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Observation of Conjunctiva-Associated Lymphoid Tissue With In Vivo Confocal Microscopy in Healthy Patients and Patients With Meibomian Gland Dysfunction

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Purpose: The purpose of this study was to assess the distribution and morphological variation of conjunctiva-associated lymphoid tissue (CALT) in healthy human subjects and patients with meibomian gland dysfunction (MGD) using laserscanningin vivo confocal microscopy.

Methods: A total of 34 healthy subjects and 32 patients with MGD were enrolled. All subjects underwent a conventional examination consisting of slitlamp biomicroscopy, tear film break-up time, and the Schirmer test. In vivo microscopy was applied to analyze the morphological changes in the diffuse lymphoid layer and lymphoid follicles in CALT. Conjunctival impression cytology (CIC) of samples of patients' palpebral conjunctiva and immunofluorescence staining of CD4 and CD8 antibodies were also performed to indicate the immune response status of CALT.

Results: In the MGD group, the density of diffuse lymphocytes ($P < 0.001$), follicles ($P < 0.001$), and perifollicular lymphocytes was higher ($P < 0.001$) and the central reflection of the follicles was stronger ($P < 0.001$) than in the control group, while there was no difference in the follicle area ($P = 0.758$). Besides, diffuse lymphocyte density was correlated with telangiectasia, and follicular center reflection intensity was correlated with plugging. CIC immunofluorescence staining showed a higher percentage of CD4$^+$ ($P < 0.001$) and CD8$^+$ ($P < 0.001$) cells in the MGD group than in the control group.

Conclusions: Using laser scanning in vivo confocal microscopy and CIC immunofluorescence staining, we observed the activation of CALT in patients with MGD, and some CALT-related parameters correlated with the lid margin findings of patients with MGD.

Key Words: conjunctiva-associated lymphoid tissue, meibomian gland dysfunction, in vivo confocal microscopy, mucosal immune

Meibomian gland dysfunction (MGD) is a subtype of dry eye disease (DED).[1] A deficiency of lipid components leads to increased tear evaporation rates, tear hyperosmolarity, and damage to the underlying ocular structure.[2] The incidence of MGD increases with age, reaching 74.5% in people older than 50 years, as shown in a recent study.[3] Patients may have signs, such as conjunctival hyperemia and superficial punctate keratitis, which seriously affect vision and quality of life. Therefore, MGD-related pathogenesis has attracted increasing attention from ophthalmologists. Factors including hormone levels, medication use, ocular surface flora, and dietary composition are risk factors for MGD.[4–8]

Many researchers have shown that the immune response plays an important role in DED. The mucosal immune system plays an important defensive role in systemic immunity. Mucosal tissues are in close contact with the outside world, and they work with mucosa-associated lymphoid tissue (MALT) to maintain the normal state of the body and prevent disease. Eye-associated lymphoid tissue comprises the lacrimal gland, lacrimal drainage-associated lymphoid tissue , and conjunctiva-associated lymphoid tissue (CALT).[9] Similar to other types of MALTs, CALT consists of induction sites and effector sites.[10] Lymphoid follicles are the induction sites of CALT; these lenticular structures measure approximately 0.3 mm in diameter and have bright germinal centers.

The function of CALT has not been fully studied. Previous studies have shown that CALT is a normal structure in healthy eyes.[9,11] However, most of these previous studies were based on autopsy and animal models. It is difficult to observe real-time changes in CALT in humans under physiological and pathological conditions. In recent years, with the advancement of technical methods, laser scanning in vivo confocal microscopy (IVCM) has become an important tool for clinical examination and scientific research because it enables noninvasive, real-time, dynamic observation of changes in the ocular surface at the cellular level.

Agnifili et al confirmed the existence of CALT using IVCM in the eyes of 108 normal healthy volunteers, verifying the findings of previous autopsy research and clarifying that IVCM can be used as an important tool for observing CALT.[11] However, the role of CALT in ocular diseases is still not fully understood.

The purpose of this study was to determine the features of CALT in MGD. Images of CALT-inducing and CALT-effecting sites from both healthy people and patients with MGD were collected and analyzed to determine the morphological changes in CALT. In addition, conjunctival impression cytology (CIC) and immunofluorescence staining of CD4$^+$ T cells and CD8$^+$ T cells were performed to confirm the functional changes in CALT. To date, this is the first article to study the relationship between CALT and MGD; this study may help to further clarify the pathogenesis of this common ocular surface disease.

MATERIALS AND METHODS

Patients

This study was an observational cross-sectional study. Subjects with MGD in the Outpatient Department of the First Affiliated Hospital of Harbin Medical University (Harbin, China) between 2018 and 2020 were recruited. The research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University and adhered to the principles of the Declaration of Helsinki.

A total of 32 patients with a diagnosis of MGD were enrolled in this study and were compared with 34 normal age-matched and sex-matched healthy control subjects. This experiment enrolled patients who were diagnosed with MGD according to their clinical symptoms (foreign body sensation, photophobia, blurred vision, and conjunctival congestion) and 2 or more abnormal signs (redness and thickening of the lid margins, reduced secretion, abnormal quality of secretion, and obstruction of meibomian glands).[12] All healthy subjects underwent careful inquiries into their medical history before enrolment and received adequate examinations, including slitlamp biomicroscopy, fundoscopy, intraocular pressure measurement, and tear break-up time examination. Those with no abnormal signs after adequate examination were enrolled as healthy subjects. About the previous scoring standards, we scored the performance of the eyelid margins of patients with MGD.[13] The degree of telangiectasia was rated from 0 to 3; irregularity and thickening of the eyelid margin was rated from 0 to 213.

The exclusion criteria were as follows: people younger than 18 years; pregnant women; people wearing contact lenses; people suffering from diabetes or autoimmune diseases, ocular surface infections, or ocular surface allergies; and people who had received eye drops or eye surgery in the past 24 months.

In Vivo Laser Confocal Microscopy Examination

IVCM [Heidelberg Retinal Tomograph 3 with the Rostock Cornea Module (HRT3/RCM); Heidelberg Engineering GmbH, Heidelberg, Germany] was applied to examine the palpebral conjunctiva of both groups. The HRT3/RCM IVCM apparatus provides a 400-μm^2 field of view with lateral and vertical resolutions of 1 μm. Before the lens of the confocal microscope was covered with a cap (Tomo-Cap; Heidelberg Engineering), gel was added between the lens and the cap to establish coupling. Based on the results of Agnifili et al,[11] the most distributed CALT region of the conjunctiva, the superior tarsal conjunctiva, was selected for examination. Both eyes of each patient were anesthetized by applying 0.4% oxybuprocaine hydrochloride eye drops (Benoxil 0.4%, Santen Pharmaceutical Co, Ltd, Japan) for surface anesthesia. The patients' upper eyelids were gently flipped, and they were instructed to sit. During the procedure, the patients were instructed to look down. The examination was performed from the conjunctiva epithelial layer to the deepest layer of the tarsal conjunctiva; the scanning methods used were the same as described previously.[11] The layers with the best imaging quality were selected for collection and analysis; at least 10 clear images were taken for each part.

During examination, we observed both the diffuse lymphoid tissue and the lymphoid follicles of the CALT; the depth of the illuminated plane was determined by the depth reading of the confocal microscope. The diffuse lymphoid tissue is located in the subconjunctival epithelium and is composed of spot-like, highly reflective cells that are presumed to be lymphocytes. Lymphoid follicles were oval in shape, and the layers with the largest follicle diameters were captured for further analysis. The counting software that accompanied the Heidelberg in vivo confocal microscope was used to determine the density of follicles, the density of parafollicular lymphocytes, and the cell density of diffuse lymphoid tissue. ImageJ was used to calculate the area of the follicle, and the gray value module was used to analyze the reflection of the follicular center. Three clearly collected images of diffuse lymphoid tissue and follicles from each eye of the patients were used for the analysis of the corresponding data.

CIC and Immunofluorescence Staining

We performed CIC on the subjects' superior tarsal conjunctiva. The CIC samples were collected at the center of the superior tarsal conjunctiva. In preparation for CIC, subjects underwent local anesthesia with 0.4% oxybuprocaine hydrochloride eye drops. Subsequently, a sterile membrane (0.45 μm, Millipore, Boston, MA) was placed on the surface of the palpebral conjunctiva and pressed for a few seconds. To increase the number of collected cells, the palpebral conjunctiva was gently wiped with cotton swabs to keep it dry.

Indirect immunofluorescence was used to observe the staining of CD4$^+$ and CD8$^+$ cells in CIC samples. After fixing the CIC sample with 4% paraformaldehyde, antibodies against CD4 (A0362, 1:200, Abclonal, Wuhan, China) or CD8 (A11033, 1:200, Abclonal) were incubated at 4°C overnight. After washing, we stained CD4 with an Alexa Fluor 488-conjugated anti-rabbit antibody (A-11034, 1:1000, Invitrogen, Shanghai, China) or CD8 with an Alexa Fluor

594-conjugated anti-rabbit antibody (A-11037, 1:1000, Invitrogen) at room temperature for 50 minutes. The cells were observed with a Leica fluorescence microscope (LEICA DMi8, Leica Microsystems, Wetzlar, Germany). Three different fields of each sample were observed and evaluated to count the positively stained cells.

Statistics

The Mann–Whitney *U* test was used to test for differences in parameters of diffuse lymphocyte density, parafollicular lymphocyte density, follicles, follicular reflex intensity, and follicular area between the MGD group and the control group. Correlations of CALT-related parameters and lid margin findings were analyzed with Pearson correlation analysis. The independent-samples *t* test was used to compare the percentages of CD4$^+$ and CD8$^+$ cells between the groups. Statistical analysis was performed using Microsoft Excel 2007 and SPSS 22.0 software. $P < 0.05$ was taken as the statistically significant threshold.

RESULTS

In this study, the MGD group included 32 patients (10 men and 22 women) and the median age was 43 years (Q25–Q75 = 32–59 years). The control group included 34 healthy subjects (11 men and 23 women), and the median age was 44 years (Q25–Q75 = 28–53 years). There was no significant difference in sex ($P = 0.923$) or age ($P = 0.695$) distribution between the MGD group and the control group (Table 1). We observed and compared the CALT-related parameters of both eyes in the control group and the MGD group, and the results showed that there was no significant difference between the left and right eyes. Therefore, we randomly selected the right eye for analysis of related parameters. In the following study, we chose the right eye of each group to analyze the results. The anterior segment photographs of patients with MGD are shown in Figure 1.

Diffuse Lymphoid Tissue

Diffuse lymphoid tissue is composed of presumed lymphocytes with hyperreflective puncta within and under the conjunctival epithelium. We compared the density of diffuse lymphocytes in the right eyes of the MGD group and the control group. The lymphocyte density in the right eye was significantly higher in the MGD group than in the control group [474 cells/mm^2 (Q25–Q75: 268–786 cells/

mm^2) versus 177 cells/mm^2 (Q25–Q75: 146–258 cells/mm^2)] ($P < 0.001$) (Fig. 2).

Lymphoid Follicles
Lymphoid Follicle Density

The lymphoid follicle is an oval structure with a germinal center in the center and surrounding lymphocytes. The density of lymphoid follicles was significantly higher in the MGD group than in the control group ($P < 0.001$). The density of lymphoid follicles in the right eye of the MGD group was 32 follicles/mm^2 (Q25–Q75: 28–37 follicles/mm^2), and the density of lymphoid follicles in the right eye of the control group was 26 follicles/mm^2 (Q25–Q75: 21–31 follicles/mm^2) (Fig. 3).

Lymphoid Follicle Reflection Intensity

When we compared the reflection intensity of the lymphoid follicle centers of the MGD group and the control group using ImageJ, we found that the intensity of the follicular reflection of the MGD group was significantly stronger than that of the control group ($P < 0.001$). The follicle center reflection intensity of the right eye in the MGD group was 115 (Q25–Q75: 102–126), whereas the follicle center reflection intensity of the right eye in the control group was 97 (Q25–Q75: 89–110) (Fig. 4).

Parafollicular Lymphocyte Density

Parafollicular lymphocytes are highly reflective cells distributed around lymphoid follicles. After comparison, we found that the density of parafollicular lymphocytes in the MGD group was significantly higher than in the control group ($P < 0.001$). In the MGD group, the density of parafollicular lymphocytes in the right eye was 276 cells/mm^2 (Q25–Q75: 206–327 cells/mm^2), whereas in the control group, the density of parafollicular lymphocytes in the right eye was 146 cells/mm^2 (Q25–Q75: 107–187 cells/mm^2) (Fig. 5).

Follicular Area

With the application of ImageJ software, we calculated and compared the follicular area of the right eye in the MGD group and the control group. In the MGD group, the follicular area was 23,485 mm^2 (Q25–Q75: 18,382–29,195 mm^2),
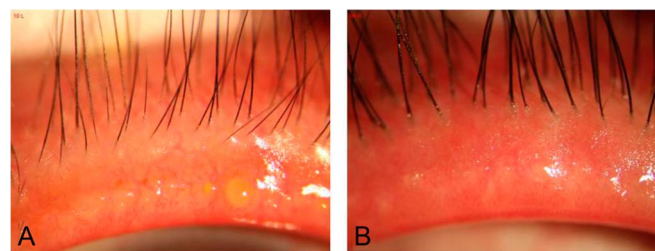

FIGURE 1. The palpebral margins of enrolled patients with MGD. Signs of lid margin congestion and telangiectasia, clogged meibomian gland orifices, and abnormal qualities of the expressed meibum.

TABLE 1. Demographics and Clinical Parameters of the 2 Groups

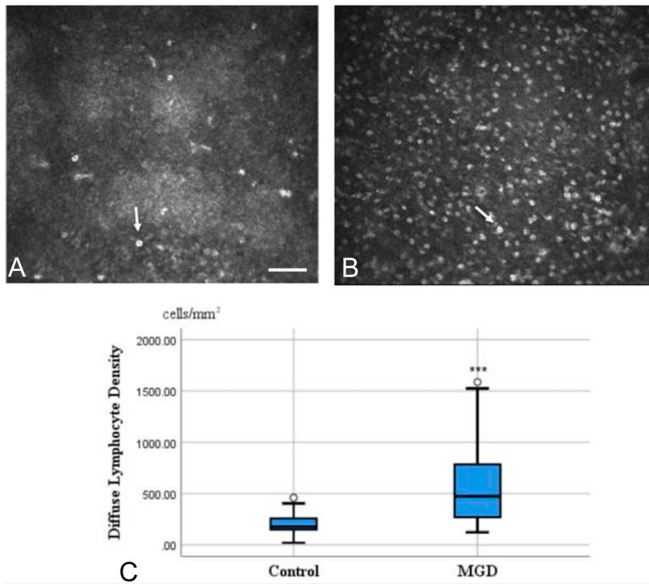| Parameter	Normal (n = 34)	MGD (n = 32)	P
Age (yr)	32 (43,59)	28 (44,53)	0.695
Sex (M/F)	11/23	10/22	0.923
BUT (s)	10.97 ± 2.70	5.37 ± 3.08	<0.001
Schirmer test (mm)	8.23 ± 3.26	9.94 ± 6.32	0.193

FIGURE 2. Diffuse lymphoid tissue in CALT. Highly reflective cells are presumed to be lymphocytes (indicated by arrows). A, Diffuse lymphoid tissue in the control group. B, Diffuse lymphoid tissue in the MGD group. C, The level of highly reflective inflammatory cell infiltration in the MGD group is significantly increased (***significant at $P < 0.001$). Scale bar: 50 μm. (The full color version of this figure is available at www.corneajrnl.com.)

whereas the follicular area of the right eye in the control group was 22,902 mm² (Q25–Q75: 20,815–29,766 mm²). There was no significant difference between the groups ($P = 0.758$).

Immunofluorescence Staining

We performed CD4+ and CD8+ immunofluorescence staining of CIC samples from the MGD group and the control group. The results showed that the percentages of CD4+ cells and CD8+ cells were significantly higher in the MGD group than in the control group (44.0 ± 15.1% vs. 22.9 ± 12.1%, $P < 0.001$ and 49.4 ± 15.5% vs. 17.3% ± 12.8%, $P < 0.001$, respectively) (Figs. 6 and 7).

CALT-Related Parameters and Lid Margin Findings

We analyzed the correlation of CALT-related parameters and lid margin findings. We found that the severity of lid margin telangiectasia was related to the density of diffuse lymphocytes ($P < 0.01$), and the follicular center reflection intensity was correlated with plugging ($P = 0.028$). With severe lid margin telangiectasia, the density of diffuse lymphocytes increases. As the signs of plugging worsen, the intensity of follicular center reflection increases (Table 2).
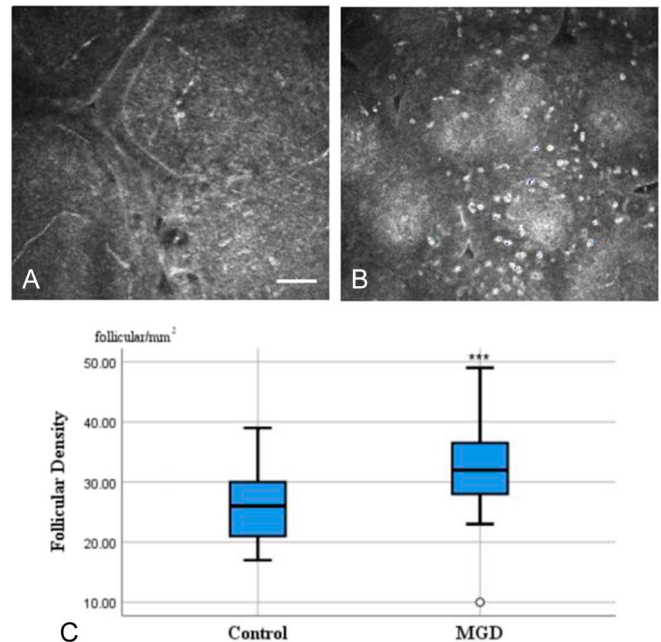

FIGURE 3. Lymphoid follicles in CALT. The follicular germinal center is surrounded by highly reflective parafollicular lymphocytes. A, Lymphoid follicles in the control group, with a lower follicle density. B, Lymphoid follicles in the MGD group, with a higher follicle density. C, The density of lymphoid follicles in the MGD group is significantly increased (***significant at $P < 0.001$). Scale bar: 50 μm. (The full color version of this figure is available at www.corneajrnl.com.)

DISCUSSION

Using IVCM, our study showed that CALT-related parameters, such as the lymphocyte density in diffuse lymphoid tissue, follicular center reflection intensity, follicular density, and parafollicular lymphocyte density, were increased in patients with MGD compared with the control group. In addition, CIC examination of the patients' conjunctiva revealed that the proportions of CD4+ and CD8+ cells were increased. These results indicated that CALT was activated in patients with MGD.

In recent years, CALT, part of the MALT, has gradually begun to attract interest from researchers. CALT has been shown to exist in animals such as mice and rabbits.[14,15] However, from previous studies, it remains controversial whether CALT is a normal part of the structure of the human eye.[14] The Knop group performed an autopsy of conjunctival sacs from 53 human samples and found that CALT was present in healthy eyes.[15] CALT consists of diffuse lymphoid tissue, lymphoid follicles, crypt-associated lymphoid tissue, and high endothelial venules. Among them, diffuse lymphoid tissue is mainly located in the subepithelial lamina propria. As the effector sites of CALT, diffuse lymphoid tissue is mainly composed of T cells and IgA+ and IgM+ B cells. Lymphoid follicles are the afferent arms of CALT, and they are located beneath the layer of diffuse lymphoid tissue. The follicles are centered on CD45R/B220+ B cells while CD3+ T cells are distributed in the periphery.[15,16]

FIGURE 4. A, The reflection of lymphoid follicle centers is weaker in the control group. B, The reflection of lymphoid follicle centers is stronger in the MGD group. C, Follicle reflection is significantly stronger in the MGD group than in the control group (***significant at $P < 0.001$). Scale bar: 50 μm.
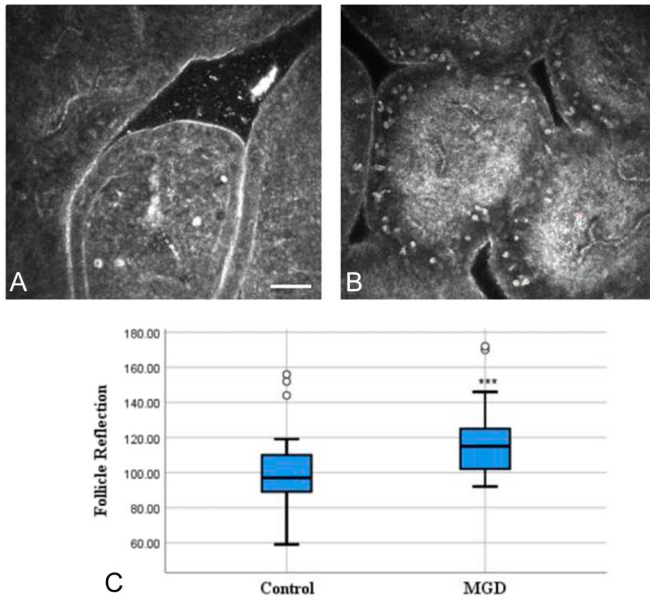

FIGURE 5 . A, The reflection of lymphoid follicle centers is weaker in the control group. B, The reflection of lymphoid follicle centers is stronger in the control group. C, The follicle reflection is significantly stronger in the MGD group than in the control group (***significant at $P < 0.001$). Scale bar: 50 μm. (The full color version of this figure is available at www.corneajrnl.com.)

At present, the role of CALT in ocular surface immunity is still not clear. Liang et al[17] observed the activation of CALT in rabbit eyes after injecting LPS subconjunctivally. After stimulation with LPS, there was a large amount of lymphocyte infiltration at the superficial site and beneath the lymphoid follicles in the conjunctiva. With IVCM, Mastropasqua et al[18] also found the same changes in the ocular surface of patients treated with anti-glaucoma drugs. These studies confirmed the feasibility of observing CALT activation through IVCM and provided evidence for the involvement of CALT in regulating ocular surface immunity.

In previous studies, many complicated factors, including ocular surface microorganisms, dietary lipid intake, and inflammation of the meibomian glands, are all closely connected with MGD.[19–22] Owing to keratinization of the epithelium and an increase in the viscosity of the meibum, the terminal duct of the meibomian gland is obstructed, resulting in atrophy and reduced secretion from the meibomian gland, which leads to a series of changes affecting the tear fluid, the margin of the eyelid, and the ocular surface.[23] The obstruction of the meibomian gland promotes the growth of bacteria, which leads to an increase in esterase, changes in the viscosity of the meibum, and aggravated inflammation.[21] In addition, Ziemanski et al found that high levels of omega-3 in the diet reduce the incidence of MGD in postmenopausal women, which may be related to inhibiting the inflammation function of omega-3.[24] Mahajan et al[20] observed both animals and patients in whom the meibomian gland orifice was blocked by aggregated neutrophil extracellular traps, which resulted in a
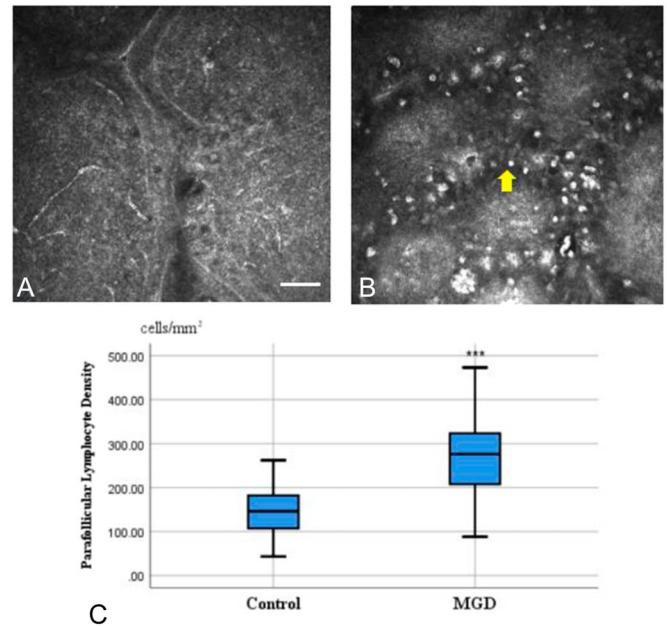
lack of lipid components on the ocular surface and destruction of the glands. Thus far, few studies have focused on the role of the ocular surface immune system.

In recent years, several studies have confirmed that inflammation is involved in the development of MGD. Using CD45 markers in the immunofluorescence staining of meibomian gland samples of patients with MGD, Nien et al[24] found that CD45+ infiltration appeared in the acinar, duct, and interstitial areas of the meibomian gland, and the degree of CD45 infiltration was significantly associated with the severity of the meibomian gland expression grade. In addition to immune cell participation in the formation of aggregated neutrophil extracellular traps to block the meibomian gland openings,[20] T lymphocytes also play an important role in the occurrence of MGD27. Using an MGD animal model, Reyes et al confirmed that accompanied by symptoms of MGD, the immune response of Th17 cells in the eyes of mice is enhanced.[27] However, there have been no signs of MGD in Il17a−/− mice.[27] Through the transfer of Th17 cells induced in vitro, mice exhibited an obstruction of the meibomian gland.[27] The research outcomings of that group indicated that T lymphocytes play a vital role in the pathogenesis of MGD.[27] As an ocular surface immune tissue containing abundant immune cells including T lymphocytes, we speculate that CALT may play an important role in the pathogenesis of MGD. Currently, there is a lack of relevant studies confirming the role of CALT in MGD, and this speculation remains to be confirmed in future, in-depth analyses.
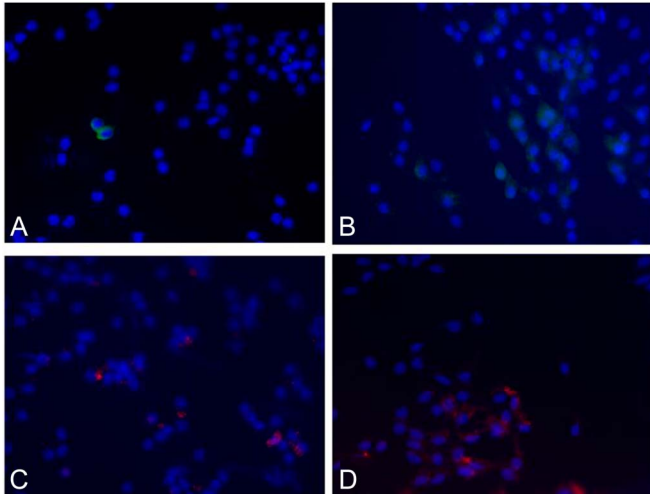
FIGURE 6. Immunofluorescence staining of CIC. A, CD4+ immunofluorescence staining of CIC samples from the control group. B, CD4+ immunofluorescence staining of CIC samples from the MGD group. C, CD8+ immunofluorescence staining of CIC samples from the control group. D, CD8+ immunofluorescence staining of CIC samples from the MGD group. Magnification: ×400. (The full color version of this figure is available at www.corneajrnl.com.)

In this study, we found that CALT-related parameters were significantly higher in the MGD group than in the control group. During the pathogenesis of MGD, CD4+ T cells can differentiate into Th1 and Th17 cells and release numerous proinflammatory cytokines, such as IFN-γ and IL-17.[27] In addition, by applying IL-23, which can stabilize Th17 and reduce the frequency of Th17, the researchers found that the degree of meibomian gland obstruction in the animal model was significantly reduced.[25] In animal models treated with lifitegrast, Singh also found that as the degree of meibomian gland obstruction was reduced, the percentage of Th17 cells decreased significantly.[26] Diffuse lymphoid tissues are mainly composed of T cells. We observed a significant activation of diffuse lymphoid tissues in the MGD group. The increase in the density of diffuse lymphocytes observed in this study is consistent with the increase in the

density of epithelial immune cells observed in patients with MGD in previous studies,[12] and both indicate that immune cell activation in the conjunctiva occurs during the pathogenesis of MGD. We refer to the cells in diffuse lymphocytes as presumed lymphocytes in reference to the research of Agnifili et al.[11] These cells are located in the subepithelial lamina propria and are highly reflective cells distributed in layers, potentially composed of lymphocytes or plasma cells.[11] In the anatomical position, they correspond to CD3+ T cells and plasma cells in CALT found during autopsy.[11,27] Because it is difficult to determine the specific cell types using IVCM, herein, they are called presumed lymphocytes.

Cain and Phillips[28] observed the CALT of rabbit eyes at different ages and found that the number of follicles significantly decreased over time. This phenomenon was accompanied by a weakened ocular surface immune function and an increased incidence of disease. This phenomenon indicates that changes in follicle-related parameters may be related to changes in mucosal immune function. In our research, we observed similar patterns in lymphoid follicle-related parameters. Compared with the control group, the MGD group showed an increased follicular density when the ocular surface was in an activated state. Activation of both the afferent and efferent arms of CALT indicated that CALT was activated during the onset of MGD.

In clinical studies of DED, changes in the CD4+/CD8+ ratio are controversial. Barabino et al[29] performed CIC and flow cytometry examinations of the ocular surface of patients with dry eye and found that the ratio of CD4+/CD8+ in the dry eye group was significantly higher than in the normal group. They believed that this characteristic indicated an important role of CD4+ T lymphocytes in the development of dry eye. However, Reinoso et al[30] found that the CD4+/CD8+ ratio was lower in the dry eye group than in the normal group after brush cytology and flow cytometry The incidence of dry eye has increased significantly among patients receiving bone marrow transplants. Rojas et al assessed the changes in immune cells in the conjunctiva of patients before and after bone marrow transplantation. They found that the number of CD4+ T lymphocytes and CD8+ T lymphocytes increased significantly. They speculated that the activation of these immune cells might be related to the high incidence of dry eye in patients receiving bone marrow
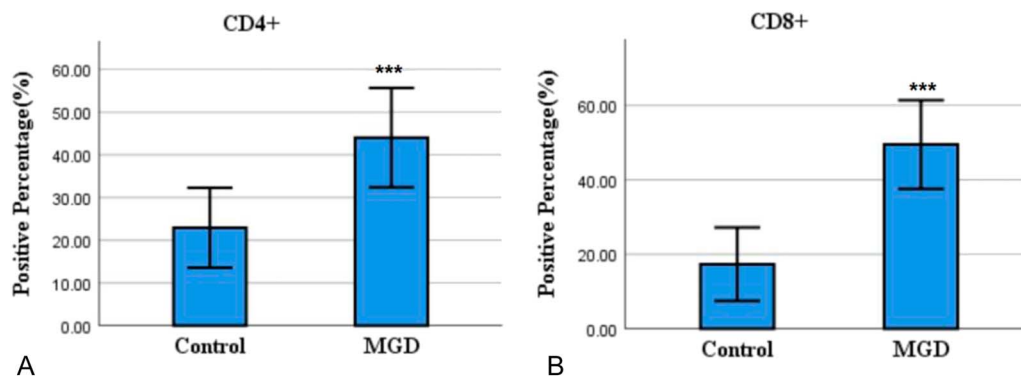

FIGURE 7. Percentages of CD4+ and CD8+ cells in the CIC samples of the MGD and control groups (***significant at *P* < 0.001). (The full color version of this figure is available at www.corneajrnl.com.)

TABLE 2. Analysis of CALT-Related Parameters and Lid Margin Signs

	Telangiectasia	Thickness	Plugging
Diffuse lymphocyte density			
Pearson correlation	0.563	0.235	0.289
P	0.001*	0.195	0.108
Follicle area			
Pearson correlation	0.134	0.172	0.302
P	0.466	0.346	0.093
Parafollicular lymphocyte density			
Pearson correlation	0.102	0.235	0.034
P	0.578	0.195	0.855
Follicular center reflection intensity			
Pearson correlation	0.32	−0.182	0.388
P	0.074	0.318	0.028†
Follicle density			
Pearson correlation	−0.285	−0.093	0.324
P	0.113	0.612	0.071

*Significant at $P < 0.01$.
†Significant at $P < 0.05$.

transplants.[31] CD4^{+} T-cell–mediated immunoreaction plays an important role in the mechanism of DED.[32] Previous studies have shown that when the CD4^{+} T cells generated in the DED model are transferred to nude mice with T-cell defects, the nude mice also exhibit ocular surface inflammation.[33] After IC and IF staining, we found that the proportions of CD4^{+} T and CD8 + T cells were higher in the MGD group than in the control group. Our experimental results further clarified the increase in CD4^{+} and CD8^{+} T lymphocytes in the conjunctiva of patients with MGD, and they provided evidence for the immunological changes in patients with MGD.

There are some limitations to our study. First, given the limited number of patients, we did not compare the degree of CALT activation according to the MGD severity. If a larger clinical study were conducted, it might become possible to provide a new grading standard for MGD from an immunological perspective and assess its correlation with the clinical symptoms of patients. Second, this study was a retrospective cross-sectional study that did not compare the degree of CALT activation in patients with MGD before and after treatment. Currently, there is no standard and quantifiable withdrawal criterion in the treatment of patients with MGD. We speculate that the degree of CALT activation is reduced after MGD treatment. If this speculation is confirmed, the activation level of CALT can be used as an important indicator and a noninvasive continuous monitoring tool for adjusting the dosages and courses of treatment.

In summary, we observed changes in CALT in patients with MGD using IVCM and IC for the first time, which confirmed that CALT was activated during the course of MGD.

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