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The CD4/CD8 Ratio in Vitreous Fluid Is of High Diagnostic Value in Sarcoidosis

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Purpose: Sarcoidosis is an idiopathic inflammatory disorder involving multiple organs, and ocular manifestation (represented by granulomatous uveitis) is one of the common features. A well-known immunologic feature in sarcoidosis is an increased CD4+ helper T-cell type 1 lymphocyte subset in bronchoalveolar lavage (BAL) fluid. The current study investigated the vitreous lymphocyte subsets of ocular sarcoidosis to elucidate the immunologic features of this disorder in the eye.

Design: Case-control study.

Participants and Controls: Fifty-one eyes of 38 patients with ocular sarcoidosis, confirmed by international diagnostic criteria, were enrolled in this study. Twenty-seven eyes of 26 patients with other causes of uveitis were enrolled as nonsarcoid controls.

Methods: Evaluation of diagnostic tests for cell profiles of ocular sarcoidosis. Lymphocytes in the vitreous samples were analyzed by cytology, polymerase chain reaction, and flow cytometry. Peripheral blood was also obtained from each patient and analyzed in comparison with the vitreous samples.

Main Outcome Measures: CD4/CD8 ratios of vitreal and peripheral T lymphocytes.

Results: CD4/CD8 ratios of the vitreous T lymphocytes were significantly higher in ocular sarcoidosis than in nonsarcoidosis vitreous samples. In the patients with ocular sarcoidosis, the CD4/CD8 ratios of vitreal T lymphocytes were significantly higher than the CD4/CD8 ratios of peripheral T lymphocytes. No significant differences were found between the CD4/CD8 ratios of vitreal and peripheral T lymphocytes in the patients without sarcoidosis. Moreover, the CD4/CD8 ratios of peripheral T lymphocytes in the patients with ocular sarcoidosis were significantly higher than in patients without sarcoidosis. The sensitivity and specificity of the vitreal CD4/CD8 ratio were 100% and 96.3%, respectively, for the diagnosis of ocular sarcoidosis.

Conclusions: Our findings suggest that the CD4/CD8 ratio of vitreous-infiltrating lymphocytes has high diagnostic value in ocular sarcoidosis, comparable to that of the CD4/CD8 ratio in BAL fluid lymphocytosis for pulmonary sarcoidosis. Furthermore, a high CD4/CD8 ratio of peripheral blood T lymphocytes should be one of the laboratory findings for ocular sarcoidosis. Diagnostic vitrectomy using flow cytometric analysis may be a useful adjunct for the diagnosis of ocular sarcoidosis, particularly in complex cases.

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Sarcoidosis is an idiopathic inflammatory disorder characterized by noncaseating epithelioid cell granulomas in multiple tissues and organs.[1,2] The disease most commonly affects the lung, but other tissues (e.g., the heart, central nervous system, and spleen) may also be affected.[3] Although the exact cause of sarcoidosis is currently unknown, studies have linked the disease with genetic, environmental, or age-related heterogeneity.[4,5] Both endogenous and pathogenic bacterial species have been detected in sarcoid lesions, although their association with disease etiology remains unclear.[6] It has been surmised that genetic predispositions, compounded by other factors, may trigger granulomatous responses to endogenous or infectious microbes in sarcoidosis.[5,7]

The basic diagnostic criteria for sarcoidosis include the clinical and radiologic presentation, histopathologic verification of noncaseating epithelioid cell granulomas, and exclusion of other diseases with granuloma formation reactions (e.g., tuberculosis).[3] Clinical features suggestive of sarcoidosis include uveitis in the eye, erythema nodosum, and maculopapular skin lesions; chest radiography may reveal bilateral hilar lymphadenopathy (BHL) or pulmonary infiltration. Previous reports have shown that fiberoptic bronchoscopy can be used to enable a transbronchial lung biopsy for the diagnosis of sarcoidosis.[8,9]

Immunologic findings are typical in sarcoidosis.[3] An increased CD4+ helper T-cell type 1 lymphocyte subset (lymphocytosis) in bronchoalveolar lavage (BAL) fluid and a CD4/CD8 ratio greater than 3.5 are helpful for the diagnosis of sarcoidosis. Depression of delayed-type hypersensitivity is also known to be a common feature of sarcoidosis.[10] Bronchoalveolar lavage lymphocytosis (CD4:CD8 >3.5) and other lab-

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oratory features highly consistent with sarcoidosis, such as high serum levels of angiotensin-converting enzyme, can be reliable indicators when diagnosing sarcoidosis in cases without histology.[11,12]

It is reported that 30% to 60% of patients with sarcoidosis have ocular involvement.[13–16] Bilateral anterior or posterior uveitis is common, but the conjunctiva, lacrimal gland, and orbit of the eye also can be affected. The clinical presentation of sarcoid uveitis is characteristically marked by iris nodules, mutton-fat keratic precipitates, and tent-shaped peripheral anterior synechia in the anterior segment of the eye. Phlebitis and vitreitis, resulting in snowball-like vitreous opacity, are the common posterior segment findings. Chronic uveitis can result in the formation of an epiretinal membrane and cystoid macular edema, leading to severe visual impairment.[17] Uveitis is commonly treated with topical (or occasionally systemic) corticosteroids, but in cases accompanied by the formation of an epiretinal membrane or long-standing vitreous opacity, surgical intervention may be necessary.

For the diagnosis of sarcoidosis with ocular involvement, internationally acknowledged criteria have been established.[18] Although it is generally not difficult to diagnose ocular sarcoidosis with typical clinical findings, a considerable number of sarcoidosis cases present with nonspecific vitreal opacity, thus making a differential diagnosis difficult.[19]

Analysis of a vitreous sample is reported to be useful in diagnosing uveitis or other diseases with vitreal opacity. Vitreous samples may be analyzed by cytology, cell surface marker analysis by cytokine measurement, and polymerase chain reaction (PCR) for detection of infectious origins, such as viral, fungal, or bacterial DNA.[20,21] Flow cytometric analysis is also reported to be useful in diagnosing primary intraocular lymphoma[22] and uveitis.[23]

The current study found that the CD4/CD8 ratios of lymphocytes obtained from vitrectomy specimens are significantly higher in sarcoidosis compared with nonsarcoid uveitis. We also discovered that a CD4/CD8 ratio greater than 3.5 of vitreous-infiltrating lymphocytes is highly specific to ocular sarcoidosis and is efficient for the differential diagnosis of the disease. Moreover, a vitreal CD4/CD8 ratio greater than 3.5 was found to have high sensitivity and specificity in the diagnosis of ocular sarcoidosis. This was comparable to a CD4/CD8 ratio greater than 3.5 from BAL fluid for the diagnosis of pulmonary sarcoidosis.

Materials and Methods

The study was designed as a prospective study. Patients who met inclusion criteria were identified in the uveitis outpatient clinic at Kyoto Prefectural University of Medicine and invited to participate in the study. Details of patients and controls are provided in Tables 1 and 2 (available at http://aaojournal.org). Included were patients with uveitis with visual disturbance due to prolonged vitreous opacity or epiretinal membrane that was refractory to conventional steroid therapy; enrolled patients agreed to receive pars plana vitrectomy and to participate in the study. Patients were excluded from the ocular sarcoidosis group if they had a history of intraocular surgery or other active systemic disease. Patients in the study were categorized into 2 groups (sarcoidosis and nonsarcoidosis) according to international criteria for the diagnosis of ocular sarcoidosis established at the International Workshop on Ocular Sarcoidosis (IWOS).[18] The IWOS criteria have 4 classifications of ocular sarcoidosis based on suggestive clinical signs, appropriate laboratory investigations, and biopsy results. Briefly, biopsy-supported diagnosis with a compatible uveitis is classified as "definite" ocular sarcoidosis; presence of BHL and compatible uveitis but without biopsy is classified as "presumed" ocular sarcoidosis; presence of suggestive intraocular signs and 2 positive investigational test results (negative tuberculin skin test result, elevated angiotensin-converting enzyme, elevated liver enzymes, and chest computed tomography) without BHL and biopsy is classified as "probable" ocular sarcoidosis; negative biopsy, with suggestive intraocular signs and 2 positive investigational test results, is classified as "possible" ocular sarcoidosis. Patients who met the IWOS criteria were categorized as the sarcoidosis group, and other patients were categorized as the nonsarcoidosis group.

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine. All experimental procedures were conducted in accordance with the tenets set forth in the Declaration of Helsinki. The study was registered in the University Hospital Medical Information Network Clinical Trial Registry. The purpose of the research and the experimental protocols were explained in detail to all patients, and their informed consent was obtained before participation in this study.

Participants

A total of 78 eyes of 64 patients were enrolled in this study. Of those 78 eyes, 51 eyes of 38 patients were diagnosed with ocular sarcoidosis. Of the 38 patients with ocular sarcoidosis, 15 were diagnosed with "definite" ocular sarcoidosis on the basis of positive histopathologic manifestation by skin biopsy (12 patients) and transbronchial lung biopsy (3 patients). Eleven patients with BHL but without biopsy were categorized as having "presumed" ocular sarcoidosis, and 12 patients without BHL and biopsy were diagnosed with "probable" ocular sarcoidosis on the basis of laboratory investigation. There were no patients diagnosed with "possible" ocular sarcoidosis. The remaining 27 eyes (of 26 patients) were grouped as nonsarcoid controls; in these cases, uveitis was associated with endophthalmitis, acute retinal necrosis, primary intraocular lymphoma, or idiopathic uveitis. The basic characteristics of all patients are listed in Tables 1 and 2 (available at http://aaojournal.org).

Sample Collection Procedures

From each patient, a vitreous specimen was obtained at the start of a conventional 20- or 25-gauge pars plana vitrectomy operation using a CV-24000 (NIDEK, Co., Ltd., Aichi, Japan) or an Accurus (Alcon Laboratories, Inc., Fort Worth, TX) vitrectomy system. A 3-way cock was attached between the connection part of the suction-tube line of the cutter probe, and a 10-ml syringe was connected to the free end of the 3-way cock. Dry vitrectomy without balanced salt solution (Alcon Laboratories, Inc.) perfusion was conducted with a cut rate of 500 cpm so as not to damage cells infiltrating the vitreous. After collecting 1.5 to 3 ml of pure vitreous sample, additional vitrectomy was performed under balanced salt solution perfusion. All of the obtained samples were promptly stored at 4°C and then brought to the clinical laboratory of the university hospital for cell analysis (including flow cytometry). Peripheral blood samples were collected preoperatively from patients, and samples were immediately analyzed with the following experiment.

Procedure of Flow Cytometry Analysis of Vitreous and Peripheral Blood Samples

Vitreous samples were filtered with a 70-μm cell strainer (BD Falcon Cell Strainer; BD Biosciences, Bedford, MA) and then washed and resuspended with phosphate-buffered saline (Nissui, Tokyo, Japan) containing 2% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) and 0.1% sodium azide (Nacalai Tesque) at a final volume of 0.5 ml. For the flow cytometric analysis, 0.1 ml of each sample was incubated with one of the following mixtures of monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated mouse immunoglobulin-G1 (Dako Denmark AS, Glostrup, Denmark)/phycoerythrin (PE)-conjugated mouse immunoglobulin-G1 (Dako Denmark AS)/PE-Cy-Chrome5 (Cy5)–conjugated anti-CD45 (clone T/29/33; Dako Denmark AS), anti–CD3-FITC (UCHT1; Dako Denmark AS)/anti–CD19-PE (HD37; Dako Denmark AS)/anti–CD45-Cy5 (T29/33; Dako Denmark AS), or anti–CD4-FITC (T4; Beckman Coulter, Miami, FL)/anti–CD8-PE (T8; Beckman Coulter)/anti–CD3-Cy5 (UCHT1; Dako Denmark AS), respectively. After incubation for 15 minutes at room temperature in complete darkness, the samples were washed again and resuspended at a final volume of 0.5 ml. To examine the lymphocyte subsets of the vitreous samples using an Epics XL-MCL flow cytometer (Beckman Coulter), we initially counted $CD45^+$ cells on CD45 versus side-scatter plots combined with forward-scatter and side-scatter. If more than 100 $CD45^+$ cells were found to be contained in 1 test tube, then further analysis was performed to examine the T-cell ($CD3^+$ $CD45^+$)/B-cell ($CD19^+$ $CD45^+$) ratio, as well as the $CD3^+$ $CD4^+$ T-cell/$CD3^+$ $CD8^+$ T-cell ratio. The remaining portion (0.2 ml) of the resuspended vitreous sample was prepared for cytology with a cytocentrifuge (Cytospin 4; Thermo Fisher Scientific, Inc., Waltham, MA).

Flow cytometry was also used to study the peripheral blood lymphocyte population. Briefly, whole blood anticoagulated with ethylenediaminetetraacetic acid was stained with the same panels of monoclonal antibodies used for vitreous samples, treated with Multi-Q-prep (Beckman Coulter) to lyse the contaminating red blood cells, washed, and resuspended at a final volume of 0.5 ml/tube.

Real-Time Polymerase Chain Reaction Analysis of Vitreous Samples

Genomic DNA of human herpes virus (HHV) in the aqueous humor and vitreous was measured through the use of 2 independent PCR assays (a qualitative multiplex PCR assay and a quantitative real-time PCR assay) as described previously.[24,25] DNA was extracted from samples using an E21 virus minikit (QIAGEN, Inc., Valencia, CA) installed on a robotic workstation for automated purification of the nucleic acids (BioRobot E21; QIAGEN, Inc.). The multiplex PCR was designed to qualitatively measure the genomic DNA of 8 types of HHV and other ocular pathogens: herpes simplex virus (HSV) type 1 (HSV-1 or HHV-1) and type 2 (HSV-2 or HHV-2), varicella zoster virus (HHV-3), Epstein–Barr virus (HHV-4), cytomegalovirus (HHV-5), HHV-6, HHV-7, HHV-8, *Propionibacterium acnes*, *Toxoplasma*, *Toxocara*, *Bartonella henselae*, *Chlamydia trachomatis*, *Treponema pallidum*, *Mycobacterium tuberculosis*, *Candida* (18s rRNA), *Aspergillus* (18s rRNA), and bacterial 16s rRNA. The PCR was performed using a LightCycler (Roche Diagnostics [Schweiz] AG, Rotkreuz, Switzerland). Primers and probes of HHV-1–8 and the PCR conditions have been described.[24] Specific primers for the virus were used with Accuprime Taq (Invitrogen, Carlsbad, CA). The products were subjected to 40 cycles of PCR amplification. Hybridization probes were then mixed with the PCR products. Subsequently, real-time PCR was performed only for the HHVs, with the genomic DNA detected by multiplex PCR. The real-time PCR was performed using Amplitaq Gold (Applied Biosystems, Foster City, CA) and the Real-Time PCR 7300 system (Applied Biosystems). All of the products obtained were subjected to 45 cycles of PCR amplification. The value of the viral copy number in the sample was considered to be significant when more than 50 copies/tube (5000 copies/ml) were observed.

Statistical Analysis

Statistical comparisons of the CD4/CD8 ratios between the groups were carried out by nonparametric analysis using the Mann–Whitney *U* test. Sensitivity and specificity were calculated for the CD4/CD8 ratio of vitreous and peripheral samples in detecting sarcoidosis. Positive predictive value was also estimated from sensitivity and specificity value:

$$\text{predictive value (\%)} = \text{pretestprobability} \times \text{sensitivity}/100/ [\text{pretest probability} \times \text{sensitivity}/100 + (100 - \text{pretest probability}) \times (100 - \text{specificity})/100]$$

Pretest probability was calculated as 17% according to the percentage of patients with ocular sarcoidosis in our uveitis outpatient clinic. Moreover, performances of CD4/CD8 ratio and its cutoff points were assessed using the receiver operator curve space. These analyses were performed using Prism software, version 5.0.1 (Graph Pad Software, San Diego, CA).

Results

Flow Cytometric Analysis of Vitreous Fluid and Peripheral Blood Samples

Flow cytometric data are summarized in Tables 1 and 2 (available at http://aaojournal.org). Representative flow cytometric data of sarcoid vitreous samples are shown in Figure 1 (sample obtained from a 59-year-old subject with confirmed ocular sarcoidosis; first line of Table 1, available at http://aaojournal.org). In the sarcoidosis group, the mean CD4/CD8 ratios of vitreous and peripheral blood sample were 40.7 (95% confidence interval [CI], 3.5–77.9) and 3.0 (95% CI, 2.5–3.5), respectively. In each classification of the ocular sarcoidosis group, the mean CD4/CD8 ratios of vitreous and peripheral blood sample were 70.0 (95% CI, −34.6 to 174.6) and 2.7 (95% CI, 1.9–3.5) for definite ocular sarcoidosis, 26.9 (95% CI, 15.4–38.4) and 3.1 (95% CI, 2.4–3.8) for presumed ocular sarcoidosis, and 22.9 (95% CI, 7.9–37.9) and 3.5 (95% CI, 2.5–4.5) for probable ocular sarcoidosis, respectively. In the nonsarcoidosis group, the mean CD4/CD8 ratios of vitreous and peripheral blood sample were 2.0 (95% CI, 1.1–2.9) and 2.0 (95% CI, 1.5–2.5), respectively. The CD4/CD8 ratios of lymphocytes obtained from the vitreous samples of patients with ocular sarcoidosis were significantly higher ($P < 0.0001$) than those in the vitreous of the nonsarcoidosis group (Fig 2A). Also, a significant difference was observed in the CD4/CD8 ratio of peripheral lymphocytes between the sarcoidosis and nonsarcoidosis groups ($P = 0.0163$) (Fig 2B). In addition, the CD4/CD8 ratios of lymphocytes obtained from the vitreous samples were significantly higher ($P < 0.0001$) than those of the peripheral blood samples in the ocular sarcoidosis group (Fig 2C). However, in the nonsarcoidosis group, no significant difference was found ($P = 0.67$) between the CD4/CD8 ratio of the vitreous samples and the peripheral samples (Fig 2D).

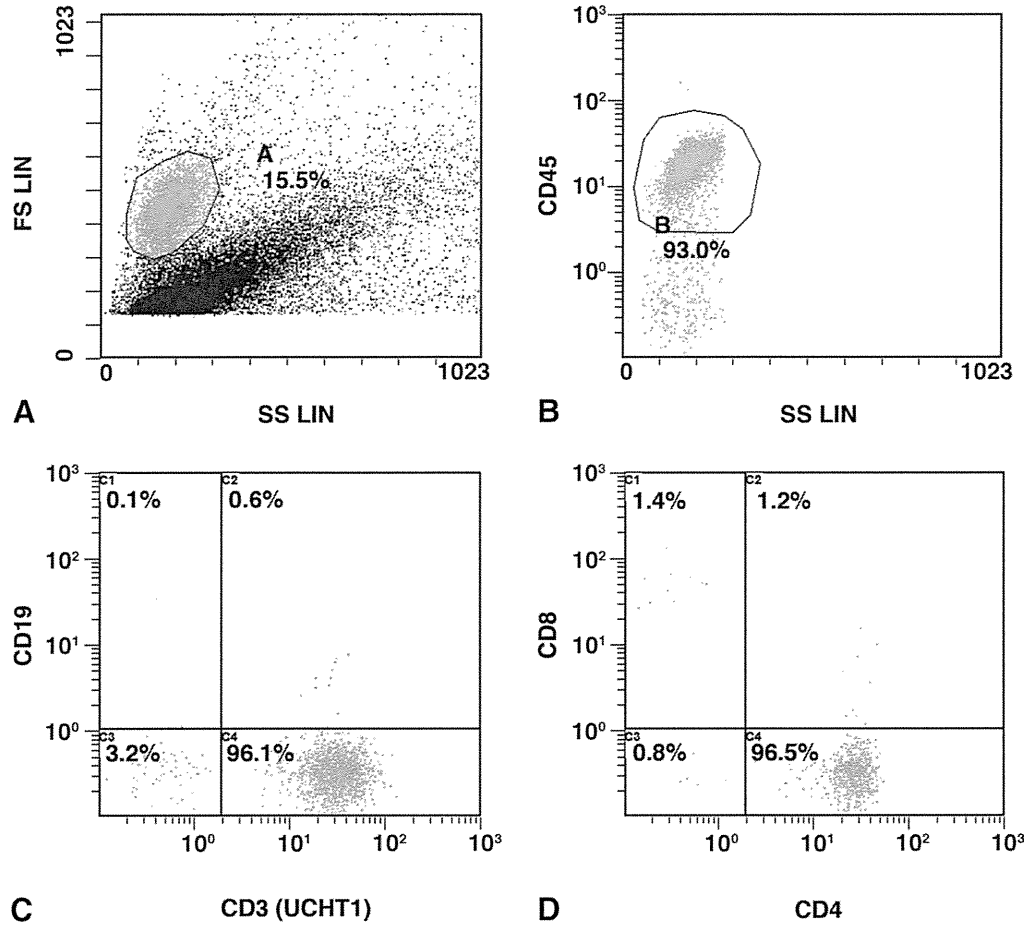

Figure 1. Representative flow cytometric data in confirmed ocular sarcoidosis. Total cells from vitreous samples (**A**) were gated with CD45+ cell population (**B**), CD3+ or CD16+CD56+ cell population (**C**), and CD4+ or CD8+ cell population (**D**). FS = forward scatter; LIN = liner; SS = side scatter.

Multiplex Polymerase Chain Reaction Analysis Results of Vitreous Samples

In the nonsarcoidosis group, multiplex PCR revealed varicella zoster virus DNA in 1 patient with an epiretinal membrane and herpes virus infection, bacterial 16s rRNA in 4 patients with endophthalmitis, and HSV-2 DNA in 3 patients with acute retinal necrosis and iritis (Table 2, available at http://aaojournal.org). Moreover, the PCR analysis detected cytomegalovirus in 2 patients. *Toxocara* DNA was not detected by PCR analysis, but the 2 positive results were elicited on *Toxocara* serology[26] using vitreous samples and peripheral blood. No viral or bacterial DNA was detected in the sarcoidosis group, patients with idiopathic uveitis, or patients with primary intraocular lymphoma.

Statistical Analysis

All of the patients in the ocular sarcoidosis group had high CD4/CD8 ratios (>3.5). Only 1 patient in the nonsarcoidosis group with endophthalmitis had a high CD4/CD8 ratio. The sensitivity and specificity of the vitreal CD4/CD8 ratio with a cutoff point of 3.5 were 100% and 96.3%, respectively, for the diagnosis of ocular sarcoidosis. The sensitivity and specificity of the peripheral CD4/CD8 ratio with a cutoff point of 2.7 were 52.6% and 84.6%, respectively, for the diagnosis of ocular sarcoidosis. The positive predictive values of vitreal and peripheral CD4/CD8 ratios were 84.2% and 41.2%, respectively. We compared the diagnostic performance of vitreal and peripheral CD4/CD8 ratio by plotting their performance in receiver operator curve space (Fig 3). The vitreal CD4/CD8 ratio was shown to have better diagnostic performance than the peripheral CD4/CD8 ratio by calculating the area under the curve. The best cutoff points for vitreal and peripheral CD4/CD8 ratio were 3.5 and 2.7, respectively.

In all patients involved in this study, the clinical course was favorable, with no adverse events resulting from the surgical procedures, including the collection of the vitreous samples.

Discussion

To the best of our knowledge, this is the first report to describe the unique immunologic features of vitreous lymphocytosis in ocular sarcoidosis characterized by a high CD4/CD8 ratio. A CD4/CD8 ratio of vitreous-infiltrating lymphocytes greater than 3.5 provided a diagnosis of ocular sarcoidosis with a sensitivity of 100% and a specificity of 96.3%, which are both remarkably high values. A CD4/CD8 ratio greater than 3.5 in lymphocytes obtained from BAL fluid had a sensitivity of 53% and a specificity of 94%.

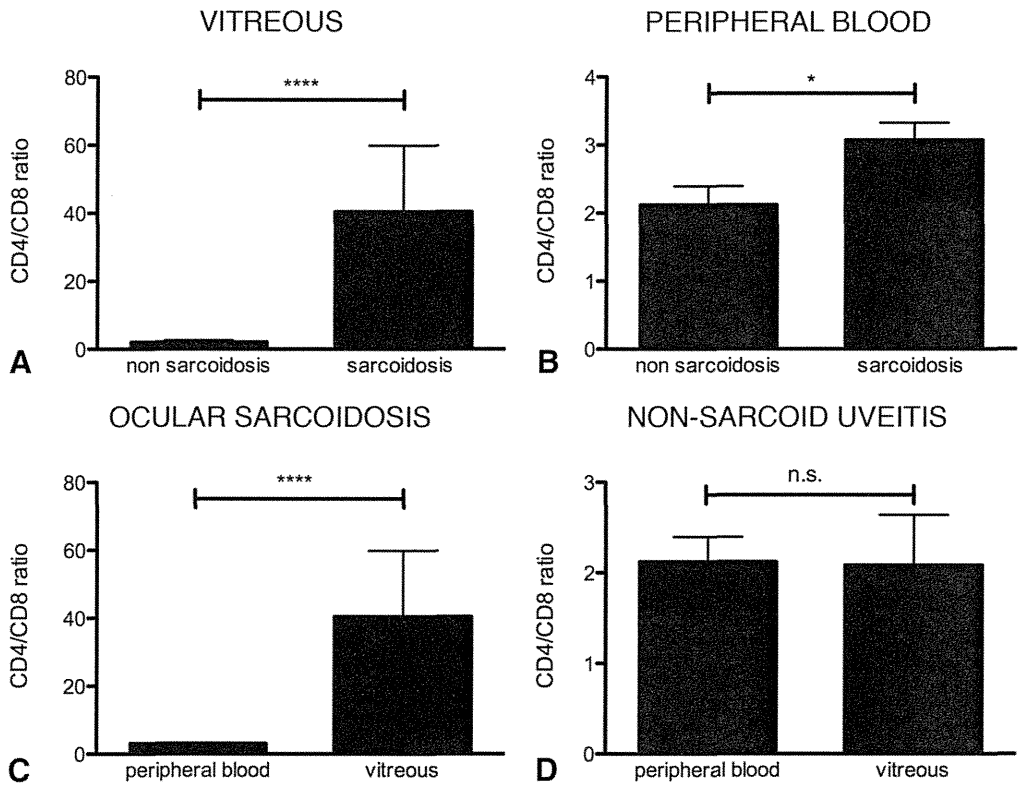

Figure 2. Quantitative analysis of CD4/CD8 ratios in vitreous and peripheral blood samples of sarcoidosis and nonsarcoidosis groups. **A,** Vitreous: T lymphocytes obtained from the ocular sarcoidosis vitreous samples were significantly higher (****$P < 0.0001$) than those of nonsarcoidosis vitreous samples. **B,** Peripheral blood: A significant difference was observed in the CD4/CD8 ratio of peripheral lymphocytes between the sarcoidosis and nonsarcoidosis groups (*$P = 0.0163$). **C,** Ocular sarcoidosis: CD4/CD8 ratio of T lymphocytes between the vitreous and peripheral blood samples in the sarcoidosis group (****$P < 0.0001$). **D,** Nonsarcoid uveitis: In the nonsarcoidosis group, there was no significant difference between the vitreal and peripheral blood CD4/CD8 ratios of T lymphocytes. n.s. = not significant.

Thus, our results indicate that a high CD4/CD8 ratio of lymphocytes obtained from the vitreous has a high diagnostic value, comparable to that of a high CD4/CD8 ratio in BAL fluid lymphocytes.

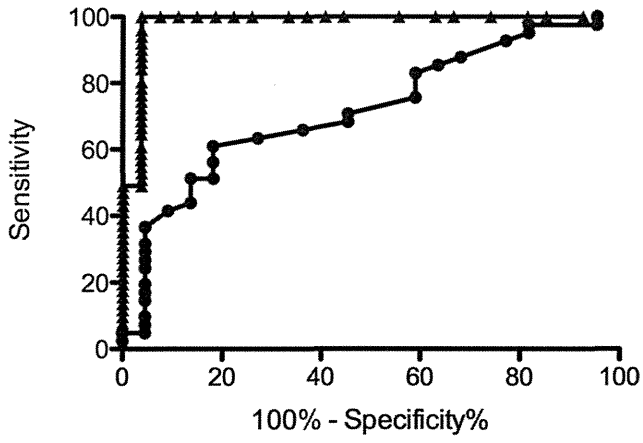

Figure 3. Performance of CD4/CD8 ratios in vitreous and peripheral blood samples discriminating sarcoidosis and nonsarcoidosis groups in receiver operator curve space. The comparison of diagnostic performance with vitreal and peripheral CD4/CD8 ratio by plotting their performance in receiver operator curve space.

Patients with sarcoidosis first present in the clinical setting with a variety of symptoms, and ocular symptoms account for a relatively high percentage. It has been reported that approximately 30% to 60% of patients with sarcoidosis manifest ocular involvement.[13–16] Kataoka[27] reported that ocular symptoms are the most frequently seen symptom at first presentation in Japanese patients. At the present time, diagnosis of sarcoidosis presenting with ocular manifestation requires other systemic features to confirm diagnosis. However, there are considerable numbers of suspected ocular sarcoidosis cases with a negative result in the systemic search.[28] Transbronchial lung biopsy and BAL are said to be relatively less-invasive procedures, yet there is currently disagreement as to whether these procedures should be performed in patients without clinical and radiologic pulmonary presentation. Ocular sarcoidosis manifests peculiar focal lymphocytosis characterized by an elevated CD4/CD8 ratio, and this unique CD4/CD8 pattern is significantly higher than that of peripheral blood. This immunologic dissociation between ocular and peripheral lymphocytes is an important aspect for the diagnosis of ocular sarcoidosis, because this feature is not observed in other types of uveitis. Therefore, it is rational to use this immunologic information to diagnose sarcoidosis with an active lesion localized in the eye to enable an early and less-invasive diagnosis. Of note,

the CD4/CD8 ratio in the peripheral blood was not so high in patients with sarcoidosis but still significantly different than in the patients without sarcoidosis. This may account for the systemic aspect of this disorder, showing that this immunologic feature is not only limited to the active lesion site.

Likewise, the hallmark of sarcoidosis in other organs is the identification of a noncaseating epithelioid cell granuloma. There are reports of identifying granulomata in conjunctiva[29] and the Schlemm's canal,[30] but presentation of granuloma in the conjunctiva is a rare case, and a Schlemm's-canal biopsy is only possible at the time of trabeculectomy surgery, which is only performed when sarcoidosis-induced glaucoma cannot be controlled by topical medication. Because it is rare to find a granuloma in the vitreous, and retinal biopsies are accompanied with the risk of serious complications such as retinal detachment, flow cytometric analysis of vitreous-infiltrating lymphocytes can be considered as a helpful and safer diagnostic tool.

Diagnostic vitrectomy for chronic uveitis of unknown cause is widely performed.[20,21] Davis et al[23] reported the usefulness of vitreous specimen analysis, including flow cytometry, for the differential diagnosis of intraocular lymphoma, chronic infectious endophthalmitis, and atypical chorioretinitis. Our results show that through cytometric analysis of a vitrectomy specimen, it is possible to distinguish sarcoidosis from other types of uveitis. Multiplex PCR is also helpful for ruling out other diagnoses, such as infection. There are reports that have suggested an association between sarcoidosis and propionibacterial infection.[6,30] There is no evident explanation among propionibacterial infection, noncaseating granuloma, and lymphocyte subsets at the present time, but it is safe to say that ocular sarcoidosis and pulmonary sarcoidosis have identical pathologic features. Further experiments are warranted to resolve this kind of discrepancy.

Basic treatment for ocular sarcoidosis involves corticosteroid medication, but vitrectomy for chronic uveitis-like sarcoidosis, especially for those cases resistant to pharmacologic treatment, is well documented in the published literature. Good outcomes of surgical treatment for sarcoidosis are reported for vitreous opacity resistant to medical treatment,[31] cystoid macular edema,[32] and epiretinal membrane.[33] Likewise, our operating procedures (including vitreous sample extraction) had no adverse events, indicating that this diagnostic procedure is safe for the patient. Clinical course was favorable for all the patients, and good visual acuity was achieved during the follow-up period (data not shown). Because of this good clinical outcome, we believe that a diagnostic vitrectomy should be recommended whenever a patient with suspected sarcoidosis has symptoms associated with vitreous opacity or epiretinal membrane.

In conclusion, in patients with systemic sarcoidosis (based on the international classification), diagnostic vitrectomy with flow cytometric lymphocyte analysis is useful as an adjunct to the standard diagnosis of ocular sarcoidosis. In these patients, information obtained from vitreous samples, especially a CD4/CD8 ratio greater than 3.5, has high diagnostic value, comparable to the CD4/CD8 ratio in BAL fluid lymphocytosis in pulmonary sarcoidosis. The role of diagnostic vitrectomy with CD4/CD8 analysis in subjects with questionable systemic diagnoses remains a topic for additional study.

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Ocular surface inflammation is regulated by innate immunity

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ABSTRACT

On the ocular surface, as in the intestines and airway, the surface epithelium serves a critical function as the front-line defense of the mucosal innate immune system. Although the detection of microbes is arguably the most important task of the immune system, an exaggerated epithelial host defense reaction to endogenous bacteria may initiate and perpetuate inflammatory mucosal responses.

In this review we first describe commensal bacteria found on the ocular surface, which is in contact with the ocular surface epithelium. We also discuss the innate immunity of the ocular surface epithelium and we present the allergic reaction regulated by ocular surface epithelial cells. We address ocular surface inflammation due to disordered innate immunity and we present our hypothesis that the onset of Stevens-Johnson syndrome (SJS) with severe ocular surface complications, a devastating ocular surface inflammatory disease, is strongly associated with abnormality of the innate immune system.

In this review we raise the possibility that some ocular surface inflammatory diseases are pathogenetically related with a disordered innate immune response.

Focusing on the innate immunity of the ocular surface might help to elucidate the pathogenesis of various ocular surface diseases.

Contents

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1. Introduction

On the ocular surface, as in the intestines and airway, the surface epithelium serves a critical function as the front-line defense of the mucosal innate immune system (Haynes et al., 1999; Streilein, 2003). Epithelial cells lining mucosal surfaces play a pivotal role in innate immunity; upon challenge they secrete chemokines and other immune mediators. The ocular surface epithelium also features all isoforms of human beta defensins and can produce inflammatory cytokines such as interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)α, IL-6, and IL-8. Anti-microbial molecules such as IgA, lysozyme, and lactoferrin are found in tear fluids. Goblet cells in conjunctival epithelium produce mucin. Thus, the ocular surface possesses many non-specific defense mechanisms against microbes (Fig. 1).

Although the detection of microbes is arguably the most important task of the immune system, an exaggerated epithelial host defense reaction to endogenous bacteria may initiate and perpetuate inflammatory mucosal responses (Bouma and Strober, 2003; Strober, 2004; Strober et al., 2002).

In this review we first describe commensal bacteria found on the ocular surface, which is in contact with the ocular surface epithelium. We also discuss the innate immunity of the ocular surface epithelium and we present the allergic reaction regulated by ocular surface epithelial cells. We address ocular surface inflammation due to disordered innate immunity and we present our hypothesis that the onset of Stevens-Johnson syndrome (SJS) with severe ocular surface complications, a devastating ocular surface inflammatory disease, is strongly associated with abnormality of the innate immune system.

We propose that the pathogenesis of some human ocular surface inflammatory diseases is related to a disordered innate immune response.

2. Commensal bacteria on the ocular surface

2.1. Commensal bacteria

Bacterial flora comprised of gram-positive and gram-negative organisms can be found on the skin and in mucosal tissues. The ocular surface and other mucosal tissue are host to commensal bacteria (Doyle et al., 1995; Hara et al., 1997; Ueta et al., 2007a). To examine these organisms present on the ocular surface we harvested commensal bacteria from the lower conjunctival sacs of 42 healthy volunteers using CultureSwab (Becton Dickinson, Brescia, Italy) without touching the lids. *Staphylococcus epidermidis* (*S. epidermidis*) was isolated from 45% of the volunteers and *Propionibacterium acnes* (*P. acnes*) from 31% (Ueta et al., 2007a). Interestingly, although the ocular surface epithelium is in constant contact with bacteria and bacterial products, the healthy ocular surface is not in an inflammatory state. The ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes (Fig. 2).

2.2. Polyclonality of S. epidermidis

In humans, the predominant staphylococcus species *S. epidermidis* is widely distributed over the body surface (Kloos and Musselwhite, 1975). When we used pulsed-field gel electrophoresis (PFGE) to examine the diverse genetic background of *S. epidermidis* isolated from the ocular surface of healthy donors we found that the bacterium was polyclonal (Ueta et al., 2007a) (Fig. 3A).

For analysis, DNA bands were compared by visual inspection and interpreted according to Tenover et al. (1995). Based on the number of DNA fragments that exhibited different pulse patterns, strains with identical pulse patterns, and those with 2–3 or 4–6 fragments of different patterns were considered indistinguishable, closely related, and possibly related, respectively. When more than 6 DNA fragments manifested a different migration pattern, the isolates were considered to be unrelated.

We analyzed commensal bacteria isolated from the conjunctival sac, upper and lower lid margins, and upper and lower meibomian glands of another 40 healthy volunteers. *S. epidermidis* was isolated from 12 individuals; 7 harbored these bacteria at multiple ocular surface sites. Interestingly, *S. epidermidis* organisms isolated from multiple sites in single subjects were polyclonal. When we analyzed *S. epidermidis* isolated from the conjunctival sac of the same donor at different time points, we also found that the organisms were polyclonal and yielded multiple colonies. At some sampling points there was a change in the dominant strain (Ueta et al., 2007a).

Although *S. epidermidis* is a common component of the normal ocular flora, it can lead to chronic blepharitis, conjunctivitis, and keratitis, especially in immunocompromised hosts (Baum, 1978; Pinna et al., 1999), suggesting that opportunistic infection with *S. epidermidis* is reflective of the status of the host. We encountered one instance in which monoclonal *S. epidermidis* was isolated from multiple sites in both eyes (Fig. 3B). The host was an immunocompromised patient who had undergone bone marrow transplantation. Disruption of the balance between *S. epidermidis* and the immune status of this host may have resulted in the monoclonality of these bacteria. Based on these observations we postulated that a balance between commensal bacteria and the host mucosal immunity maintains the polyclonality of *S. epidermidis*, which may contribute to homeostasis of the commensal organisms, and that a weakened host mucosal immune status may contribute to their pathogenicity. When the host mucosal immunity is normal, commensal bacteria are in a symbiotic relationship with the host, however, if the host's mucosal immunity is abnormal, commensal bacteria can become pathogenic (Ueta et al., 2007a) (Fig. 3C).

The finding of Seal et al. (1985) that a specific strain of *S. epidermidis* could increase in the lids of blepharitis patients and manifest pathogenicity on the ocular surface may indicate that the role of *S. epidermidis* on the ocular surface requires further investigation.

2.3. Hypersensitivity to bacteria

Ocular surface inflammations such as catarrhal ulcers (marginal keratitis) and phlyctenular keratitis are thought to reflect

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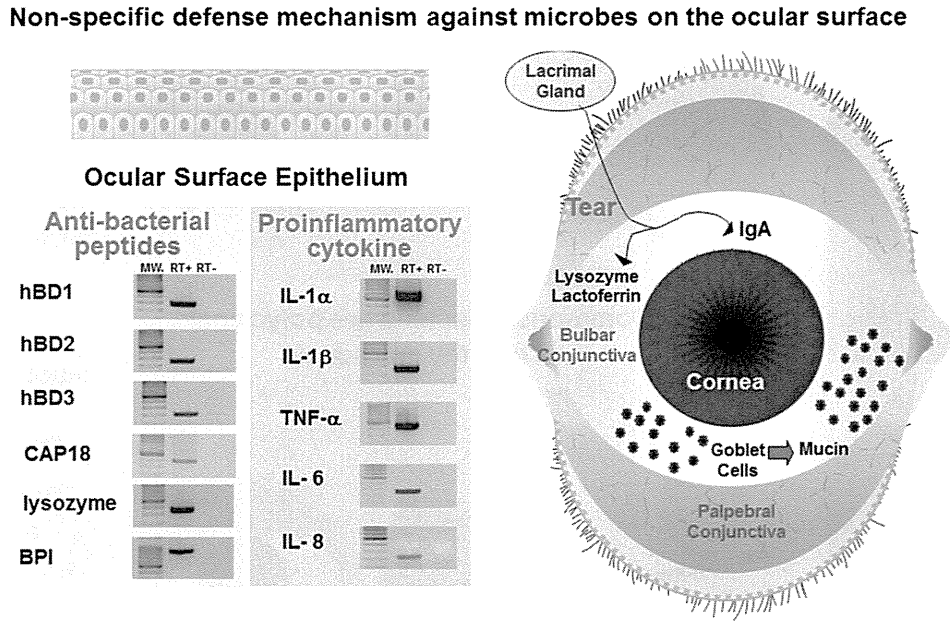

Fig. 1. Non-specific defense mechanism against microbes on the ocular surface. The ocular surface epithelium harbors all isoforms of human beta defensins and can produce inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6, and IL-8 (left: RT-PCR, human corneal epithelial cells.). Tear fluids contain anti-microbial molecules such as IgA, lysozyme and lactoferrin. Goblet cells in conjunctival epithelium produce mucin (right).

a hypersensitivity to bacteria (Mondino et al., 1978, 1981; 1982; Seal et al., 1985).

In rabbits immunized with *Staphylococcus aureus* (*S. aureus*) cell walls, Mondino and Kowalski (1982) observed vascularized, elevated nodular infiltrates of the cornea; topical challenge with viable *S. aureus* produced peripheral corneal infiltrates separated from the limbus by al lucid interval. Patients with symptomatic marginal keratitis requiring treatment with steroids manifested enhanced delayed hypersensitivity to *S. aureus* cell wall antigens (Ficker et al., 1989). Catarrhal ulcers are usually a complication of long-standing staphylococcal blepharitis, conjunctivitis, or meibomitis (Smolin and Okumoto, 1977; Thygeson, 1969) that may be subclinical. Cultures from the lid margins of patients with long-standing staphylococcal blepharitis, conjunctivitis, or meibomitis, usually yield colonies of *S. aureus* (Thygeson, 1969), although the lid margin of normal eyes does not usually harbor *S. aureus* (Hara et al., 1997; Doyle et al., 1995). Because corneal cultures tend to be negative for the organisms, it has been suggested that catarrhal ulcers are not the result of direct infection of the cornea but rather derive from an antigen–antibody reaction with complement activation and neutrophil infiltration in patients sensitized to staphylococcal antigens (Mondino et al., 1978, 1981; Smolin and Okumoto, 1977).

We used PFGE to analyze the relationship between catarrhal ulcers and the presence of *S. aureus*. The diagnosis of catarrhal ulcer

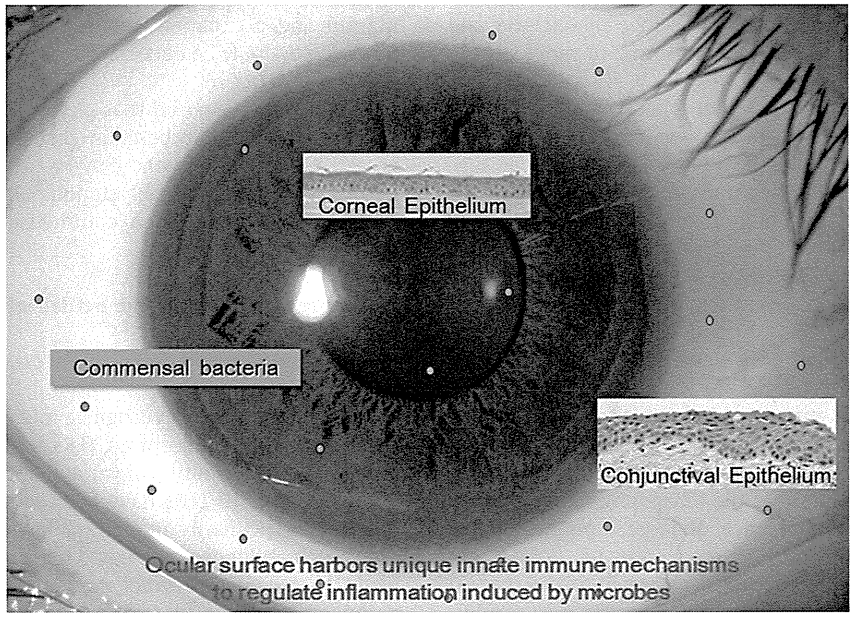

Fig. 2. Healthy ocular surface. The healthy ocular surface is not in an inflammatory state, although the ocular surface epithelium is in constant contact with bacteria and bacterial products. Ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes.

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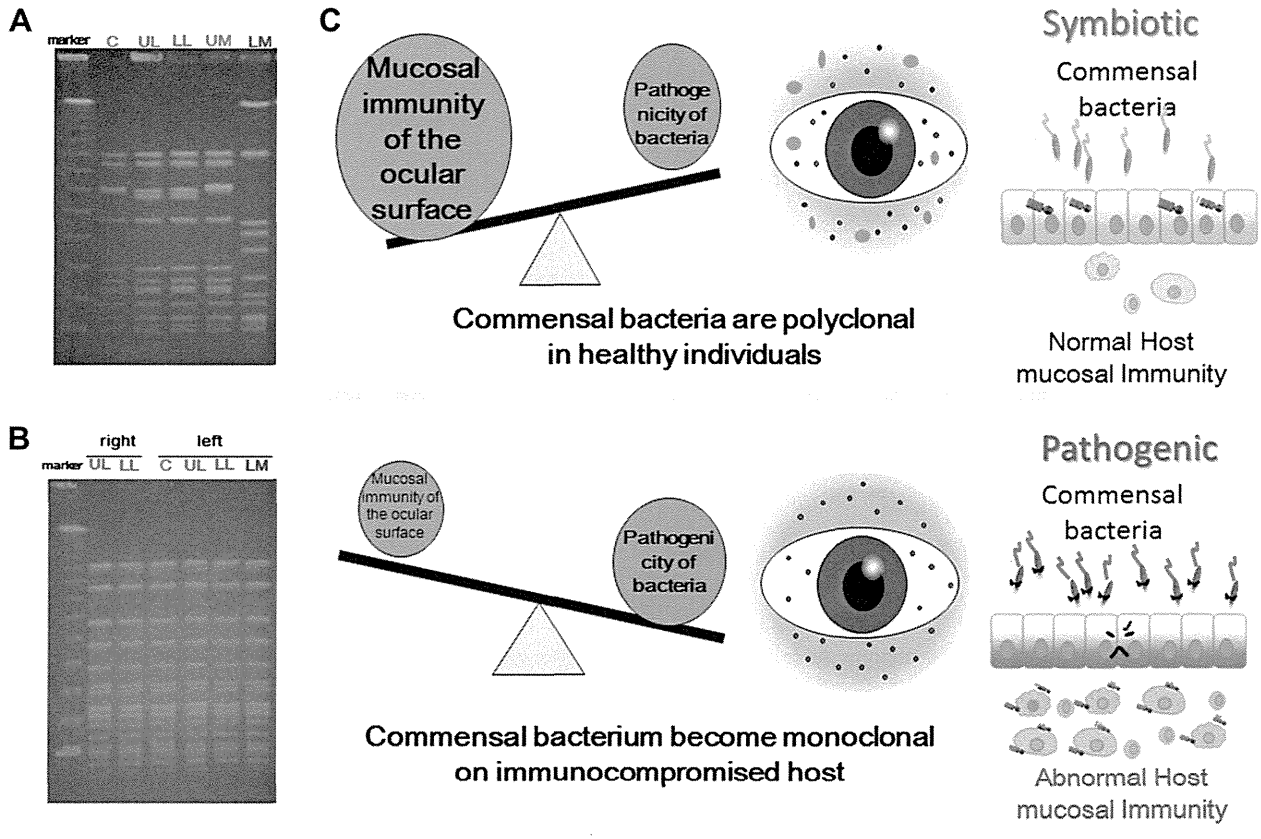

Fig. 3. Importance of the balance between mucosal immunity of the ocular surface and pathogenicity of bacteria. A. The PFGE patterns of *S. epidermidis* on healthy ocular surfaces were polyclonal. B. The PFGE patterns of *S. epidermidis* isolated from multiple sites in both eyes of an immunocompromised patient were monoclonal. (PFGE: pulsed-field gel electrophoresis, C: conjunctiva, UL: upper lid margin, LL: lower lid margin, UM: upper meibomian gland, LM: lower meibomian gland). A&B; Reprinted with permission from Ueta et al. (Ueta et al., 2007a). C. A good balance between commensal bacteria and host immunity maintains the polyclonality of *S. epidermidis*. A weakened host immune status may contribute to the bacterium's pathogenicity. When the host mucosal immunity is normal, commensal bacteria are in a symbiotic relationship with their host. However, if the host mucosal immunity is abnormal, commensal bacteria can be pathogenic.

was based on ocular surface manifestations. Clinical examinations revealed oval infiltrates, ulcers separated from the limbus by a distinct lucid border, and adjacent conjunctival inflammation (Fig. 4A). We examined 3 ocular sites (the conjunctival sac and the upper and lower lid margins) for the presence of bacteria and compared the *S. aureus* organisms isolated from 2 or more sites in each patient.

The colonization by *S. aureus* is shown schematically in Fig. 4B. In case 1, *S. aureus* was detected in the lower lid margin of the affected- and the conjunctiva of the unaffected-eye; PFGE suggested that these *S. aureus* were the same clone. In case 2, *S. aureus* was detected in the upper lid margin and conjunctiva of the affected- and in the lower lid margin of the unaffected eye; PFGE again suggested that these organisms were the same clone. In case 3, *S. aureus* was detected in the lower lid margin of the affected eye (Ueta et al., 2009b).

Although our study included only a small number of patients, we found *S. aureus* in the lid margin of eyes affected by catarrhal ulcers. This suggests that its presence at that site rather than the conjunctival sac is important for the development of catarrhal ulcers. As we were able to detect all *S. aureus* organisms in enrichment cultures, it appears that the development of catarrhal ulcers does not require the presence of large amounts of the bacterium (Ueta et al., 2009b).

Interestingly, in case 2 we also found *S. aureus* in the lid margin of the unaffected eye. Thus, even if a patient sensitized to staphylococcal antigens harbors *S. aureus* on both eyes, catarrhal ulcers may develop on only one eye. Moreover, our PFGE analysis showed that *S. aureus* detected in both eyes might be derived from the same clone, suggesting that the kind of the *S. aureus* clone is not necessarily important for the initiation of catarrhal ulcers. These findings raise the possibility that besides the presence of *S. aureus* on the lid margin and the patient's sensitivity to staphylococcal antigens, other factors may be necessary for the initiation of catarrhal ulcers (Ueta et al., 2009b).

It has been proposed that phlyctenular keratitis is reflective of hypersensitivity to tuberculin protein or *S. aureus* (Beauchamp et al., 1981; Neiberg and Sowka, 2008). Others (Suzuki et al., 2005) reported that phlyctenular keratitis in young patients might involve *P. acnes*, a commensal bacterium on the ocular surface, but not *S. aureus*.

3. Innate immunity of the ocular surface epithelium

3.1. Toll-like receptors (TLRs) of the ocular surface epithelium

The ability of cells to recognize microbial motifs is attributable to pattern recognition receptors, TLRs, important molecules associated with innate immunity (Kawai and Akira, 2007; Medzhitov et al., 1997). To date, 10 TLRs have been identified in humans; they are investigated primarily on mammalian host immune-competent cell types such as dendritic cells and macrophages. These cells are most likely to come into direct contact, via mucosal epithelia, with pathogens from the environment (Hornung et al., 2002).

The ability of cells to recognize pathogen-associated molecular patterns may depend on the expression of a family of TLRs whose

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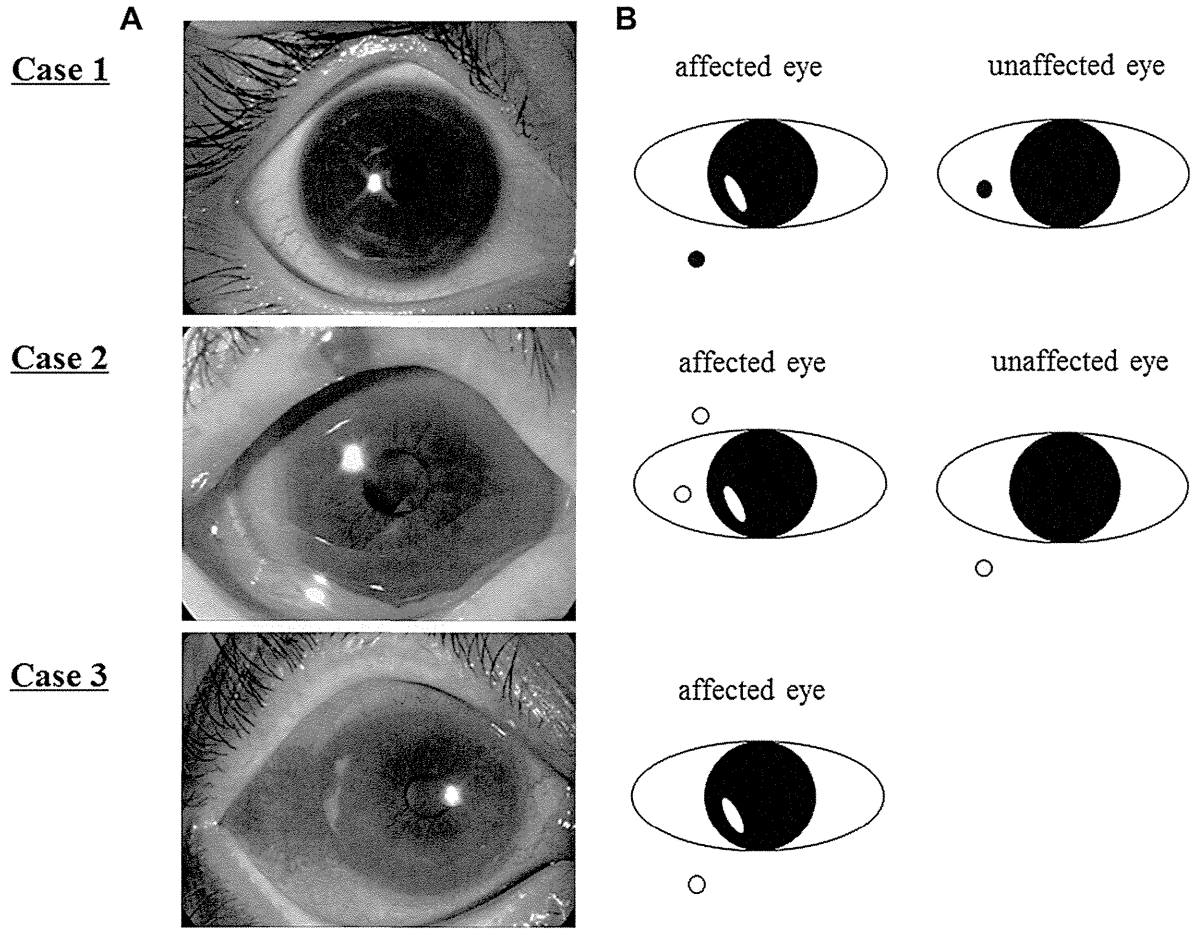

Fig. 4. Relationship between catarrhal ulcers and the presence of *S. aureus*. A. The diagnosis of catarrhal ulcer was based on ocular surface manifestations, i.e. oval infiltrates, ulcers separated from the limbus by a distinct lucid border, and adjacent conjunctival inflammation. B. The presence of *S. aureus* on the lid margin is important for the development of catarrhal ulcers. In case 1, *S. aureus* were detected on the lower lid margin of the affected- and the conjunctiva of the unaffected-eye. This suggested involvement of the same clone. In case 2, *S. aureus* were detected on the upper lid margin and the conjunctiva of the affected- and in the lower lid margin of the unaffected-eye, again suggesting involvement of the same clone. In case 3, *S. aureus* were detected on the lower lid margin of the affected eye. A&B; Reprinted with permission from Ueta et al. (Ueta et al., 2009b).

triggering results in the secretion of pro-inflammatory cytokines and interferon (IFN) α/β (Kawai and Akira, 2007; Medzhitov et al., 1997). For example, TLR2 recognizes peptidoglycan (PGN) or lipoprotein, components of the gram-positive bacterial cell wall, and forms a heterodimer with TLR1 or TLR6. TLR3 recognizes viral double-stranded (ds) RNA, which is mimicked by polyinosine-polycytidylic acid (polyI:C). Lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, is recognized by TLR4 and flagellin, a component of bacterial flagellae, by TLR5. TLR7 or TLR8 recognizes viral single-stranded RNA, and TLR9 recognizes bacterial and viral deoxy-cytidylate-phosphate-deoxy-guanylate (CpG) DNA. Bacterial and viral CpG DNA acts as a pathogen-associated molecular pattern by virtue of a 20-fold greater frequency of unmethylated CG dinucleotides in microbial-compared to vertebrate-DNA.

The function of TLR10 remains to be fully elucidated (Kawai and Akira, 2007) (Fig. 5).

TLR expression is not restricted to phagocytic cell types, rather, it appears that the majority of cells in the body including mucosal epithelial cells express TLRs (Zhang et al., 2004). Ocular surface epithelial cells are in constant contact with bacteria and bacterial products and they form a structural and functional barrier against numerous pathogenic and nonpathogenic bacteria.

Using reverse transcription-polymerase chain reaction (RT-PCR) assays we first examined whether the human ocular surface epithelium expresses mRNA specific for TLR1–TLR10. We found mRNA expression specific for TLR1–TLR10 in the human conjunctival epithelium; mRNA from all TLRs except TLR8 was present in human corneal epithelium (Ueta, 2008; Ueta and Kinoshita, 2010a) (Fig. 5B).

TLR3 recognizes viral dsRNA, which is synthesized by almost all viruses at the time of duplication. We used polyI:C in our experiments to stimulate both human peripheral mononuclear cells and primary human ocular surface epithelial cells (corneal and conjunctival epithelial cells), since viral dsRNA is mimicked by polyI:C, the ligand for TLR3. In human peripheral mononuclear cells polyI:C stimulation did not increase the production of IL-6 and IL-8. On the other hand, in human ocular surface epithelial cells, polyI:C stimulation significantly induced the secretion of IL-6 and IL-8. Since interferon (IFN)-β is controlled by TLR3 signaling, IFN-β-specific mRNA was significantly increased in polyI:C-stimulated cells. Quite surprisingly, IFN-β-specific mRNA expression was markedly higher in human corneal and conjunctival epithelial cells than peripheral mononuclear cells (Fig. 6) (Ueta et al., 2005a). Redfern et al. (2011) reported that the TLR3 agonist up-regulated the expression of the antimicrobial peptides, hBD-2 and hCAP-18, in primary human corneal epithelial cells.

TLR4 recognizes LPS, a component of the cell wall of gram-negative bacteria. In human peripheral mononuclear cells, LPS stimulation significantly increased the production of IL-6 and IL-8;

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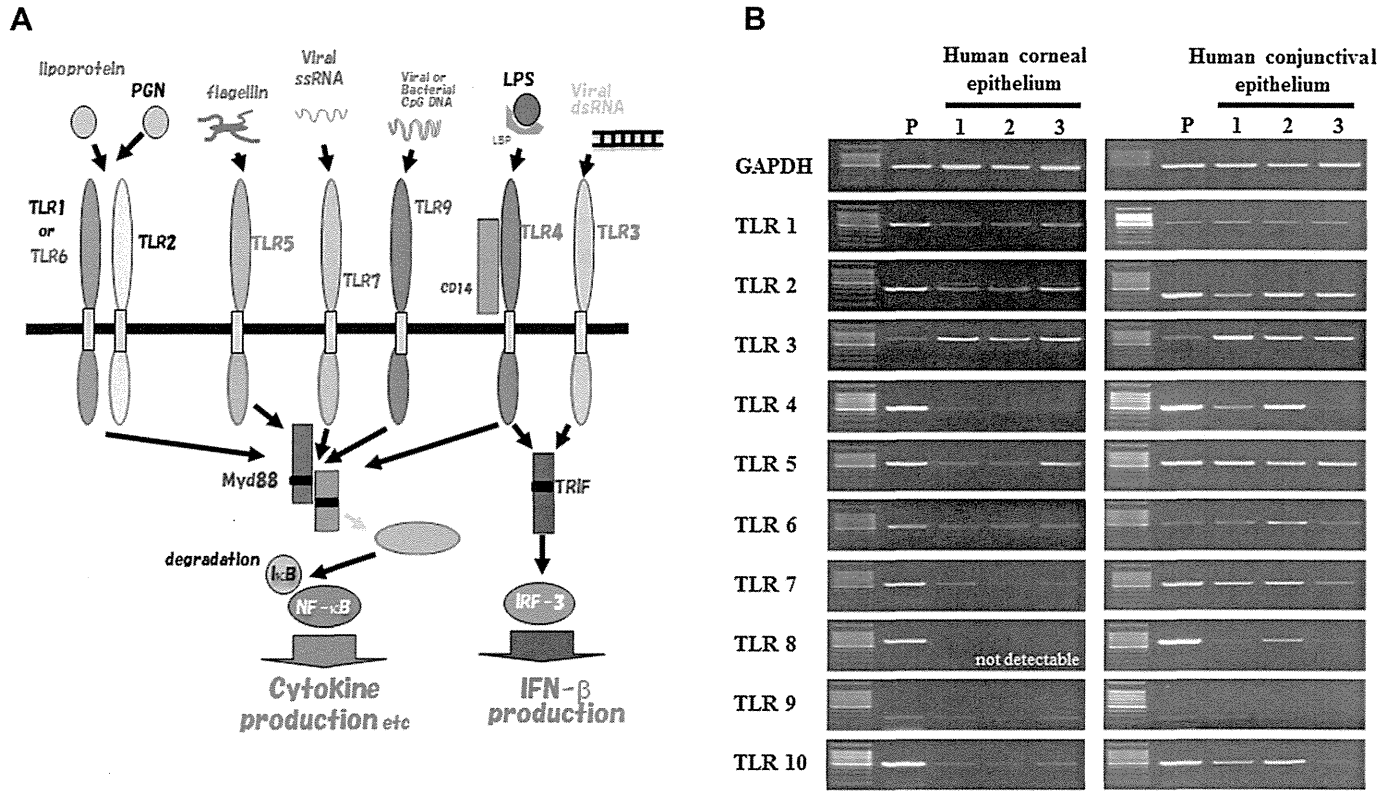

Fig. 5. A. Function of Toll-like receptors (TLRs) PGN: peptidoglycan LPS: lipopolysaccharides Myd88: myeloid differentiation factor 88 TRIF: TIR domain-containing adaptor-inducing IFN-β IRF-3: interferon regulatory factor 3. B. Human ocular surface epithelium expresses TLR-specific mRNA. The positive control (P) was human mononuclear cells. In human conjunctival epithelium we detected the expression of mRNA specific for TLR1–TLR10; mRNA from all TLRs except TLR8 was present in human corneal epithelium. (1,2,3 show samples from different individuals.). Reprinted with permission from Ueta et al. (Ueta, 2008).

in human ocular surface epithelial cells it did not induce the secretion of IL-6 and IL-8 (Fig. 6) (Ueta et al., 2004). Zhang et al. (2008) suggested that the LPS unresponsiveness of human corneal epithelial cells might be due to the deficient expression of MD-2, an essential component for LPS-TLR4 signaling. On the other hand, other groups reported that TLR4 of corneal epithelium could respond to their ligands (Johnson et al., 2005).

TLR5 recognizes flagellin, the protein of bacterial flagellae and *Pseudomonas aeruginosa* (*P. aeruginosa*) contributes to the inflammatory response of human corneal epithelium (Zhang et al., 2003). Flagellae are present mainly on gram-negative bacteria such as *P. aeruginosa*. Ocular surface-related bacteria with flagellae include pathogenic *P. aeruginosa* and non-pathogenic *Bacillus subtilis* (*B. subtilis*). We stimulated human peripheral mononuclear cells and primary human corneal and conjunctival epithelial cells with different kinds of flagellin as the ligand of TLR5. We used flagellin derived from the ocular surface pathogen *P. aeruginosa*, from the ocular surface non-pathogen *B. subtilis*, and from the intestinal pathogen *Salmonella typhimurium* (*S. typhimurium*). All flagellin stimulation of human peripheral mononuclear cells significantly increased the production of IL-6 and IL-8 (Fig. 7A). On the other hand, in human corneal and conjunctival epithelial cells only *P. aeruginosa*-derived flagellin significantly induced the secretion of IL-6 and IL-8; *B. subtilis*- and *S. typhimurium*-derived flagellin did not (Fig. 7A) (Hozono et al., 2006; Kojima et al., 2008; Ueta, 2008).

Our immunohistochemical studies showed that TLR5 protein was consistently and abundantly expressed only at basal- and wing-sites in stratified corneal and conjunctival epithelium, indicating a spatially selective presence on the basolateral- but not the apical-side (Fig. 7B) (Hozono et al., 2006; Kojima et al., 2008; Ueta, 2008). Although ocular surface epithelial cells respond to flagellin derived from ocular pathogenic bacteria through TLR5 and produce inflammatory cytokines, superficial ocular surface epithelial cells do not express TLR5. Therefore, it is reasonable to speculate that TLR5 of the ocular surface epithelium cannot function on the healthy ocular surface without epithelial defects (Hozono et al., 2006; Kojima et al., 2008; Ueta, 2008).

According to Kumar et al. (2007), pre-exposure of human corneal epithelial cells to low-dose flagellin induced a state of tolerance characterized by the reduced production of IL-8 and TNF-α upon subsequent challenge with a high dose of flagellin; they noted Pseudomonas-induced up-regulation of antimicrobial genes such as hBD-2 and LL-37.

In summary, ocular surface (corneal and conjunctival) epithelial cells selectively respond to microbial components and induce limited inflammation. Immune-competent cells such as macrophages, on the other hand, recognize various microbial components through different TLRs, induce inflammation, and then exclude the microbes. The difference between macrophages and ocular surface epithelial cells may be ascribable to dissimilarities due to the latter's coexistence with commensal bacteria. The unique innate immune response of the ocular surface epithelium might contribute to its ability to coexist with commensal bacteria (Ueta, 2008; Ueta and Kinoshita, 2010a).

3.2. Function of TLR3 in the ocular surface epithelium

Stimulation with polyI:C, a TLR3 ligand, elicited an increase in the mRNA expression of IL-6, IL-8, and IFN-β in human ocular epithelial cells (corneal and conjunctival epithelial cells) (Ueta,

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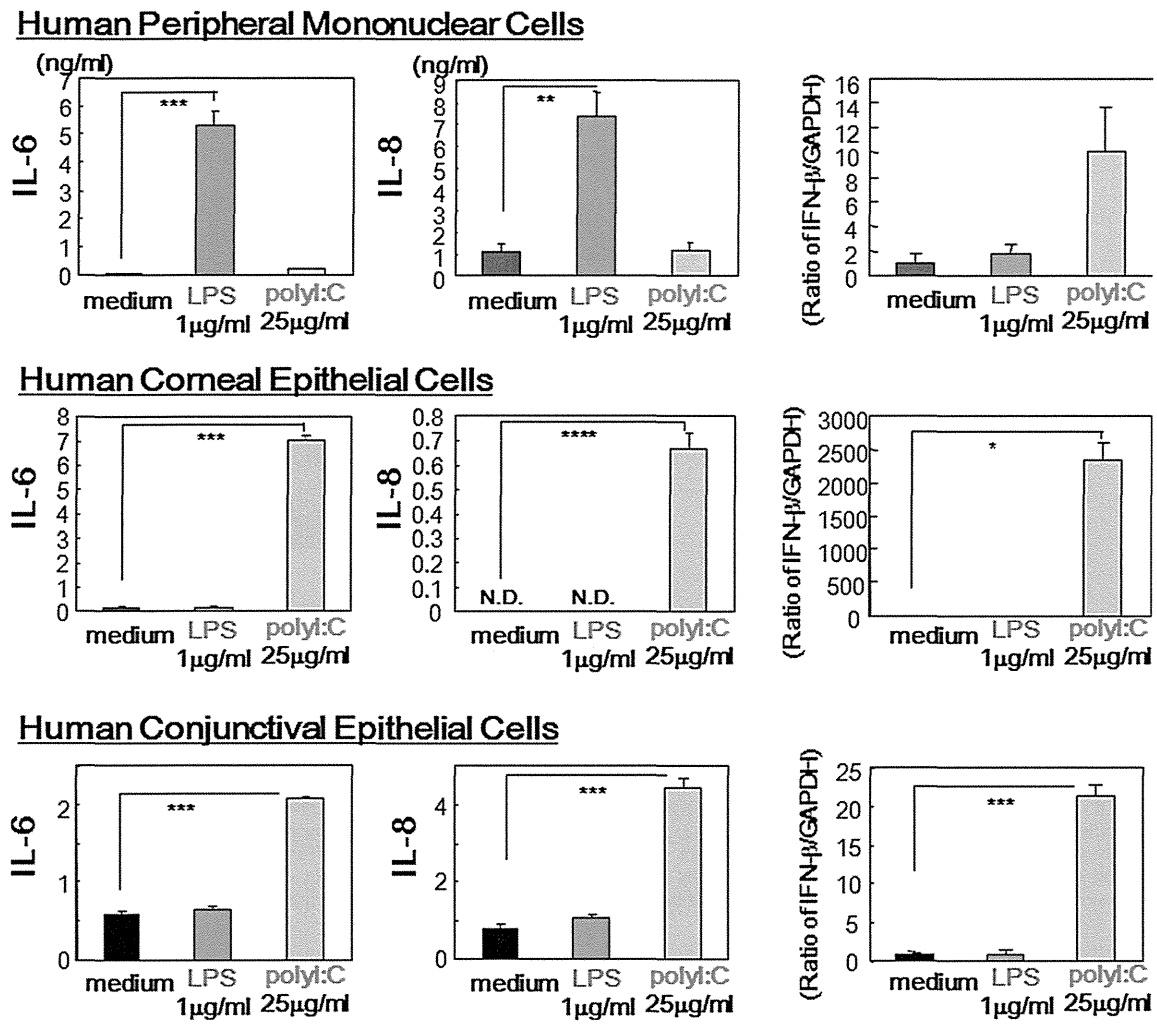

Fig. 6. *Response of human peripheral mononuclear- and of primary human corneal- and -conjunctival epithelial cells to polyI:C, the TLR3 ligand, and LPS, the TLR4 ligand.* Cultured cells were left untreated or exposed to polyI:C (25 μg/ml) or LPS from *P. aeruginosa* (1 μg/ml) for 24 h and assayed for the production of IL-6 and IL-8, or they were incubated for 3hr and assayed for the expression of IFN-β mRNA. For all cell types, the ratio of IFN-β/GAPDH mRNA (right-most column) shows an increase in specific mRNA over unstimulated cells. Data show the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$); evaluation was with Student's *t*-test using the Excel program. Modified with permission from Ueta et al. (Ueta et al., 2005a; Ueta and Kinoshita, 2010a).

2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a). PolyI:C stimulation also up-regulated the mRNA expression of the antiviral chemokine IFN-γ inducible protein 10 (IP-10), myxovirus resistance gene A, and 2′,5′-oligoadenylate synthetase (Kumar et al., 2006).

To examine the comprehensive effects of polyI:C stimulation of primary human conjunctival epithelial cells we subjected cells that had, or had not been cultured with polyI:C to gene expression analysis. We found that polyI:C stimulation induced the up-regulation of many transcripts: 150 were up-regulated more than 3-fold and 47 were up-regulated more than 10-fold. Quantitative RT-PCR confirmed the up-regulation of 11 of these transcripts, i.e. CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, RIG-I, and MDA-5 (Fig. 8) (Ueta et al., 2010b).

Although they are also innate-immune-response-related genes, CXCL11, CXCL10 (Klunker et al., 2003; Ying et al., 2008), IL28A (Bullens et al., 2008), CCL5, CCL4, and CCL20 (Gros et al., 2009) have been reported to be up-regulated in allergic diseases. TSLP (Soumelis et al., 2002; Ying et al., 2005), IL7R (Ziegler and Liu, 2006), and ICAM-1 (Hingorani et al., 1998) are allergy-related genes. At least 9 of the 47 transcripts that we found to be up-regulated more than 10-fold upon polyI:C stimulation of primary human conjunctival epithelial cells may be associated with allergy. Our results show that TLR3 of the human conjunctival epithelium might not only induce anti-viral innate immune responses, but also regulate allergic reactions.

Among TLR1–TLR10, TLR3 is the most intensely expressed TLR in ocular surface epithelial cells (Ueta, 2008; Ueta and Kinoshita, 2010a). However, we found that RIG-I and MDA-5, reported to be implicated in viral dsRNA recognition (Kawai and Akira, 2009), were also remarkably up-regulated by polyI:C stimulation of primary human conjunctival epithelial cells.

Quantitative RT-PCR assay showed that 11 transcripts (CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, RIG-I and MDA-5) could be up-regulated upon polyI:C stimulation in not only primary human conjunctival epithelial cells but also primary human corneal epithelial cells (Ueta and Kinoshita, 2010b). As polyI:C stimulation up-regulated these 11 transcripts in human ocular surface epithelial cells, these cells can be induced by polyI:C stimulation to express many transcripts that include not only transcripts of anti-viral innate immune response-related but also of allergy-related genes.

3.3. RIG-I and MDA-5 of the ocular surface epithelium

The TLR family detects pathogen-associated molecular patterns on the surface of cells and in the lumina of intracellular vesicles

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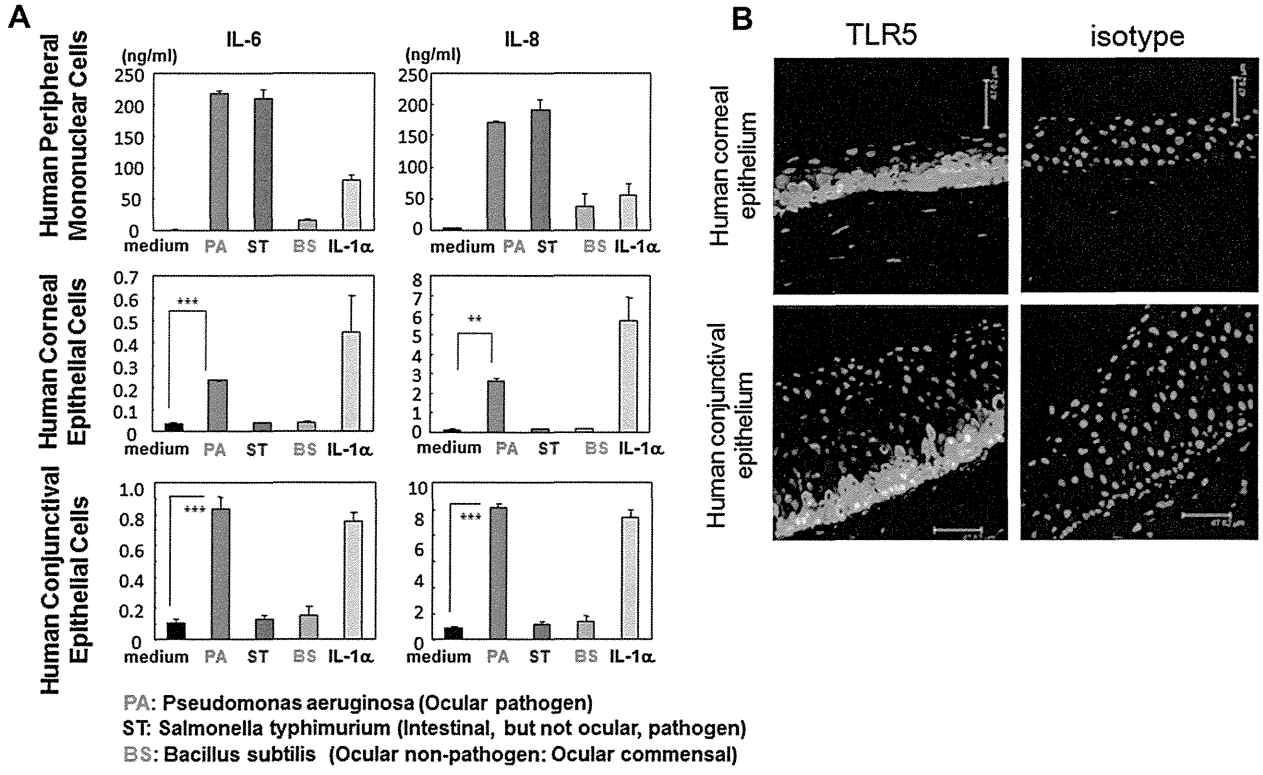

Fig. 7. Function of TLR5 in ocular surface. A. Responsiveness to various flagellins, which are TLR5 ligands, of human peripheral mononuclear cells and primary human corneal- and -conjunctival epithelial cells. Cultured cells were left untreated or exposed for 24 h to different flagellins (100 ng/ml). Data show the mean ± SEM (**, $p < 0.01$; ***, $p < 0.005$); evaluation was with Student's *t*-test using the Excel program. Modified with permission from Ueta et al. (Hozono et al., 2006; Kojima et al., 2008). B. Immunolocalization of TLR5 in human corneal and conjunctival tissue detected by immunofluorescence staining. Frozen cryostat sections were incubated with anti-TLR5 antibody or under isotype-control conditions. Bound antibodies were visualized after incubation with Alexa Fluor 488 goat anti-mouse IgG; nuclei were stained with propidium iodide.

such as endosomes or lysosomes. The existence of a cytosolic system for detecting intracellular pathogen-associated molecular patterns has also been confirmed. The cytosolic pattern recognition receptors include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). Thus, the ability of cells to recognize pathogen-associated molecular patterns depends on the expression of a family of TLRs, NLRs, and RLRs.

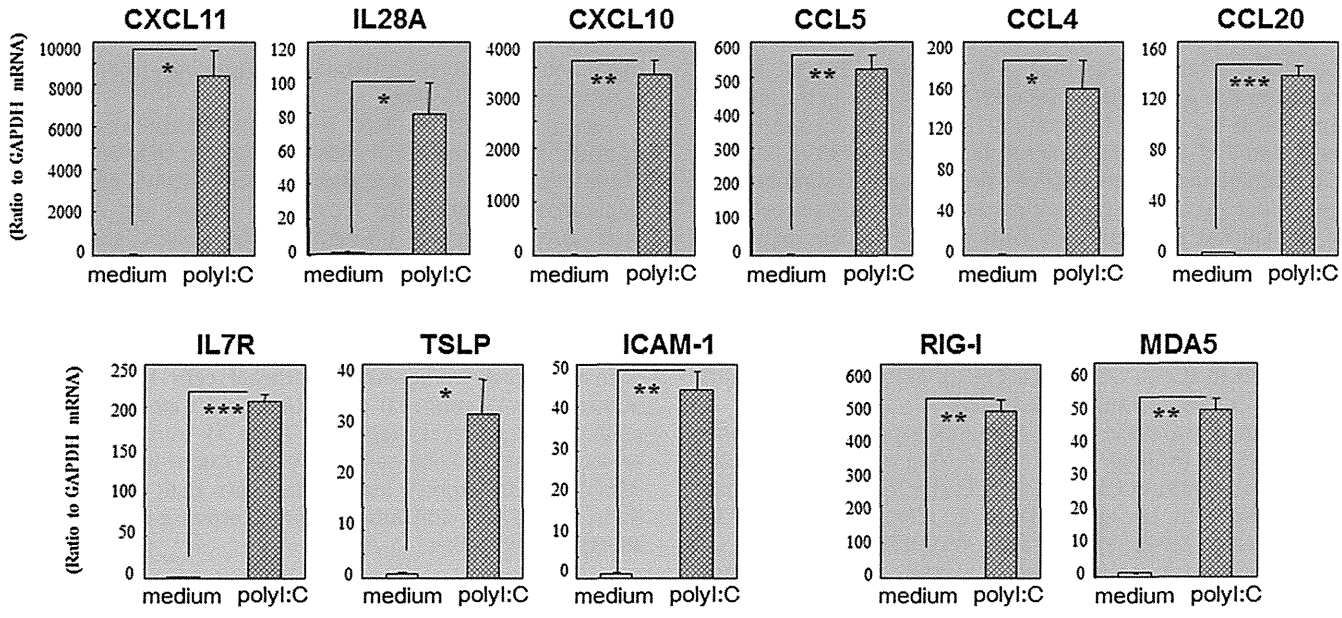

Fig. 8. mRNA expression of 11 transcripts in primary human conjunctival epithelial cells. The cells were exposed for 6 h to 25 μg/ml polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$). Reprinted with permission from Ueta et al. (Ueta et al., 2010b).

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RLRs belong to the RNA helicase family that specifically detects virus-derived RNA species in the cytoplasm. They coordinate anti-viral responses via the induction of type I IFN. RIG-I and MDA-5, which were up-regulated in primary human conjunctival epithelial cells upon polyI:C stimulation, are RLRs (Kawai and Akira, 2009) (Fig. 9A).

The human ocular surface epithelium expresses TLR3, which recognizes dsRNA mimicking polyI:C, a synthetic dsRNA (Alexopoulou et al., 2001). PolyI:C stimulation induces the secretion of inflammatory cytokines such as IL-6 and IL-8, and type I IFN such as IFN-β (Ueta, 2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a). Moreover, our gene expression analysis of primary human conjunctival epithelial cells using oligonucleotide microarrays to examine the comprehensive effects of polyI:C stimulation showed that transcripts including *CXCL11*, *IL28A*, *CXCL10*, *CCL5*, *CCL4*, *IL7R*, *TSLP*, *CCL20*, and *ICAM-1* were up-regulated more than 10-fold (Ueta et al., 2010b). In addition, new receptors that recognize dsRNA and polyI:C, RIG-I and MDA-5, are also up-regulated upon polyI:C stimulation in primary human conjunctival epithelial cells (Ueta et al., 2010b).

We examined the expression of RIG-I and MDA-5 in human conjunctival epithelium because not only TLR3, but also RIG-I and MDA-5 detect viral dsRNA. Moreover, to determine whether RIG-I and/or MDA-5 contribute to polyI:C-inducible responses in conjunctival epithelium we investigated the function of IPS-1, an adaptor molecule common to RIG-I and MDA-5 (Kawai et al., 2005), using IPS-1-knock-out (KO) mice.

Human conjunctival epithelial cells express RIG-I-, MDA-5-, and TLR3 mRNA (Fig. 9B) and the expression of mRNA in *RIG-I-*, *MDA-5-*, *and TLR3* genes was up-regulated by polyI:C stimulation. This up-regulation was particularly pronounced in *RIG-I* and *MDA-5* (Fig. 9C) (Ueta et al., 2011a). The protein expression of RIG-I and MDA-5 but not of TLR3 was markedly up-regulated in polyI:C-stimulated primary human conjunctival epithelial cells (Fig. 9D) (Ueta et al., 2011a).

We examined the function of IPS-1 and TLR3 in the conjunctival epithelium of IPS-1-KO- (Kawai et al., 2005) and TLR3-KO-mice. For the *in vivo* analysis of murine conjunctival epithelial cells we delivered a polyI:C solution subconjunctivally and as eyedrops, then we subjected these cells to gene expression analysis. Compared to control mice, *Mx2* (myxovirus (influenza virus) resistance 2), *Rsad2* (radical S-adenosyl methionine domain containing 2), *Cmpk2* (cytidine monophosphate (UMP-CMP) kinase 2), *Cxcl10* (chemokine (C-X-C motif) ligand 10), *Mx1* (myxovirus (influenza virus) resistance 1), *Ifi44* (interferon-induced protein 44), *Ifi203* (interferon-activated gene 203), *Iigp2* (interferon-inducible GTPase 2), and *Rtp4* (receptor transporter protein 4) were significantly down-regulated in conjunctival epithelial cells of IPS-1-KO mice (Ueta et al., 2011a). Moreover, *Mx2, Rsad2, Cmpk2*, and *Ccl5* (chemokine (C–C motif) ligand 5), but not *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2, and Rtp4* were significantly down-regulated in the conjunctival epithelium of TLR3-KO- compared to wild-type-mice (Ueta et al., 2011a). Thus, not only TLR3 but also RIG-I and/or MDA-5 contribute to polyI:C-inducible immune responses in the conjunctival epithelium (Ueta et al., 2011a).

Mx2 is an interferon-regulated gene that selectively inhibits hanta virus replication (Jin et al., 2001). *Rsad2* is an interferon-inducible protein that inhibits many DNA and RNA viruses (Shaveta et al., 2010). *Cmpk2*, a pyrimidine nucleoside monophosphate kinase, is thought to be involved in macrophage activation and inflammatory responses (Xu et al., 2008). In conjunctival epithelial cells, *Mx2* and *Rsad2*, which exert anti-viral actions, and *Cmpk2*, which is involved in inflammatory responses, were regulated by TLR3 and IPS-1 (RIG-I or/and MDA-5) (Ueta et al., 2011a).

Ifi44 is associated with hepatitis C virus infection although its function is unknown (Hallen et al., 2007). *Iigp2* plays a cell-

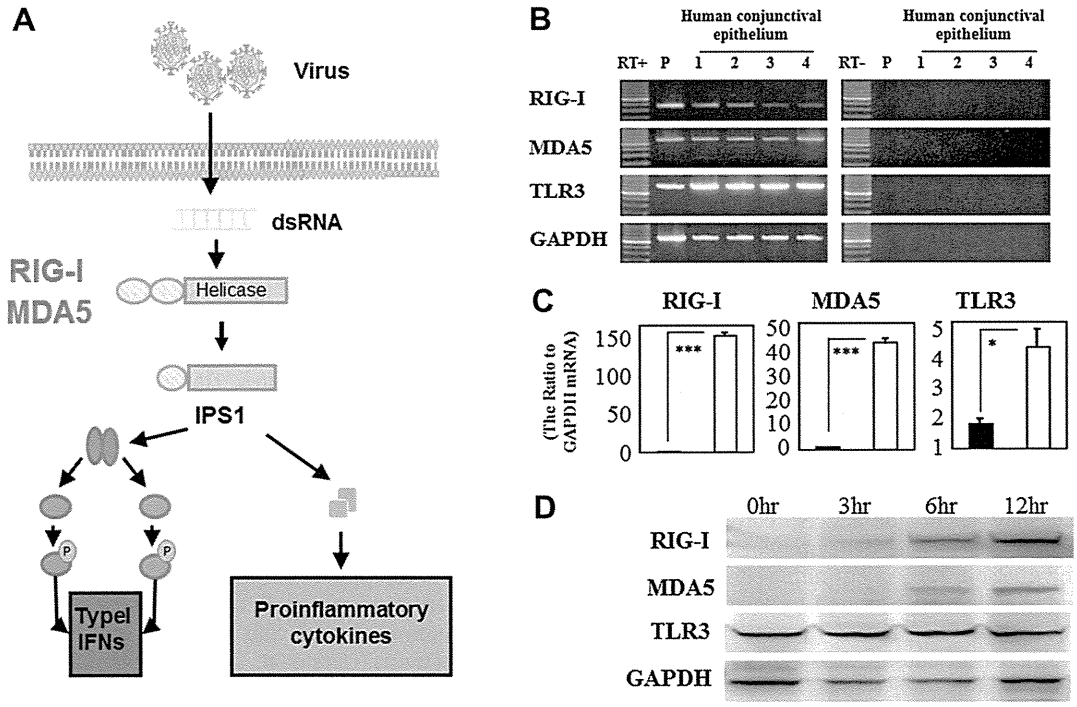

Fig. 9. A. RIG-I and MDA-5 are retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). B. Expression of RIG-I, MDA-5, and TLR3 mRNA in human conjunctival epithelial cells. The positive control (P) was mRNA isolated from human mononuclear cells (*, $p < 0.05$; ***, $p < 0.0005$). C. Up-regulation of RIG-I, MDA-5, and TLR3 mRNA expression in human conjunctival epithelial cells stimulated with polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. D. Up-regulation of the protein expression of RIG-I and MDA-5 in primary human conjunctival epithelial cells by polyI:C stimulation. Reprinted with permission from Ueta et al. (Ueta et al., 2011a).

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autonomous role in IFN-γ-mediated chlamydia inhibition (Miyairi et al., 2007). *Mx1* is an interferon-regulated gene that selectively interferes with the multiplication of influenza viruses (Horisberger, 1995). *Cxcl10* is expressed primarily in response to a wide range of DNA and RNA viruses and plays a role in the recruitment of leukocytes during inflammation (Farber, 1997). The expression of *Cxcl10* is also increased in allergic diseases; it was elevated in the epidermis of patients with atopic dermatitis (Klunker et al., 2003) and in the bronchoalveolar lavage fluid of patients with severe asthma (Ying et al., 2008). *Ifi203*, a member of the *Ifi-200* gene family, is induced by type I and II interferons; it has been reported as a regulator of cell proliferation and differentiation, and plays a role in apoptotic and inflammatory processes (Mondini et al., 2010). *Rtp4* is a member of the receptor transport protein (RTP) family and participates in the export of odorant and taste receptors (Saito et al., 2004). As these 6 transcripts are dominantly regulated by IPS-1 (RIG-I or/and MDA-5), it is evident that not only TLR3 but also RIG-I and MDA-5 contribute to the polyI:C-induced innate immune response (Ueta et al., 2011a).

Ccl5 is up-regulated in the presence of viral infection (Prehaud et al., 2005) and in the skin lesions of patients with chronic atopic dermatitis (Gros et al., 2009). *Ccl5*, which plays a role in inflammation and allergy, was dominantly regulated by TLR3 in conjunctival epithelial cells (Ueta et al., 2011a). We reported that TLR3 regulated the late-phase reaction of experimental allergic conjunctivitis (EAC) in a mouse model; eosinophilic conjunctival inflammation was reduced in TLR3-KO- and exacerbated in TLR3 transgenic-mice (Ueta et al., 2009c). These findings suggest that TLR3 in conjunctival epithelial cells can induce anti-viral innate immune responses and that it exerts other functions such as the regulation of allergic reactions. It has been reported that in the absence of viral infection TLR3 amplified immune responses during acute inflammatory processes, a phenomenon that may involve TLR3 stimulation by endogenous RNA from necrotic cells (Cavassani et al., 2008). Thus, innate immunity can respond to endogenous molecules released by host cells as a result of necrosis, pathogen infection, damage, injury, and certain pathological conditions that are directly or indirectly recognized by TLRs, NLRs, and RLRs and by yet to be identified sensors (Kawai and Akira, 2009). Endogenous RNA from tissues or cells might stimulate not only TLR3 but also RIG-I or/and MDA-5.

4. Allergic conjunctivitis may be regulated by epithelial cells

4.1. Allergic conjunctivitis

Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions. It is accompanied by characteristic symptoms (itching, conjunctival edema, redness, and tearing) during the early phase; eosinophils infiltrate the conjunctivae during the late phase. The signs and symptoms of allergic conjunctivitis have a significant deleterious effect on the patients' health, comfort, and quality of life. Current treatments are not curative and may elicit side-effects; corticosteroids place patients at increased risk for the development of glaucoma and cataracts (Ono and Abelson, 2005). Continuing efforts are needed to better understand allergic responses and to develop effective and safer drugs.

The allergic response in conjunctivitis is typically elicited by ocular exposure to allergens such as grass or tree pollen that leads to the crosslinkage of membrane-bound IgE. This in turn triggers mast cell degranulation and a release of a cascade of allergic and inflammatory mediators. The rapid release of histamine from mast cells within minutes of exposure to allergens is important in early-phase reactions. In addition, mediators released by mast cells during this phase may contribute to the development of late-phase reactions in which eosinophils are recruited to tissue sites affected by allergic inflammation (Broide, 2007). T cells (Fukushima, 2007) and fibroblasts (Fukuda et al., 2006) have been reported to contribute to the development of late-phase reactions (Fig. 10).

4.2. Development of eosinophilic conjunctival inflammation during the late-phase reaction in mast cell-deficient mice

Mast cells and the mediators they release are thought to contribute to the development of allergic conjunctivitis which is triggered by IgE cross-linking on mast cells; their mediators produce early-phase reactions in the conjunctiva (Graziano et al., 2001). Preformed or newly synthesized mediators, including histamine, are released from mast cells in the acute phase of allergic reaction. This results in clinical manifestations such as conjunctival redness, eye itching, and increased tearing.

Although mast cells play a central role in immediate allergic reactions and in the early phase of allergic conjunctivitis (Graziano et al., 2001), their role in the late-phase response is not clearly defined. The magnitude of eosinophil infiltration into the conjunctiva reflects the severity of the late-phase reaction. Using genetically mast cell-deficient (W/W^v) mice and our C57BL/6 mouse model of allergic conjunctivitis (Ueta et al., 2007b) we directly assessed the role of mast cells in conjunctival eosinophil infiltration.

We compared eosinophil infiltration in congenic WBB6F1-normal- (+/+) and mast cell-deficient ([WB–W/+ × C57BL/6–W^v/+]F1; W/W^v) mice. In mice sensitized and challenged by ragweed (RW), the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in both mast cell-deficient mice and their congenic littermates, although no sensitization and sensitization without challenge did not affect the number of eosinophils. There was no difference between mast cell-deficient and -sufficient mice (Fig. 11) (Ueta et al., 2007b).

We next compared the expression of eotaxin-specific mRNA in the eyelids of WBB6F1-normal- (+/+) and mast cell-deficient (W/W^v)-mice because chemokines such as eotaxin recruit eosinophils. Sensitization and challenge by RW significantly increased the expression of eotaxin-specific mRNA compared with sensitization alone in both mast cell-deficient and -sufficient mice (Ueta et al., 2007b). After RW sensitization, the level of serum total IgE, anti-RW IgE, and anti-RW IgG_1 was comparable in mast cell-deficient mice and their congenic littermates (Ueta et al., 2007b).

Sensitization with challenge of mast cell-deficient mice produced an increase in IgE and IgG_l antigen-specific antibody responses, conjunctival eosinophils, and eotaxin-specific mRNA in the eyelids. In this respect, these mice were indistinguishable from their congenic littermates. However, mast cells were identified histologically in the submucosa of WBB6F1-normal- (+/+) but not of W/W^v-mice, suggesting that the development of eosinophilic conjunctival inflammation in the late phase of allergic conjunctivitis is not dependent on the presence of functional mast cells (Ueta et al., 2007b).

Our findings indicate that mast cells do not play an essential role in the development of eosinophilic conjunctival inflammation in mice sensitized and challenged. However, this does not exclude the contribution of mast cells to other aspects of late-phase allergic conjunctivitis (Ueta et al., 2007b).

4.3. TLR3 and allergy

TLR3 recognizes dsRNA, a component of the life-cycle of most viruses, mimicking polyI:C (Alexopoulou et al., 2001). Among TLR1–TLR10, TLR3 is expressed most intensely in the ocular surface

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