Evaluation of Chemokine mRNA Expression to Assess Allergic Inflammation of the Ocular Surface in Chronic Allergic Conjunctival Diseases

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Evaluation of Chemokine mRNA Expression to Assess Allergic Inflammation of the Ocular Surface in Chronic Allergic Conjunctival Diseases

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Purpose: We validated the use of chemokine messenger RNA (mRNA) expression analysis for the assessment of ocular surface allergic inflammation in chronic allergic conjunctival diseases (ACDs) with proliferative lesions, including giant papillae and gelatinous infiltration of the limbus.

Methods: This prospective sectional study included 19 patients with chronic ACDs and 10 healthy volunteers as controls. Patients with chronic ACDs were divided into 2 subgroups according to the severity of the clinical score: active stage ACD subgroup (n = 9) and stable stage ACD subgroup (n = 10). Impression cytology using a filter paper for each upper tarsal conjunctiva of the patients with chronic ACDs and control subjects was performed, and the expression levels of *IL1A*, *CXCL8*, *IL16*, and *CCL24* mRNAs encoding interleukin (IL)-1α, CXCL8/IL-8, IL-16, and CCL24/ eotaxin-2, respectively, were determined by quantitative real-time polymerase chain reaction using impression cytology specimens.

Results: *CCL24* and *IL16* mRNA levels in the active ACD subgroup were significantly higher than those in the control group ($P = 0.003$ and 0.004, respectively). *IL1A* and *CXCL8* expression levels in the active ACD subgroup were significantly higher than those in the stable ACD ($P = 0.008$ and 0.029, respectively) and control ($P = 0.008$ and 0.014, respectively) subgroups. Furthermore, significant correlations were detected between *IL16* and *CCL24* mRNA levels ($r = 0.76$, $P = 0.0001$) and between *IL1A* and *CXCL8* ($r = 0.67$, $P = 0.0004$).

Conclusions: At least 2 kinds of inflammatory reactions, IL-1α- and CXCL8-associated inflammation and CCL24- and IL-16-associated inflammation, may be involved in the exacerbation of chronic ACDs.

Key Words: chronic allergic conjunctival diseases, interleukin-1α, interleukin-8, interleukin-16, eotaxin-2

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Allergic conjunctival diseases (ACDs), including allergic conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis, are inflammatory diseases of the conjunctiva caused by the predominantly immunoglobulin E (IgE)-mediated immediate hypersensitivity response and accompanied by conjunctival eosinophilic inflammation. Furthermore, in refractory ACD with severe eosinophilic inflammation, other mechanisms, including neutrophilic inflammation, interleukin (IL)-17 producing helper T cell (Th17) response, IL-1α producing macrophage response, and innate immunity, have also been implicated.[1–5] This etiological variety implies a very complex immunological basis of ACD.

ACDs are associated with various clinical findings, such as conjunctival hyperemia, conjunctival edema, limbal swelling, and papillary or giant papillary formation due to direct exposure of the conjunctiva to the allergen.[1,6,7] ACDs comprise 5 different clinical forms: seasonal allergic conjunctivitis, perennial allergic conjunctivitis, atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and giant papillary conjunctivitis.[8,9] Chronic ACDs, such as AKC and VKC, are particularly severe and refractory forms; their characteristic clinical findings are giant papillae, limbal gelatinous infiltration, and shield ulcer, developing because of severe allergic inflammation of the ocular surface. The allergic inflammation of the conjunctiva plays a crucial role in major pathological conditions of ACDs, but each clinical form has different clinical characteristics. Therefore, in addition to the immediate hypersensitivity reaction, several pathological conditions, including neutrophilic inflammation and exaggerated innate immune response, are believed to be associated with conjunctival allergic inflammation in patients with ACDs.[5]

In the clinical tests for ACDs, allergy-associated factors in tear samples and impression cytology specimens are usually investigated in the laboratory to assess the extent of allergic inflammation in the conjunctiva.[10] The assay of the total IgE and antigen-specific IgE antibody levels in tears is believed to be a useful tool for diagnosing ACDs and detecting

ACD-associated allergens.[11,12] Because the invasion of eosinophils and type 2 helper T (Th2) cells is a major pathological condition of allergic inflammation in the ocular surface, the levels of the eosinophil cationic protein (ECP), eosinophil-associated and Th2-associated cytokines and chemokines, in tears are reportedly useful markers of the clinical severity of ACDs.[2,13,14] Moreover, simultaneous measurements of various cytokines and chemokines in tears are helpful in the differential diagnosis of ocular surface inflammation that develops because of allergy, autoimmunity, or infection.

In impression cytology (membrane biopsy), a method of cytological diagnosis in the ocular surface, goblet cell density, and keratinization of conjunctival epithelial cells are histologically evaluated.[3] The real-time reverse transcriptase polymerase chain reaction (RT-PCR) method has recently improved, and mRNA expression levels can be evaluated on the ocular surface by using impression cytology specimens. Previously, we reported that impression cytology using a filter paper was a useful clinical test with low invasiveness for the patients. It enabled evaluation of mRNA expression levels of allergy-associated factors contained in the ocular surface lining fluid filled with epithelial cells, mucin, tear, and inflammatory cells.[15]

In allergic disorders such as bronchial asthma, allergic rhinitis, and atopic dermatitis, cytokines and chemokines secreted by epithelial cells and invading inflammatory cells are associated with the immune response and inflammatory reaction in cutaneous and mucosal tissues. IL-1α produced by the epithelial cells of the bronchus induces an innate immune response in human lung fibroblasts.[16] Furthermore, in asthma, the CXCL8/IL-8 concentration in airway tissues increases with neutrophilia in sputum and is recognized as an aggravation factor in patients with a severe form of the disease.[17] The increased chemotactic activity of CD4$^+$ T cells and eosinophils in patients with asthma is mainly attributable to IL-16.[18,19] Activation of the CCL24/eotaxin-2-dependent pathway reportedly exacerbates eosinophilic airway inflammation.[20] In ACDs, we reported that CCL24 and IL-16 protein amounts increase in patients' tears,[21] and *CCL24* mRNA expression is upregulated in conjunctival epithelial cells in patients with VKC.[14] Therefore, simultaneous measurements of protein and mRNA levels of different functional cytokines and chemokines, for example, IL-1α, CXCL8, IL-16, and CCL24, on the ocular surface may be a valuable clinical laboratory test for evaluating the activity and severity of chronic ACDs in patients with various inflammatory reactions that accompany the allergic hypersensitivity reaction.

To verify this assumption, in this study, we investigated mRNA expression levels of inflammatory chemokines in the fluid lining the ocular surface of patients with chronic ACDs with severe and stable allergic inflammation.

MATERIALS AND METHODS

The study protocol, approved by the Institutional Review Board of the Nihon University School of Medicine (approval number: RK-120511-11), adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Subjects

In total, 19 consecutive patients diagnosed with AKC or VKC at the Department of Ophthalmology of the Nihon University Itabashi Hospital (Tokyo, Japan) between July 2015 and November 2016 were included in this study as the chronic ACD group. This group was further divided into 2 subgroups according to the clinical severity of AKC and VKC: active ACD subgroup and stable ACD subgroup. Demographic data for the subjects are shown in Table 1. Only patients who had not received treatment or were treated with anti-allergic ophthalmic solutions, including mast cell stabilizers, histamine H$_1$ receptor antagonists, corticosteroids, or immunosuppressive agents such as cyclosporine and tacrolimus, were included in the study (Table 1). Patients who used oral medicines or received injections for the treatment of allergic disease or immunotherapy were excluded. Patients with ocular surface disorders other than ACDs—including lagophthalmos, blepharospasm, conjunctival chalasis, dry eye, infectious conjunctivitis, infectious keratitis, Stevens–Johnson syndrome, or ocular pemphigoid—and those who could not provide a sufficient amount of tear sample were excluded. AKC and VKC were diagnosed according to the Japanese ACD guidelines.[8] Healthy volunteers without any allergic diathesis or history of wearing contact lenses were recruited as controls (n = 10).

Clinical Staging of Chronic Allergic Conjunctival Diseases

Clinical severity of chronic ACDs was scored using the 5-5-5 exacerbation grading scale for ACDs.[7] In this method, 100 points are assigned for each of the 5 severe clinical findings (active giant papillae, gelatinous infiltrates of the limbus, exfoliative epithelial keratopathy, shield ulcer, and papillary proliferation at lower palpebral conjunctiva); 10 points are assigned for each of the 5 moderate clinical findings (blepharitis, papillary proliferation with velvety

TABLE 1. Demographic Data of Study Subjects

	Control	ACD (Stable Stage)	ACD (Active Stage)	*P*
No. of patients (cases)	10	10	9	
Age [mean ± SD (yrs)]	24.7 ± 3.47	21.8 ± 12.3	29.1 ± 14.9	0.371
Gender (male:female)	5:5	8:2	9:0	0.024
VKC/AKC ratio	—	6/4	4/5	0.656
Clinical score* [median (points)]	—	12	114	0.0002
Topical treatment				
Anti-allergy drugs	—	9	8	1.000
Corticosteroids	—	1	3	0.303
Immunosuppressive agents (C:T ratio†)	—	9 (1:8)	9 (0:9)	1.000
None	—	0	0	NA

N/A, not applicable.
*Score on the 5-5-5 exacerbation grading scale for allergic conjunctivitis.
†C:T ratio: cyclosporine ophthalmic solution 0.1%: tacrolimus ophthalmic suspension 0.1% ratio.

TABLE 2. Relative mRNA Levels of *CCL24*, *CXCL8*, *IL16*, and *IL1A* in the Control Group

	CCL24	*CXCL8*	*IL16*	*IL1A*
Median (ΔΔCT)	1.7	0.88	0.48	0.0098
95th percentile (ΔΔCT)	7.4	2.8	2.0	4.0
Fifth percentile (ΔΔCT)	0.0017	0.021	0.038	0.001

appearance, Horner–Trantas spots, edema of bulbar conjunctiva, and superficial punctate keratopathy); and 1 point is given for each of the 5 mild clinical findings (papillae at the upper palpebral conjunctiva, follicular lesions at the lower palpebral conjunctiva, hyperemia of palpebral conjunctiva, hyperemia of bulbar conjunctiva, and lacrimal effusion). The sum of total points in each grade determined the severity score on the 5-5-5 exacerbation grading scale.

According to the results of the 5-5-5 exacerbation grading scale for ACDs, the active stage subgroup had 9 patients with a clinical score of ≥100 points, whereas the stable stage subgroup comprised 10 patients with a clinical score of <100 points.

Sample Collection from the Ocular Surface

The membrane biopsy of the ocular surface was performed by a method identical to that of impression cytology but using the 5-mm tip of Schirmer test papers (Schirmer Tear Production Measuring Strips; Ayumi Pharmaceutical Corporation, Tokyo, Japan) instead of the nitrocellulose membrane. The Schirmer test paper was applied to the upper tarsal conjunctiva without local anesthesia or washing the eye, pressed gently using a glass rod, then removed, and preserved in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) until RT-PCR analysis.

Measurements of mRNA Levels of IL1A, CXCL8, IL16, and CCL24 on the Ocular Surface

Expression levels of *IL1A*, *CXCL8*, *IL16*, and *CCL24* mRNAs encoding IL-1α, CXCL8, IL-16, and CCL24, respectively, on the ocular surface of control subjects and patients with chronic ACDs were evaluated using membrane biopsy samples obtained from the affected eye in unilateral cases, from the more severely affected eye in bilateral cases of chronic ACDs, and from the right eye of control subjects.

TABLE 3. Two-Factor Analysis of *CXCL8* and *CCL24* mRNA Levels in Control Group

	CCL24	
Control (n = 10)	**High (>7.4)**	**Low (<7.4)**
CXCL8		
High (>2.8)	0	1
Low (<2.8)	1	8

TABLE 4. Two-Factor Analysis of *CXCL8* and *CCL24* mRNA Levels in ACD Group

	CCL24	
ACD Group (n = 19)	**High (>7.4)**	**Low (<7.4)**
CXCL8		
High (>2.8)	10	1
Low (<2.8)	4	4

To detect *IL1A*, *CXCL8*, *IL16*, and *CCL24* expression by real-time RT-PCR, total RNA from each sample was extracted with a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and used to synthesize cDNA with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Japan, Tokyo, Japan). Real-time RT-PCR was performed using the TaqMan gene expression assay (Life Technologies Japan, Tokyo, Japan) and predesigned primers/probes Hs00174092_m1 (*IL1A*), Hs99999034_m1 (*CXCL8*), Hs00189606_ml (*IL16*), and Hs00171082_ml (*CCL24*) (Life Technologies Japan, Tokyo, Japan) on a Step One Plus system (Life Technologies Japan, Tokyo, Japan). Target Ct values were normalized to those of *GAPDH* (Hs99999905_m1) from the same sample. Expression levels were determined by the comparative threshold cycle (ΔΔCT) method.

Statistical Analyses

Statistical analyses were performed using MAC Toukei–Kaiseki v.2 software (Esumi, Tokyo, Japan). Differences between active and stable stage subgroups of the chronic ACD group and the control group were evaluated with the χ^2 test and Kruskal–Wallis H test. *IL1A*, *CXCL8*, *IL16*, and *CCL24* mRNA expression levels were compared between the stable and active stage subgroups of the chronic ACD group and control group by the Steel test. Assessments of the relationships between *IL1A*, *CXCL8*, *IL16*, and *CCL24* expression levels were performed using the partial correlation coefficient test. Correlations between the expression levels of *IL1A* and *CXCL8* or between the levels of *IL16* and *CCL24* in chronic ACD group were analyzed by calculating the Spearman rank correlation coefficient. Differences were considered statistically significant if $P < 0.05$.

RESULTS

IL1A, CXCL8, IL-16, and CCL24 mRNA Expression Levels on the Ocular Surface Increase Depending on the Clinical Severity of Chronic Conjunctival Diseases

The median, 95th percentile, and 5th percentile of *IL1A*, *CXCL8*, *IL16*, and *CCL24* mRNA expression levels on the ocular surface of control subjects are shown in Table 2. The cutoff value of ocular surface mRNA expression for each cytokine/chemokine was set up based on the 95th percentile value for the respective cytokine/chemokine in the control group, so that if the sample's expression level was equal to or larger than the cutoff value, the expression was considered to

TABLE 5. Characteristics of CCL24^{high} CXCL8^{high} and CCL24^{high} CXCL8^{low} Subgroups

	CCL24^{high}-IL-8^{high} Subgroup	CCL24^{high}-IL-8^{low} Subgroup	P
No. of patients	10	4	
Gender (%male)	90%	75%	0.505
Age (median)	14	20	0.322
VKC/AKC ratio	3/1	5/5	0.581
Clinical score (median) (range)	62.5 (3–223)	63 (3–213)	0.887
Medication			
Anti-allergic drug	9/10	3/4	0.505
Tacrolimus	10/10	4/4	NA
Steroid	4/10	0/4	0.251
Recurrence count* (median) (range)	1 (0–4)	0 (0)	0.016

*The recurrence was calculated by the number of recurrences for the past 1 year.
NA, not applicable.

be "high," whereas if the expression level was less than the cutoff value, the expression was considered to be "low." The 2×2 contingency tables for *CXCL8* and *CCL24* in control and ACD groups are given in Tables 3 and 4. The combined expression patterns of *CXCL8* and *CCL24* on the ocular surface were significantly different in the control and ACD groups ($P = 0.0063$, χ^2 test). The characteristics of combined expression patterns of *CXCL8* and *CCL24* mRNAs on the ocular surface were as follows. There were 10 samples with high levels of both *CXCL8* and *CCL24* mRNAs in the ACD group, and none in the control group. Furthermore, there were 4 and 8 samples with low levels of both *CXCL8* and *CCL24*

mRNAs in ACD and control groups, respectively. In addition, there were 4 samples and 1 sample with low levels of *CXCL8* and high levels of *CCL24* in ACD and control groups, respectively (Tables 3 and 4). The clinical characteristics of *CCL24*^{high} *CXCL8*^{high} and *CCL24*^{high} *CXCL8*^{low} subgroups are shown in Table 5. The recurrence was assessed by the number of recurrent episodes during the past year. The *CCL24*^{high} *CXCL8*^{high} subgroup had significantly more recurrences than the *CCL24*^{high} *CXCL8*^{low} subgroup.

For all cytokines/chemokines examined, mRNA expression levels were directly proportional to the severity of the condition (Fig. 1). The expression levels of *CCL24* and *IL16* mRNA in the active ACD subgroup were significantly higher than those in the control group ($P = 0.003$ and 0.004, respectively; Figs. 1A, B, respectively). Furthermore, the expression levels of *IL1A* and *CXCL8* in the active ACD subgroup were significantly higher than those in the stable ACD subgroup ($P = 0.008$ and 0.029, respectively) and control ($P = 0.008$ and 0.014, respectively) groups (Figs. 1C, D, respectively).

Correlation Between IL1A and CXCL8 and Between IL16 and CCL24 mRNA Expression Levels on the Ocular Surface

Partial correlation analysis revealed significant correlations between mRNA levels of *IL16* and *CCL24* ($P = 0.002$), and of *IL1A* and *CXCL8* ($P = 5.4 \times 10^{-11}$) in all subjects (combined ACD and control groups; Table 6). Significant correlations between *CCL24* and *IL16* mRNA levels ($\rho = 0.76$, $P = 0.0001$, Spearman correlation coefficient; Fig. 2A) and between *IL1A* and *CXCL8* mRNA levels ($\rho = 0.67$, $P = 0.0004$, Spearman correlation coefficient; Fig. 2B) were observed.

FIGURE 1. Expression levels of *CCL24*, *IL16*, *IL1A*, and *CXCL8* mRNA on the ocular surface in the control group and in patients with ACDs. Patients with ACD were subdivided into individuals with stable ACD and active ACD. mRNA expression levels were evaluated from the amount of the gene amplified by real-time polymerase chain reaction, which was calculated by the CT method. Expression levels of *CCL24* (A), *IL16* (B), *IL1A* (C), and *CXCL8* (D) mRNAs were expressed in ratios. Expression levels of *CCL24* (A) and *IL16* (B) in the active ACD group were significantly higher than those in the control group. Expression levels of *IL1A* (C) and *CXCL8* (D) mRNAs in the active ACD group were significantly higher than those in the stable ACD and control groups. Data are presented as box-whisker plots. Statistical significance of differences is indicated as follows: *$P < 0.05$; **$P < 0.01$.
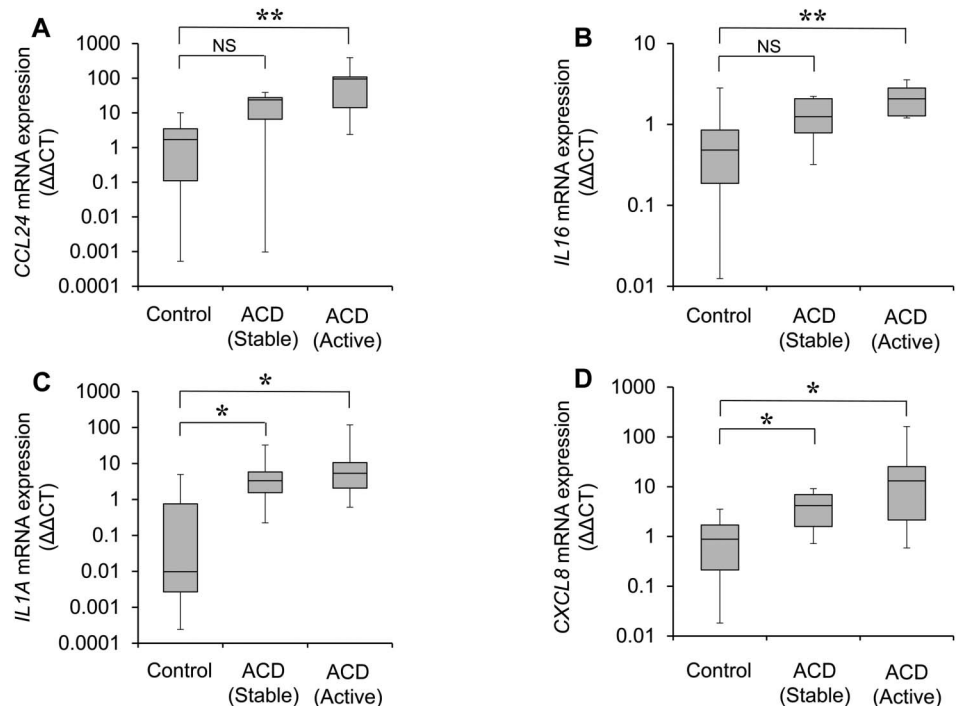
TABLE 6. Values of Partial Correlation Coefficient *r* Indicating the Extent of Correlation Between Expression Levels of Different Cytokines

|	CCL24	CXCL8	IL16	IL1A
CCL24	1			
CXCL8	0.002	1		
IL16	0.564*	0.206	1	
IL1A	0.007	0.909**	−0.076	1

*P < 0.005; **P < 0.0001.

DISCUSSION

In an attempt to expand the range of available methods to assess allergic inflammation of the ocular surface in chronic ACDs, we determined the mRNA levels of cytokines/chemokines in impression cytology specimens. We found that mRNA expression levels of the cytokines IL-1α, CXCL8, IL-16, and CCL24 increased in patients with chronic ACDs in the active stage compared with their levels in patients with chronic ACDs in the stable stage and in control subjects. Furthermore, alterations in the expression pattern of cytokines concerned 2 systems: the CXCL8/IL-1α axis and the IL-16/CCL24 axis. These results mean that there are likely 2 different types of pathophysiological events associated with the exacerbation of allergic inflammation in the conjunctiva of patients with chronic ACDs.

ACDs are believed to be conjunctival inflammatory disorders that exhibit the immediate hypersensitivity response as a basic pathological condition.[1,8,9] However, some pathological conjunctival manifestations of AKC and VKC, such as giant papillae and limbal gelatinous infiltration, cannot be explained only by the immediate hypersensitivity response. Thus, the details of the pathological conditions that cause conjunctival proliferative alterations are not fully understood. Because severe infiltration of eosinophils, basophils, and CD4⁺ cells, including Th2 cells, in conjunctival tissues was reported in a histopathological study of giant papillae,[22] it was suggested that AKC and VKC pathological conditions are strongly associated with the late-phase reaction of the immediate hypersensitivity response. In addition, depending on the assay of the allergy-associated factors in tears, increases in antigen-specific IgE antibodies, ECP, Th2 cytokine, CCL11/eotaxin-1, CCL24, soluble IL-6 receptor, CCL20, and CXCL8 were reported,[11,12,21,23–29] and it has been shown that VKC-associated conditions involve complex immune responses. Therefore, we investigated the expression of *IL1A*, *CXCL8*, *IL16*, and *CCL24* mRNAs on the ocular surface because cytokines encoded by these genes might be associated with inflammation of the conjunctiva of patients with chronic ACDs. Furthermore, the noninvasive membrane biopsy method with a filter paper was employed to obtain ocular surface samples, which could be useful for the quantitative follow-up of patients with chronic ACDs. We are convinced that a prognostic ocular surface marker that can predict the therapeutic effect would be a useful clinical tool in the treatment of chronic ACDs.

One of the major pathophysiological features of allergic inflammation in the conjunctiva is eosinophilic inflammation. Eosinophils express the C-C chemokine receptor CCR3 on their cell surface, and CCR3 ligands, including CCL11, CCL24, CCL26/eotaxin-3, MCP-4, and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), are crucial for the infiltration of eosinophils in allergically inflamed tissues.[30] We previously reported that ECP levels in tears in VKC and AKC groups were significantly increased compared with those in a control group.[13,31,32] In addition, ECP levels in tears significantly correlated with the severity of clinical findings.[2] Furthermore, we reported that out of all mRNAs encoding the members of the eotaxin family, *CCL24* mRNA was most abundantly expressed on the ocular surface[33] and its level significantly correlated with tear ECP levels in the patients with ACDs.[14] Therefore, *CCL24* mRNA expression on the ocular surface may be a useful biomarker of eosinophilic inflammation of the conjunctiva. It has been reported that allergic inflammation is characterized mainly by the infiltration of eosinophils and CD4⁺ lymphocytes.[10] Th2 cells that express CD4 on their cell surface are a major type of inflammatory cells mediating allergic inflammation in the conjunctiva. IL-16 is a chemokine associated with the migration of CD4⁺ cells and may be also implicated in the migration of eosinophils.[19] Therefore, our data that were obtained from membrane biopsies and suggested that *CCL24* mRNA level significantly correlated with that of *IL16* on the ocular surface are quite expected in chronic ACD patients with allergic inflammation in the conjunctiva. Accordingly, these results showed that allergic inflammation caused by the immediate hypersensitivity response enhanced the cooperative expression of CCL24 and IL-16 in the ocular surface; therefore, the

FIGURE 2. Evaluation of the correlation between *CCL24* and *IL16* mRNA expression levels, and between *IL1A* and *CXCL8* mRNA expression levels on the ocular surface in all subjects. To examine the correlation, data from the ACD group were merged with those from the control group. Spearman correlation coefficients showed significant correlation between *CCL24* and *IL16* mRNA expression levels (ρ = 0.757, P = 0.0001), and between *IL1A* and *CXCL8* (ρ = 0.670, P = 0.0004).
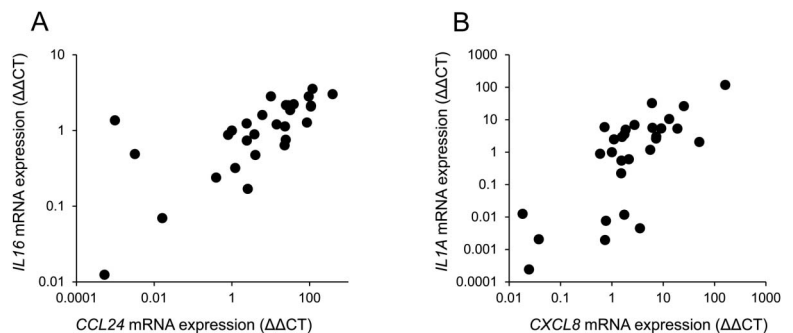
combined levels of their mRNAs and/or proteins may be a suitable biomarker of allergic inflammation in the conjunctiva.

IL-1α is known as the main inflammatory cytokine of innate immunity and one of the alarmins released from damaged epithelium.[34] In addition, IL-1α secreted by epithelial cells reportedly upregulated expression levels of IL-6, CXCL8, monocyte chemotactic protein-1, and granulocyte macrophage colony-stimulating factor in cultured primary human lung fibroblasts.[16] Moreover, IL-1α released from necrotic corneal epithelial cells was shown to increase the expression levels of IL-6 and CXCL8 in intact cultured human epithelial cells.[35,36] In addition, in the present study, we showed that *CXCL8* mRNA expression on the ocular surface of the patients with chronic ACDs significantly correlated with the expression level of *IL1A* mRNA. Therefore, these results strongly suggest that the proinflammatory cytokine or danger signal associated with innate immunity and not allergen-specific IgE antibody-dependent immediate hypersensitivity may be involved in the pathological condition of the chronic ACD.

We established the cut-off levels of *CCL24* and *CXCL8* mRNAs on the basis of the 95th percentile levels of the control group and classified patients with chronic ACDs into 4 subgroups. As a result, in addition to the most numerous *CCL24*^{high}-*CXCL8*^{high} subgroup and *CCL24*^{low}-*CXCL8*^{low} subgroup, there were patients with the *CCL24*^{low}-*CXCL8*^{high} and *CCL24*^{high}-*CXCL8*^{low} expression profiles. These results indicate that it may be helpful to examine the background factors of the patients with such binary inflammation profiles. The common characteristics of the 4 cases with *CCL24*^{high}-*CXCL8*^{low} expression profile included refractory AKC or VKC; although tacrolimus eye drops were continuously administered to those patients, inflammatory manifestations in the conjunctiva sometimes recurred. Furthermore, the recurrence count in the *CCL24*^{high}-*CXCL8*^{low} subgroup was significantly lower than that in the *CCL24*^{high}-*CXCL8*^{high} subgroup. Therefore, patients with high *CCL24* mRNA expression and low *CXCL8* mRNA expression on the ocular surface may have persistent eosinophilic inflammation in the conjunctiva despite treatment with immunosuppressive drugs. Therefore, it is necessary to be careful about therapeutic drug selections in common practice for these patients to avoid chronic ACD recurrence.

This study had several limitations. First, this study is a cross-sectional study for outpatients with ACD, and, therefore, the impact of interpatient differences on the cytokine profiles in tears could not be prevented. Second, mRNA expression levels of the selected cytokines and chemokines in this study could not ascertain the difference between AKC and VKC samples. The clinical utility of the suggested ocular allergy test for evaluating disease progression and severity should be improved considering medical treatment and cytokine profiles in tears in each clinical form of ACD. Third, the male-to-female ratio in our control group was 1:1, although men predominated in stable ACD and active ACD groups in our study, which is consistent with numerous reports in the literature. Although we believe that this circumstance does not diminish the utility of the selected cytokines/chemokines as biomarkers, the impact of sex is an important factor that will be clarified in our future studies. Fourth, we did not evaluate whether our ocular allergy test that utilized an impression cytology method correlated with changes in inflammatory cells associated with allergic inflammation in the conjunctiva. However, our method can estimate the variability and severity of allergic inflammation simultaneously if the ocular allergy test using real-time RT-PCR substitutes for conjunctival cytology. Further studies of the correlation between cytokine/chemokine mRNA expression levels as biomarker for ACD and the type of cytological inflammation in nontreated patients with chronic ACD are needed.

In conclusion, we demonstrated correlated expression of 2 different sets of ocular surface inflammation markers, *IL16/CCL24* and *CXCL8/IL1A*, in chronic ACDs. Therefore, simultaneous monitoring of these markers may be a useful clinical test for the evaluation of inflammation severity and disease prognosis in patients with chronic ACDs.

REFERENCES

1. Leonardi A, Motterle L, Bortolotti M. Allergy and the eye. *Clin Exp Immunol.* 2008;153(suppl 1):17–21.
2. Pelikan Z. Cytokine profiles in tears accompany the secondary conjunctival responses induced by nasal allergy. *Curr Eye Res.* 2014;39:120–132.
3. Shoji M, Shoji J, Inada N. Clinical severity and biomarkers, eosinophil cationic protein and CCL23, in chronic allergic conjunctival diseases. *Semin Ophthalmol.* 2018;33:325–330.
4. Chen X, Deng R, Chi W, et al. IL-27 signaling deficiency develops Th17-enhanced Th2-dominant inflammation in murine allergic conjunctivitis model. *Allergy.* 2018. doi: 10.1111/all.13691 [epub ahead of print].
5. Sugita J, Asada Y, Ishida W, et al. Contribution of interleukin-33 and TSLP in a papain-soaked contact lens-induced mouse conjunctival inflammation model. *Immun Inflamm Dis.* 2017;5:515–525.
6. Leonardi A, Bogacka E, Fauquert JL, et al. Ocular allergy: recognizing and diagnosing hypersensitivity disorders of the ocular surface. *Allergy.* 2012;67:1327–1337.
7. Shoji J, Inada N, Sawa M. Evaluation of novel scoring system using 5-5-5 exacerbation grading scale for allergic conjunctivitis disease. *Allergol Int.* 2009;58:591–597.
8. Takamura E, Uchio E, Ebihara N, et al. Japanese guideline for allergic conjunctival diseases. *Allergol Int.* 2011;60:191–203.
9. Trocme SD, Sra KK. Spectrum of ocular allergy. *Curr Opin Allergy Clin Immunol.* 2002;2:423–427.
10. Leonardi A Allergy and allergic mediators in tears. *Exp Eye Res.* 2013; 117:106–117.
11. Mimura T, Yamagami S, Kamei Y, et al. Specific IgE in tear fluid and features of allergic conjunctivitis. *Curr Eye Res.* 2013;38:917–925.
12. Leonardi A, Borghesan F, Faggian D, et al. Microarray-based IgE detection in tears of patients with vernal keratoconjunctivitis. *Pediatr Allergy Immunol.* 2015;26:641–645.
13. Shoji J, Kitazawa M, Inada N, et al. Efficacy of tear eosinophil cationic protein level measurement using filter paper for diagnosing allergic conjunctival disorders. *Jpn J Ophthalmol.* 2003;47:64–68.
14. Shiraki Y, Shoji J, Inada N. Clinical usefulness of monitoring expression levels of CCL24 (eotaxin-2) mRNA on ocular surface in patients with vernal keratoconjunctivitis and atopic keratoconjunctivitis. *J Ophthalmol.* 2016;2016:3573142.
15. Doughty MJ. A systematic assessment of goblet cell sampling of bulbar conjunctiva by impression cytology. *Exp Eye Res.* 2015;136:16–28.
16. Inada N, Shoji J, Shiraki Y, et al. Histamine H1 and H4 receptor expression on ocular surface of patients with chronic allergic conjunctival diseases. *Allergol Int.* 2017;66:586–593.
17. Suwara MI, Green NJ, Borthwick LA, et al. IL-1α released from damaged epithelial cells is sufficient and essential to trigger inflammatory responses in human lung fibroblasts. *Mucosal Immunol.* 2014;7:694–693.

18. Shannon J, Ernst P, Yamauchi Y, et al. Difference in airway cytokine profile in severe asthma compared to moderate asthma. *Chest.* 2008;133: 420–426.

19. Cruikshank WW, Center DM, Nisar M, et al. Molecular and functional analysis of a lymphocyte chemoattractant factor: association of biologic function with CD4 expression. *Proc Natl Acad Sci USA.* 1994;91:5109–5113.

20. Ferland C, Flamand N, Davoine F, et al. IL-16 activates plasminogen-plasmin system and promotes human eosinophil migration into extracellular matrix via CCR3-chemokine-mediated signaling and by modulating CD4 eosinophil expression. *J Immunol.* 2004;173:4417–4424.

21. Dai C, Yao X, Gordon EM, et al. A CCL24-dependent pathway augments eosinophilic airway inflammation in house dust mite-challenged Cd163(−/−) mice. *Mucosal Immunol.* 2016;9:702–717.

22. Shoji J, Aso H, Inada N. Clinical usefulness of simultaneous measurement of the tear levels of CCL17, CCL24, and IL-16 for the biomarkers of allergic conjunctival diseases. *Curr Eye Res.* 2017;42:677–684.

23. Matsuda A, Ebihara N, Yokoi N, et al. Basophils in the giant papillae of chronic allergic keratoconjunctivitis. *Br J Ophthalmol.* 2010;94:513–518.

24. Bacon AS, Ahluwalia P, Irani AM, et al. Tear and conjunctival changes during the allergen-induced early- and late-phase responses. *J Allergy Clin Immunol.* 2000;106:948–954.

25. Pelikan Z. Inflammatory mediator profiles in tears accompanying keratoconjunctival responses induced by nasal allergy. *Br J Ophthalmol.* 2013;97:820–828.

26. Leonardi A, Jose PJ, Zhan H, et al. Tear and mucus eotaxin-1 and eotaxin-2 in allergic keratoconjunctivitis. *Ophthalmology.* 2003;110: 487–492.

27. Fukagawa K, Nakajima T, Tsubota K, et al. Presence of eotaxin in tears of patients with atopic keratoconjunctivitis with severe corneal damage. *J Allergy Clin Immunol.* 1999;103:1220–1221.

28. Shoji J, Inada N, Sawa M. Antibody array-generated cytokine profiles of tears of patients with vernal keratoconjunctivitis or giant papillary conjunctivitis. *Jpn J Ophthalmol.* 2006;50:195–204.

29. Shoji J, Kawaguchi A, Gotoh A, et al. Concentration of soluble interleukin-6 receptors in tears of allergic conjunctival disease. *Jpn J Ophthalmol.* 2007;51:332–337.

30. Leonardi A, Curnow SJ, Zhan H, et al. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy.* 2006;36:777–784.

31. Pope SM, Zimmermann N, Stringer KF, et al. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J Immunol.* 2005;175:5341–5350.

32. Montan PG, van Hage-Hamsten M, Zetterström O. Sustained eosinophil cationic protein release into tears after a single high-dose conjunctival allergen challenge. *Clin Exp Allergy.* 1996;26:1125–1130.

33. Secchi A, Leonardi A, Abelson M. The role of eosinophil cationic protein (ECP) and histamine in vernal keratoconjunctivitis. *Ocul Immunol Inflamm.* 1995;3:23–28.

34. Shoji J, Inada N, Sawa M. Evaluation of eotaxin-1, -2, -3 protein production and messenger RNA expression in patients with vernal keratoconjunctivitis. *Jpn J Ophthalmol.* 2009;53:92–99.

35. DiPaolo NC, Shayakhmetov DM. Interleukin 1α and the inflammatory process. *Nat Immunol.* 2016;17:906–913.

36. Fukuda K, Ishida W, Miura Y, et al. Cytokine expression and barrier disruption in human corneal epithelial cells induced by alarmin released from necrotic cells. *Jpn J Ophthalmol.* 2017;61:415–422.