dendritiform cell density in the central cornea of CL compared to normal control eyes (78 $\pm$ 25 vs. 34 $\pm$ 3 cells/mm^2), but they also showed that dendritiform cell density significantly decreased with the duration of CL use. Nevertheless, in order to rule out this possible effect of CL use on contralateral eyes, we included a second control group of CL users. In the current study the dendritiform cell density in the control group of CL users was significantly lower than the dendritiform cell density noted both in the infected eye and in the contralateral unaffected eyes, suggesting that alterations observed in contralateral eyes are likely due to the infectious/inflammatory process and not due to CL use.

Previous studies have shown corneal nerve reduction in MK patients but have not demonstrated functional nerve changes or decreased corneal sensation after infectious keratitis.[5] Our study demonstrates a significant reduction in corneal sensitivity in the affected eyes of MK patients, a novel finding in MK that is not due to HSV or HZO. Moreover, our results show a significant positive correlation between corneal sensitivity loss and corneal nerve damage. Interestingly, although the diminishment of the subbasal nerve plexus was noted in both the affected and contralateral clinically unaffected eyes of patients with MK, corneal sensitivity diminishment reached statistically significance only in the infected eye. This could be explained by the fact that the magnitude of the nerve loss in the contralateral unaffected eyes was not as severe as in the eyes affected with acute MK. We have demonstrated in previous studies of HSK patients[6] and HZO patients[7] that the threshold of total nerve length for a patient to have normal sensation is only approximately 1000 μm/frame (or 7490 μm/mm^2) by slit-scanning confocal microscopy: That is, the fact that abnormal sensation is noted by Cochet-Bonnet esthesiometer only with a substantial nerve reduction could explain why the sensation in the contralateral clinically unaffected eyes was perceived as normal despite a significant decrease in corneal innervation. Another possible explanation is that the Cochet-Bonnet esthesiometer, although considered the gold standard for measuring corneal sensation, stimulates only mechanical nociceptors; it is a very gross method of measuring corneal sensitivity and is less sensitive than measurements with the Belmonte esthesiometer.[42] For example, a study by Gallar et al.[43] performed with the Belmonte esthesiometer in the contralateral eye of patients with HSK infection showed a slightly decreased corneal sensitivity that did not reach statistical significance, but a significantly decreased heat sensation.[43]

In conclusion, a coordinated bilateral interaction between the nervous system and the immune system takes place during unilateral corneal diseases. These systems share many similar molecular recognition pathways and can directly communicate with one another to have an integrated protective role. In the study presented herein, we demonstrate a sympathetic subclinical involvement in the contralateral clinically unaffected eye in patients with unilateral acute MK. In vivo confocal microscopy reveals not only a diminishment of the subbasal corneal nerves and sensation, but also an increase in dendritiform cell density in the contralateral unaffected eyes of MK patients. These contralateral responses may be important confounders in clinical trials, where investigators should use the contralateral clinically unaffected eye as control with caution. Moreover, if the bilateral increase in dendritiform cells can be seen as a sign of inflammation or immune activation, patients with unilateral disease would be at risk of developing bilateral ocular surface disease. However, long-term implications of the contralateral sympathetic immune response remain unclear. Thus, future longitudinal studies are required to assess the persistence or resolution of these changes, to elucidate whether they are disease specific, and to reveal the mechanisms and consequences of this phenomenon. Since these patients had localized infections, it would be particularly interesting to perform an assessment of the systemic immune response in future studies.

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References

1. Cruzat A, Pavan-Langston D, Hamrah P. In vivo confocal microscopy of corneal nerves: analysis and clinical correlation. *Semin Ophthalmol*. 2010;25:171–177.
2. Hamrah P, Huq SO, Liu Y, Zhang Q, Dana MR. Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. *J Leukoc Biol*. 2003;74:172–178.
3. Hamrah P, Zhang Q, Liu Y, Dana MR. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci*. 2002; 43:639–646.
4. Hamrah P, Liu Y, Zhang Q, Dana MR. Alterations in corneal stromal dendritic cell phenotype and distribution in inflammation. *Arch Ophthalmol*. 2003;121:1132–1140.
5. Cruzat A, Witkin D, Baniasadi N, et al. Inflammation and the nervous system: the connection in the cornea in patients with infectious keratitis. *Invest Ophthalmol Vis Sci*. 2011;52:5136–5143.
6. Hamrah P, Cruzat A, Dastjerdi MH, et al. Corneal sensation and subbasal nerve alterations in patients with herpes simplex keratitis: an in vivo confocal microscopy study. *Ophthalmology*. 2010;117:1930–1936.
7. Hamrah P, Cruzat A, Dastjerdi MH, et al. Unilateral herpes zoster ophthalmicus results in bilateral corneal nerve alteration: an in vivo confocal microscopy study. *Ophthalmology*. 2013;120:40–47.
8. Mayer WJ, Mackert MJ, Kranebitter N, et al. Distribution of antigen presenting cells in the human cornea: correlation of in

vivo confocal microscopy and immunohistochemistry in different pathologic entities. *Curr Eye Res*. 2012;37:1012–1018.

9. Lin H, Li W, Dong N, et al. Changes in corneal epithelial layer inflammatory cells in aqueous tear-deficient dry eye. *Invest Ophthalmol Vis Sci*. 2010;51:122–128.
10. Mastropasqua L, Nubile M, Lanzini M, et al. Epithelial dendritic cell distribution in normal and inflamed human cornea: in vivo confocal microscopy study. *Am J Ophthalmol*. 2006;142:736–744.
11. Wang Y, Zhao F, Zhu W, Xu J, Zheng T, Sun X. In vivo confocal microscopic evaluation of morphologic changes and dendritic cell distribution in pterygium. *Am J Ophthalmol*. 2010;150:650–655.
12. Müller LJ, Marfurt CF, Kruse F, Tervo TMT. Corneal nerves: structure, contents and function. *Exp Eye Res*. 2003;76:521–542.
13. Ueno H, Ferrari G, Hattori T, et al. Dependence of corneal stem/progenitor cells on ocular surface innervation. *Invest Ophthalmol Vis Sci*. 2012;53:867–872.
14. Yamaguchi T, Turhan A, Harris DL, et al. Bilateral nerve alterations in a unilateral experimental neurotrophic keratopathy model: a lateral conjunctival approach for trigeminal axotomy. *PLoS One*. 2013;8:e70908.
15. Meijering E, Jacob M, Sarria J-CF, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry A*. 2004;58:167–176.
16. Pettit DA, Williamson J, Cabral GA, Marciano-Cabral F. In vitro destruction of nerve cell cultures by Acanthamoeba spp.: a transmission and scanning electron microscopy study. *J Parasitol*. 1996;82:769–777.
17. Johns KJ, Head WS, O'Day DM. Corneal toxicity of propamidine. *Arch Ophthalmol*. 1988;106:68–69.
18. Berry M, Gurung A, Easty DL. Toxicity of antibiotics and antifungals on cultured human corneal cells: effect of mixing, exposure and concentration. *Eye (Lond)*. 1995;9(pt 1):110–115.
19. Lee J-E, Oum BS, Choi HY, Yu HS, Lee JS. Cysticidal effect on acanthamoeba and toxicity on human keratocytes by polyhexamethylene biguanide and chlorhexidine. *Cornea*. 2007;26:736–741.
20. Chiu IM, von Hehn CA, Woolf CJ. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. *Nat Neurosci*. 2012;15:1063–1067.
21. Agro A, Stanisz AM. Neuroimmunomodulation: classical and non-classical cellular activation. *Adv Neuroimmunol*. 1995;5:311–319.
22. Wallengren J, Ekman R, Möller H. Substance P and vasoactive intestinal peptide in bullous and inflammatory skin disease. *Acta Derm Venereol*. 1986;66:23–28.
23. Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience*. 1988;24:739–768.
24. Ding W, Stohl LL, Wagner JA, Granstein RD. Calcitonin gene-related peptide biases Langerhans cells toward Th2-type immunity. *J Immunol*. 2008;181:6020–6026.
25. Hosoi J, Murphy GF, Egan CL, et al. Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. *Nature*. 1993;363:159–163.
26. Koltzenburg M, Wall PD, McMahon SB. Does the right side know what the left is doing? *Trends Neurosci*. 1999;22:122–127.
27. Shenker N, Haigh R, Roberts E, Mapp P, Harris N, Blake D. A review of contralateral responses to a unilateral inflammatory lesion. *Rheumatology (Oxford)*. 2003;42:1279–1286.
28. Twining CM, Sloane EM, Milligan ED, et al. Peri-sciatic proinflammatory cytokines, reactive oxygen species, and complement induce mirror-image neuropathic pain in rats. *Pain*. 2004;110:299–309.
29. Streilein JW, Dana MR, Ksander BR. Immunity causing blindness: five different paths to herpes stromal keratitis. *Immunol Today*. 1997;18:443–449.
30. Simard-Lebrun A, Boisjoly H, Al-Saadi A, Choremis J, Mabon M, Chagnon M. Association between unilateral quiescent stromal herpetic keratitis and bilateral dry eyes. *Cornea*. 2010;29:1291–1295.
31. Nakamura T, Ishikawa F, Sonoda K-H, et al. Characterization and distribution of bone marrow-derived cells in mouse cornea. *Invest Ophthalmol Vis Sci*. 2005;46:497–503.
32. Knickelbein JE, Watkins SC, McMenamin PG, Hendricks RL. Stratification of antigen-presenting cells within the normal cornea. *Ophthalmol Eye Dis*. 2009;1:45–54.
33. Yamagami S, Yokoo S, Usui T, Yamagami H, Amano S, Ebihara N. Distinct populations of dendritic cells in the normal human donor corneal epithelium. *Invest Ophthalmol Vis Sci*. 2005;46:4489–4494.
34. Mayer WJ, Irschick UM, Moser P, et al. Characterization of antigen-presenting cells in fresh and cultured human corneas using novel dendritic cell markers. *Invest Ophthalmol Vis Sci*. 2007;48:4459–4467.
35. Knickelbein JE, Buela K-A, Hendricks RL. Antigen-presenting cells are stratified within normal human corneas and are rapidly mobilized during ex vivo viral infection. *Invest Ophthalmol Vis Sci*. 2014;55:1118–1123.
36. Guthoff RF, Zhivov A, Stachs O. In vivo confocal microscopy, an inner vision of the cornea - a major review. *Clin Experiment Ophthalmol*. 2009;37:100–117.
37. Kidd BL, Cruwys SC, Garrett NE, Mapp PI, Jolliffe VA, Blake DR. Neurogenic influences on contralateral responses during experimental rat monoarthritis. *Brain Res*. 1995;688:72–76.
38. Bileviciute I, Lundeberg T, Ekblom A, Theodorsson E. Bilateral changes of substance P-, neurokinin A-, calcitonin gene-related peptide- and neuropeptide Y-like immunoreactivity in rat knee joint synovial fluid during acute monoarthritis. *Neurosci Lett*. 1993;153:37–40.
39. Patel SV, McLaren JW, Hodge DO, Bourne WM. Confocal microscopy in vivo in corneas of long-term contact lens wearers. *Invest Ophthalmol Vis Sci*. 2002;43:995–1003.
40. Oliveira-Soto L, Efron N. Morphology of corneal nerves in soft contact lens wear. A comparative study using confocal microscopy. *Ophthalmic Physiol Opt*. 2003;23:163–174.
41. Zhivov A, Stave J, Vollmar B, Guthoff R. In vivo confocal microscopic evaluation of langerhans cell density and distribution in the corneal epithelium of healthy volunteers and contact lens wearers. *Cornea*. 2007;26:47–54.
42. Belmonte C, Acosta MC, Schmelz M, Gallar J. Measurement of corneal sensitivity to mechanical and chemical stimulation with a CO2 esthesiometer. *Invest Ophthalmol Vis Sci*. 1999;40:513–519.
43. Gallar J, Tervo TMT, Neira W, et al. Selective changes in human corneal sensation associated with herpes simplex virus keratitis. *Invest Ophthalmol Vis Sci*. 2010;51:4516–4522.

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