Changes in Corneal Epithelial Layer Inflammatory Cells in Aqueous Tear–Deficient Dry Eye

Hui Lin, Wei Li, Nuo Dong, Wensheng Chen, Jing Liu, Lelei Chen, Hongxia Yuan, Zbixin Geng, and Zuguo Liu

Purpose. To investigate the morphology, distribution, and density of inflammatory cells in the corneal epithelium of aqueous tear–deficient dry eye.

Methods. Thirty-two patients with non-Sjögren’s syndrome (NSS) dry eye, 14 patients with Sjögren’s syndrome (SS) dry eye, and 33 healthy volunteers were studied. In vivo laser scanning confocal microscopy was used to investigate both Langerhans cell (LCs) and leukocyte distribution and density in the peripheral and central corneal epithelium. LC morphology was also evaluated. Multifactor regression analysis was used to assess whether there is a correlation between clinical manifestations and inflammatory cell densities.

Results. LCs were present in both central (34.9 ± 5.7 cells/mm²) and peripheral (90.7 ± 8.2 cells/mm²) parts of the normal corneal epithelium. Moreover, LC density increased dramatically in the central corneal epithelium in patients with NSS (89.8 ± 10.8 cells/mm²) and SS (127.9 ± 23.7 cells/mm²). The ratio of LCs with obvious processes was much higher in patients with dry eye than in healthy volunteers. LC density also increased in peripheral corneal epithelium in patients with SS, but not in those with NSS. Leukocyte density in normal corneal epithelium was very low, whereas it increased in the central corneal epithelium (4.6 ± 1.0 cells/mm²) in NSS and in both central (49.0 ± 12.9 cells/mm²) and peripheral (84.2 ± 36.8 cells/mm²) corneal epithelium in SS. Densities of LCs and leukocytes showed significant correlation with the severity found in clinical evaluation.

Conclusions. The LC and leukocyte changes in the corneal epithelium suggest their involvement in aqueous tear–deficient dry eye pathophysiology. In vivo dynamic assessment of central corneal inflammatory cell density may serve as an indicator of dry eye severity and provide new insight for dry eye treatment. (Invest Ophthalmol Vis Sci. 2010;51:122–128) DOI: 10.1167/iovs.09-3629

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Cornea

Dry eye is one of the most frequent eye disorders with a prevalence that ranges from ~5% to >35% at various ages of the adult population. Clinically, dry eye disease can be assigned to two major classes: aqueous tear–deficient dry eye and evaporative dry eye. Aqueous tear–deficient dry eye, the most common form, can be subdivided into Sjögren’s syndrome (SS) dry eye, a chronic inflammatory disease with an autoimmune basis affecting lacrimal and salivary glands, and non-Sjögren’s syndrome (NSS) dry eye, which encompasses other causes of tear deficiency.

Dry eye is characterized not only by tear film instability, but also by disturbance of homeostasis and structure of the ocular surface. In recent years, development of in vivo confocal microscopy provides the opportunity to observe in situ microstructure of the ocular surface. In patients with dry eye, distinct changes in corneal epithelial cell morphology, alterations of nerve fiber density, abnormal tortuosity in the sub-basal nerve plexus, and keratocyte activation have been identified by in vivo confocal microscopy.

There is increasing evidence in the healthy cornea that another major cell population besides epithelial cells, kerocytes, and endothelial cells is a heterogeneous population of antigen presenting cells (APCs). They include dendritic cells (DCs) and macrophages. On the other hand, during corneal wound healing and in other ocular surface diseases such as SS, epithelial and stromal layers exhibit dramatic changes in APCs and other inflammatory cells, such as T lymphocytes and polymorphonuclear neutrophils (PMNs). Among these cell populations, Langerhans cells (LCs) are a unique subset of APCs found at a depth of 35 to 60 μm in normal human central corneal epithelium, serving as the first-line sentinels and professional APCs in the ocular surface. Recent in vivo confocal microscopy studies of healthy volunteers identified LC density and distribution in the corneal epithelium and revealed changes subsequent to wearing contact lenses, corneal rejection, and some other immune-mediated inflammatory diseases. The coexistence of LCs and leukocytes under some immune-mediated inflammatory conditions was also identified by in vivo confocal microscopy.

Immune inflammation is most likely a mediator of dry eye pathogenesis. Few in vivo studies have found increased APCs in the central corneal epithelium, and an elevated number in the basal corneal epithelium in keratoconjunctivitis sicca. An ex vivo study found DCs infiltration into the lacrimal and salivary glands in patients with SS. Nevertheless, there are no reports describing inflammatory cell profiles, density, and distribution in the cornea of aqueous tear-deficient patients.

The objective of this study was to evaluate the densities and distribution patterns of LCs and nondonedritic leukocytes in different areas of the corneal epithelium in normal subjects and patients with aqueous tear deficiency. Such a determination was made to clarify whether there is an association among these parameters and the pathogenesis of dry eye.
Corneal Epithelial Layer Inflammatory Cells in Dry Eye

**Materials and Methods**

**Participants and Clinical Study**

The study was approved by the Ethics Committee of Clinical Investigation of Xiamen University Affiliated Xiamen Eye Center and was performed in accordance with the principles of the Declaration of Helsinki. All participants were informed of the purpose of this study, and their oral consent was obtained before confocal microscopy examination.

Forty-six consecutive patients with hyposecretory dry eye, including 14 with SS dry eye (15 women and 1 man; average age, 43.8 ± 14.7 years; range, 22–69) and 32 with NSS dry eye (23 women and 9 men; average age, 14.9 years; range, 22–73), and 33 healthy volunteers (17 women and 16 men; average age, 41.8 ± 16.8 years; range, 18–79) were enrolled. The diagnosis of SS was made according to the diagnostic criteria of the American-European consensus. The patients in the NSS group had dry eye diagnosed by Schirmer I test result <10 mm/5 minutes and symptoms of dry eye (foreign body sensation and/or dryness of the eye), not recognized as SS. All normal control subjects did not have any ocular irritation and did not use any ocular medication, with Schirmer I test values higher than 10 mm/5 minutes, tear film break-up time (TBUT) longer than 10 seconds, and negative ocular surface fluorescein staining. The exclusion criteria included the use of contact lenses, the history of ocular trauma or surgery, drug allergy, and the presence of systemic or ocular disease except dry eye and age-related cataract.

A careful case history was compiled for each participant and a slit lamp microscopic examination of the anterior segment was performed. Aqueous tear production was determined by Schirmer I test using standard protocol. TBUT was recorded as the average of three images formed. Aqueous tear production was determined by Schirmer I test values higher than 10 mm/5 minutes and symptoms of dry eye (foreign body sensation and/or dryness of the eye), not recognized as SS. All normal control subjects did not have any ocular irritation and did not use any ocular medication, with Schirmer I test values higher than 10 mm/5 minutes, tear film break-up time (TBUT) longer than 10 seconds, and negative ocular surface fluorescein staining. The exclusion criteria included the use of contact lenses, the history of ocular trauma or surgery, drug allergy, and the presence of systemic or ocular disease except dry eye and age-related cataract.

In Vivo Confocal Microscopy

In vivo confocal microscopy images were obtained (Heidelberg Retina Tomograph III Rostock Corneal Module (HRT III RCM); Heidelberg Engineering GmbH, Dossenheim, Germany) after topical anesthesia with 0.5% proparacaine hydrochloride (Alcon Laboratories, Fort Worth, TX). A drop of carbomer gel (Vidisc; Bausch & Lomb, Rochester, NY) was applied as coupling medium between the applanating lens and the cornea. The other eye fixed a flickering light source to stabilize the patient’s view. The examination time for each eye was less than 10 minutes.

In vivo confocal microscopy was performed on each eye in two different areas: central and superior peripheral cornea. The periphery was designated as representing the outer one sixth of the cornea. At least 30 nonoverlapping images were obtained in each area of the corneal epithelium. The identity of the subject from which the images were obtained was masked. Three images with the maximum number of target cell from each area were evaluated. Bright corpuscular or spicular elements appeared scattered among nerve fibers or within the corneal epithelium at the level of basal to the subbasal nerve plexus were considered to be LCs. In the present study, LCs with processes were defined as cell bodies longer than 12 μm with more than two processes, whereas LCs without processes were defined as LCs with cell bodies shorter than 12 μm and two or fewer processes. Leukocytes were recognized as hyperreflective cells of 12 to 15 μm in diameter without dendritic form, located at the level of lower wing to basal cells. The cell density in each visual field (400 μm²) was calculated using software provided with the microscope and was recorded as cells per square millimeter.

**Statistical Analysis**

Data from one randomly chosen eye per person was used for analysis (SPSS 16.0; SPSS Inc, Chicago, IL). Summary data are reported as the mean ± SE. Data distribution and homogeneity of variance were analyzed. Statistical comparisons of the mean values between different groups were performed using ANOVA or the Kruskal-Wallis test for normally distributed or skewed data, respectively. Multiple comparisons between the two groups were conducted by using the least significant difference (LSD) test in ANOVA or the Mann-Whitney U nonparametric tests with Bonferroni adjustment. Stepwise multifactor regression models were performed to identify the potential factors related to the cell density results from the variables of age, sex, duration of disease, as well as Schirmer I test/TBUT/corneal fluorescein staining scores. Partial correlation coefficients were calculated when more than one factor entered the regression model. All t-tests were two-tailed and P < 0.05 was considered statistically significant.

**Results**

**Clinical Data**

There was no significant difference in age among the NSS, SS, and healthy volunteer groups (ANOVA, P = 0.376). The clinical characteristics including TBUT, Schirmer I test, corneal staining, duration of disease, and the significance of differences between the groups are provided in Table 1. Tear secretion was significantly lower in patients with dry eye than in normal subjects, and lower in the SS group than in the NSS group. Tear film instability was noted both in the SS and NSS groups based on TBUT results. Moderate and severe corneal fluorescein staining was demonstrated in the NSS and SS groups, respectively.

**Increase of LCs in the Corneal Epithelium of Patients with SS or NSS**

LCs were found at a depth of 36 to 66 μm in the corneal epithelium, which spans the lower intermediate and basal epithelial cells, as well as subbasal nerve plexus. The LCs were found mostly within the subbasal nerve plexus in all subjects. In healthy volunteers, they were present in both central (34.9 ± 5.7 cells/mm²) and peripheral (90.7 ± 8.2 cells/mm²) areas. However, LC density dramatically increased in the cen-
Central corneal epithelium in NSS (89.8 ± 10.8 cells/mm²) and SS (127.9 ± 23.7 cells/mm²; Fig. 1A). The number of LCs in the peripheral corneal epithelium were also dramatically increased in the SS group (157.2 ± 29.7 cells/mm², P < 0.05, compared with the healthy group), whereas they were only slightly increased in the NSS group (106.9 ± 10.5 cells/mm², P > 0.05, compared with healthy group; Fig. 1B).

**Number of LCs Activated in SS Corneal Epithelium**

The activation status of LCs in patients with dry eye was determined based on the presence or absence of processes. Both types of LCs were observed in the central and peripheral corneas of healthy volunteers (Figs. 2A, 2B, respectively). LCs with processes were rare in the central cornea, but relatively frequent in the periphery (Fig. 2B). However, in patients with dry eye, activated LCs with processes increased especially in the central corneal epithelium (Figs. 2C–F). The density of LCs with processes in the central corneal epithelium was higher in NSS and even more so SS (Fig. 1A, control group, 3.2 ± 0.5 cells/mm²; NSS group, 9.9 ± 1.7 cells/mm²; SS group, 46.1 ± 17.3 cells/mm²; difference between the groups, P < 0.001; control versus NSS, P = 0.006; NSS versus SS, P = 0.017; control versus SS, P = 0.001), whereas the densities of LCs with processes in the peripheral corneal epithelium showed no significant difference (Fig. 1B, control group, 20.0 ± 2.7 cells/mm²; NSS group, 15.4 ± 2.2 cells/mm²; SS group, 27.2 ± 7.4 cells/mm²; difference among three groups, P = 0.385; control versus NSS, P = 0.222; NSS versus SS, P = 0.816; control versus SS, P = 0.293).

LCs without processes also increased similarly in the central corneal epithelium of NSS and SS eyes without any difference between them (Fig. 1A, control group, 51.7 ± 5.5 cells/mm²; control versus SS, P = 0.293).

**FIGURE 1.** Comparison of LCs densities in corneal epithelium in patients with dry eye and healthy individuals. (A) central cornea; (B) peripheral cornea. Note the higher densities of LCs with and without processes in eyes with aqueous tear deficiency compared with control eyes. *P < 0.05.

**FIGURE 2.** Representative in vivo confocal microscopic corneal epithelial images in different groups. (A) Central cornea of a healthy volunteer with LCs without processes (arrow) at a depth of 58 μm. (B) Peripheral cornea of a healthy volunteer with LCs with processes (arrowhead) interspersed with the epithelial cells at a depth of 41 μm. (C) Central cornea of a 36-year-old female patient with NSS with a higher density of LCs with processes (arrowheads) at a depth of 46 μm. Arrow: LCs without processes. (D) Peripheral cornea of a 46-year-old female patient with SS, showing LCs with (arrowhead) and without (white arrow) processes, and leukocytes (black arrow) at a depth of 42 μm. (E) The wire netting pattern of LCs with long processes (arrowhead) and leukocytes (arrow) in the central corneal epithelium of a 50-year-old female patient with SS at a depth of 66 μm. (F) High density of LCs with processes in the peripheral corneal epithelium of a 50-year-old female patient with SS at a depth of 60 μm. Arrowhead: LCs with processes; arrow: leukocytes. (G, H) Severe leukocyte infiltration in the corneal epithelium of a 59-year-old female patient with SS with diffused corneal fluorescein staining in the central cornea (arrow) at a depth of 60 μm (G) and in the peripheral cornea (arrow) at a depth of 44 μm (H). Bar, 100 μm.
Corneal Epithelial Leukocyte Infiltration in Dry Eye

Leukocytes in Corneal Epithelium

![Graph showing Leukocyte densities in the corneal epithelium of patients with dry eye and healthy individuals. Mean densities are shown above each bar. Note the dramatic difference in leukocyte density between patients with aqueous tear deficiency and healthy individuals in the central and peripheral corneal epithelium (P < 0.05).]

NSS group, 79.9 ± 10.2 cells/mm²; SS group, 81.9 ± 17.6 cells/mm²; control versus NSS, P = 0.002; control versus SS, P < 0.001; NSS versus SS, P = 0.905). Moreover, LCs without processes also increased in peripheral corneal epithelium in patients with SS, but not in NSS (control, 70.7 ± 7.4 cells/mm²; NSS, 91.5 ± 9.8 cells/mm²; SS, 130.0 ± 24.2 cells/mm²; control versus NSS, P = 0.155; control versus SS, P = 0.002; NSS versus SS, P = 0.043).

**Corneal Epithelial Leukocyte Infiltration in Dry Eye**

Corneal epithelial leukocyte density in healthy volunteers was very low (central, 1.6 ± 0.6 cells/mm²; peripheral, 4.3 ± 1.5 cells/mm²). Leukocyte infiltration differentially increased in NSS and SS eyes (Figs. 2C–H). In NSS, leukocytes slightly increased centrally (4.6 ± 1.0 cells/mm²), but not peripherally (8.4 ± 3.1 cells/mm²), whereas, there was a dramatic increase in both central (49.0 ± 12.9 cells/mm²) and peripheral (84.2 ± 36.8 cells/mm²) corneal epithelium in SS eyes (Fig. 3).

**Correlation between Clinical Manifestation and Inflammatory Cell Infiltration**

To determine whether there is a correlation between dry eye clinical manifestation and inflammatory cell infiltration, we performed multifactor regression analysis of clinical data and in vivo confocal microscopy images. The results showed that there is a close correlation among TBUT, Schirmer I test results, and fluorescein staining scores, indicating multicollinearity of the clinic characteristics, which represents the severity of dry eye. Thus, stepwise multifactor regression analysis was performed to screen the potential correlation factors with densities of inflammatory cells as a function of Schirmer I test results, TBUT, and corneal fluorescein staining scores, respectively, together with age, sex, and disease duration. The P-values and standard coefficients or partial correlation coefficients are shown in Table 2. Schirmer I test scores and TBUT showed an inverse correlation with the cell densities of LCs in the central cornea (Figs. 4A, 4B, respectively), and the cell densities of leukocytes in central and peripheral cornea. Meanwhile, TBUT also demonstrated an inverse correlation with LCs without processes in the peripheral cornea. The corneal fluorescein staining scores correlated closely with densities of LCs in the central cornea (Fig. 4C). Moreover, corneal fluorescein staining scores also closely correlate with the densities of leukocytes both in the central and peripheral cornea (Fig. 5). It should be noted that age and sex may have contributed to the density of LCs in the peripheral cornea, according to the regression model. It shows that young subjects of both sexes and female subjects may have more LCs in the peripheral cornea, which is in agreement with a previous study of healthy volunteers. A weak inverse correlation was noted between leukocytes cell density and the duration of dry eye disease in the regression model (Table 2).

**DISCUSSION**

Corneal epithelial Langerhans cells, serving as professional APCs, play an important role in immune surveillance of the

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**Table 2. Multiple Regression Analysis of Inflammatory Cells and Clinical Characteristics in All Participants**

<table>
<thead>
<tr>
<th>All Participants</th>
<th>Age</th>
<th>Sex</th>
<th>Schirmer I Test</th>
<th>TBUT</th>
<th>Corneal FL Staining Score</th>
<th>Duration</th>
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<tr>
<td>Central npLCs</td>
<td></td>
<td></td>
<td>&lt;0.001 (−0.385)</td>
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<tr>
<td>Central pLCs</td>
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<td></td>
<td>&lt;0.001 (−0.343)</td>
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<tr>
<td>Central LCs</td>
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<td></td>
<td>&lt;0.001 (−0.473)</td>
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<tr>
<td>Peripheral npLCs</td>
<td></td>
<td></td>
<td>&lt;0.001 (−0.355)</td>
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<tr>
<td>Peripheral pLCs</td>
<td></td>
<td></td>
<td>&lt;0.001 (−0.398)</td>
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<tr>
<td>Peripheral LCs</td>
<td></td>
<td></td>
<td>&lt;0.001 (−0.295)</td>
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<tr>
<td>Peripheral leukocytes</td>
<td></td>
<td></td>
<td>&lt;0.001 (−0.285)</td>
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Data presented as P-value (P, standardized coefficients/partial correlation coefficients); npLCs, Langerhans cells without processes; pLCs, Langerhans cells with processes; -, factors excluded from stepwise multiple regression analysis; -, factors with multicollinearity (Slt, TBUT, Fl score) not set for screening at the same time with other factors.
They are capable of taking up, processing, and presenting antigens, leading to the initiation of ocular surface immunoinflammatory responses. Despite their obvious importance, little is known about changes in LCs and leukocyte distribution patterns and density in aqueous tear-deficient dry eye.

Development of laser confocal microscopy made it feasible to investigate cell morphology and detect various cell populations on the ocular surface in situ at microscopic resolution and provide real time z-axis observation. Although lacking immunohistologic evidence for cell type identification when the confocal microscope was used, previous studies have described dendritic appearing cells as LCs. Studies from our group and others also clearly demonstrated that dendritic cells in corneal or limbal epithelium express LC-specific cell surface markers. However, without a specific marker, an in vivo confocal study still did not exclude that some of these cells could be other cell types (e.g., melanocyte).

In accordance with previous reports, LCs were found at levels spanning the lower intermediate cells to the subepithelial nerve plexus in normal corneal epithelium. They had a markedly higher density in the peripheral than in the central cornea. Of interest, LC density increased centrally about twofold in the NSS group whereas the rise in the SS group was about threefold. In the periphery, the LC density increased about twofold in patients with SS. In other studies, LCs increased in the central and peripheral cornea in contact lens wearers, along with immune-mediated corneal inflammation.

Figure 4. Correlation between clinical manifestation and LC density in the central corneal epithelium of patients with dry eye. (A) Schirmer I test result, (B) TBUT, and (C) corneal fluorescein staining score versus LC density in the central corneal epithelium.

Figure 5. Correlation between corneal fluorescein staining score and leukocyte density in the (A) central and (B) peripheral corneal epithelium of patients with dry eye.
tion.19 Our study provides further evidence that LCs participate in aqueous tear-deficient dry eye pathogenesis. For better differentiation of investigation allocation, only the superior part of peripheral cornea was investigated in our study, where it is less influenced by the tear film and other stimulus factors in the ocular surface, further study is needed to investigate the geographic distribution of LCs in the whole corneal epithelium.

Morphologic differences in corneal LCs are associated with variations in their functional states.15,29 Confocal microscopy revealed that mature LCs were larger and had more long processes, whereas their smaller counterparts, lacking dendrites, are immature.12 We found that LCs in the central cornea often lacked dendritic extensions, whereas some of them in the periphery had long processes in healthy individuals (Figs. 2A, 2B). However, in the NSS group, LCs in the central corneal epithelium with or without processes increased, and even more so in the SS group. LCs without processes also increased in the periphery in the SS group. Furthermore, the number of LCs in the central corneal epithelium showed a marked inverse correlation with hyposcretion status (Schirmer test) and instability of tear film (TBUT), whereas it had a positive correlation with ocular surface damage (corneal fluorescein staining). In other words, there was a positive correlation between LC density and dry eye severity. This correlation suggests that dry eye status correlates with immune response in these patients. Thus, the density and the morphologic features of LCs may provide an index for evaluating immunoinflammatory status in dry eye.

The mechanisms are not yet fully understood that underlie control of LC migration and maturation in corneal epithelium. It was assumed that their migration and maturation are activated in response to any type of corneal irritation and proinflammatory stimulations.4.50–51 Animal models using cautery burn,22 or sutures33 in the central cornea indeed induced migration and maturation of resident and recruited immature dendritic cells. Dry eye can induce the upregulation and secretion of proinflammatory mediators such as TNF-α, IL-1β, and MMP-9 into the tear film by the ocular surface epithelium.20,34–36 All these factors are known to modulate movement and maturation of DCs.30–37,59 Meanwhile, a variety of peptides and their cognate receptors that are responsible for migration and targeting of LCs,11,13,52 are also expressed in the ocular surface epithelium under dry eye condition. For instance, the chemokine receptor CCR5 is upregulated in the animal model and patients had dry eye.40,41 Our previous study demonstrated the existence of slow-cycling LCs in limbal basal epithelium, which express the stem cell marker ABCG2.26 In another study, we found that cautery stimulation could also induce LC migration from the limbal basal layer to the central cornea.15 Therefore, the higher density and more mature status of LCs in the central corneal epithelium of patients with aqueous tear deficiency may result from the inward migration of resident LCs at the limbus and peripheral cornea, and were rendered into a more mature phenotype by proinflammatory cytokines and/or chemokines. Further experimental study is needed to investigate whether LC proliferation also contributes to the increase of LC density in the limbus or central cornea.

Leukocyte infiltration is another dry eye characteristic related to ocular surface alteration found in this study. These inflammatory cells frequently exist in immune-mediated ocular surface diseases, such as herpes stromal keratitis and corneal graft rejection,19 and contribute to the repair process in corneal epithelial wound healing.15,16 We could not identify the leukocyte cell types due to the shortage of in vivo study. However, the leukocyte morphologic features (e.g., multilobular cell nuclei and nuclear size), indicate that a great number of those hyperreflective cells may be PMNs. They are the most abundant granulocytes in the blood and serve as important effector cells through their release of cytokines and granular products.43 In a previous study PMNs were detected in the tear fluid of patients with dry eye.44 In our study, patients with dry eye with more intense corneal fluorescein staining showed higher densities of leukocytes in both the central and peripheral cornea (Table 2, Fig. 3), suggesting that leukocyte infiltration could also serve as an indicator of ocular surface damage severity.

SS and NSS are similar in that hyposcretion and immunoinflammatory responsiveness are characteristic of these conditions.20 Our study showed increases in inflammatory cell number in both subtypes of aqueous tear-deficient dry eye, which is also consistent with this notion. However, it is well known that these two disease subtypes do not have the same pathogenesis.22 SS is a chronic inflammatory disease with an autoimmune basis and has a more severe clinical manifestation than NSS.1,20 Our in vivo confocal study demonstrated that in patients with SS, LCs appear at a higher density and are especially more activated. Furthermore, the presence of leukocytes in the corneal epithelium indicates that SS dry eye exhibits more severe ocular surface inflammation than does NSS dry eye.

In summary, the NSS and SS aqueous tear-deficient dry eye conditions are characterized by higher LCs densities having a more mature status. Leukocyte corneal epithelial infiltration also increased relative to that in healthy individuals. Such changes could contribute to the complex network of cellular interactions occurring in dry eye pathogenesis. In vivo confocal microscopy may provide a convenient noninvasive approach for assessing immune and inflammation status in these individuals. Such insight will be of help in evaluating dry eye severity and can guide clinical treatment.

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References


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