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Yukiko Shiraki, Jun Shoji, Noriko Inada, Akiko Tomioka, and Satoru Yamagami

Division of Ophthalmology, Department of Visual Sciences, Nihon University School of Medicine, Tokyo, Japan

Corresponding author

Satoru Yamagami MD, PhD; Division of Ophthalmology, Department of Visual Sciences, Nihon University School of Medicine, 30-1 Oyaguchi-Kamichou, Itabashi-ku, Tokyo 173-8610, Japan. Tel: +81-3-3972-8111; Fax: +81-3-5995-3495.

E-mail: yamagami.satoru@nihon-u.ac.jp

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IL-1 α antibody inhibits dose-dependent exacerbation of eosinophilic inflammation by crude house-dust-mite antigen in the conjunctiva of an atopic keratoconjunctivitis mouse model

Purpose: To investigate whether crude house-dust-mite antigen exacerbates eosinophilic inflammation in the conjunctival tissues of an atopic keratoconjunctivitis mouse model in a dose-dependent manner.

Materials and Methods: An atopic keratoconjunctivitis mouse model was established by percutaneous sensitization and crude house-dust-mite antigen application in NC/Nga mice. To assess the dose-dependent response, conjunctival specimens from groups that were administered high- (High-HDM) or low-dose house-dust-mite antigen (Low-HDM) following percutaneous sensitization and the control without house-dust-mite antigen administration (control group) were evaluated. Histological examination and immunofluorescence staining were performed to determine eosinophil density and the number of IL-13-positive cells. Polymerase chain reaction array was used to obtain adaptive and innate immunity-related factor profile, and quantitative polymerase chain reaction was used to determine *Il13*, *Il17a*, *Ccl11*, and *Ccl24* expression. Atopic keratoconjunctivitis model mice injected with anti-IL-1 α antibody (IL-1 α group) or vehicle (vehicle group) to the upper and lower eyelids before atopic keratoconjunctivitis development were evaluated.

Results: Eosinophil density in the conjunctiva increased with house-dust-mite antigen application in a dose-dependent manner. *CD4*, *CXCL10*, *CCR6*, *C3*, and

IL-13 mRNA levels increased more than 5-fold in the conjunctiva of the High-HDM group animals compared to those in control animals. mRNA expression of *Il13* and *Ccl11* in the conjunctiva of the High-HDM group animals significantly increased compared with that in the Low-HDM and control group animals. Conversely, the eosinophil density and *Il13* mRNA expression significantly decreased in the IL-1 α group compared with those in the vehicle group.

Conclusions: The house-dust-mite antigen increased eosinophilic infiltration and *Il13* mRNA expression in the conjunctiva of an atopic keratoconjunctivitis mouse model in a dose-dependent manner. These inflammatory alterations were partially alleviated by eyelid injection of anti-IL-1 α antibody. These findings indicate that IL-1 α -induced IL-13 production constitutes a major exacerbating factor for house-dust-mite antigen-induced atopic keratoconjunctivitis.

Keywords: atopic keratoconjunctivitis; mouse model; house-dust-mite; eosinophil; IL-13

Introduction

Atopic keratoconjunctivitis (AKC), first reported by Hogan in 1953¹, is a bilateral, chronic allergic disorder that develops in the face and eyelid skin of patients with atopic dermatitis. The primary symptom of AKC is seasonal or perennial ocular itching; the representative objective findings in the ocular surface include blepharitis, papillary or giant papillary formation in the upper tarsal conjunctiva, conjunctival hyperemia and

swelling, subepithelial fibrosis of the conjunctiva, superficial punctate keratitis, and shield ulcer ². The characteristic histological findings of AKC are severe subepithelial infiltration of eosinophils and lymphocytes, pseudotubule formation, and frank granuloma formation in the conjunctiva ³. Moreover, the level of eosinophil cationic protein, an eosinophil granule protein, significantly increases in patients with AKC in compared with that in normal subjects, in the tear test ⁴. Previously, we reported that the mRNA level of *CCL24*/eotaxin-2 significantly correlated with the clinical scores in patients with AKC or vernal keratoconjunctivitis (VKC) ⁵. We also demonstrated that *CCL24*/eotaxin-2 is a suitable ocular biomarker for allergic inflammation in AKC and VKC. Specifically, type 2 inflammation, which is mainly dependent on the helper T cell type 2 (Th2) response, is considered to be associated with allergic inflammation in AKC, similar to that of atopic dermatitis. However, the pathophysiology of type 2 inflammation caused by the innate immune response in AKC is not fully understood.

The pathophysiology of type 2 inflammation consists of IgE-mediated hypersensitivity and innate immunity, with Th2 cytokines interleukin 4 (IL-4), IL-5, and IL-13 playing a critical role in orchestrating the infiltration of inflammatory and immune cells ⁶. In particular, in innate immunity, IL-5 and IL-13 secreted by innate lymphocyte group 2 (ILC2) cells activated by IL-33 are associated with the

exacerbation of allergic inflammation. In addition, IL-1 α , IL-25, and thymic stromal lymphopoietin (TSLP) constitute inductive cytokines in innate immunity⁷. Thus, these innate immunity-associated cytokines may also play a crucial role in the acute exacerbation of allergic inflammation.

The HDM antigen, a major allergen for AKC, has the potential to induce type 2 inflammation-associated IgE-mediated immediate hypersensitivity and innate immunity⁸. For example, it has been reported that repeated challenge with HDM antigen caused type 2 inflammation in the bronchial mucosa of an asthma mouse model⁹. However, the relationship between the exacerbation of allergic inflammatory response on the ocular surface and the dose of invading HDM antigen in the conjunctiva remains unclear in AKC pathophysiology.

In our previous studies, NC/Nga mice subjected to percutaneous sensitization by crude house-dust-mite (HDM) antigen and application of crude HDM antigen developed clinical features similar to those of AKC; thus, they constitute an ideal model to further elucidate the pathophysiology of AKC^{10,11}. Specifically, our AKC mouse model exhibits the following characteristics: high HDM-specific IgE antibodies in the serum, atopic dermatitis-like dermatitis, and severe subepithelial infiltration of eosinophils and lymphocytes in conjunctiva, pseudotubule formation, frank granuloma

formation, and inducible conjunctiva-associated lymphoid tissue formation in the conjunctiva as evidenced histologically ^{10,11}. Moreover, the mRNA levels of helper T cell type 2 (Th2) chemokines, macrophage-derived chemokine (CCL22/MDC), and thymus and activation-regulated chemokine (CCL17/TARC) significantly increased in the conjunctival tissues of the AKC mouse model compared with those of control mice ¹¹. Therefore, in our AKC mouse model, Th2-associated inflammation is considered to play a central role in the pathogenesis of allergic inflammation on the ocular surface. However, the pathophysiology of type 2 inflammation caused by adaptive and innate immunity on the ocular surface is also poorly understood.

We hypothesized that the administration of crude HDM-antigen at varying dosages would induce coordinate alterations in the cytokine and chemokine profiles of the conjunctiva presenting adaptive and innate immunity-associated allergic inflammation in our AKC mouse model. Previously, we reported that the mRNA expression of *IL-1 α* , also known as alarmin, significantly increased on the ocular surface of patients with VKC ¹². Therefore, the effect of subcutaneous injection of anti-IL-1 α antibody before the induction of allergic inflammation in the conjunctiva as an inhibitor of conjunctival inflammation including adaptive and innate immunity-, and

helper T cell type 17 (Th17)-associated inflammation was examined in our AKC mouse model.

Materials and Methods

Mice

Eight-week-old female NC/Nga mice, reared under specific pathogen-free (SPF) conditions, were purchased from Charles River Japan (Yokohama, Japan). The mice were housed under SPF conditions. The mice were provided food and tap water ad libitum. All animal experiments were approved by the Animal Care and Use Committee of Nihon University School of Medicine. All mice were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) resolution regarding animals used in research.

Dermatologic or ophthalmologic ointment of *Dermatophagoides farinae* body extract

Dermatophagoides farinae body (Dfb) dermatologic ointment (Biostir[®]AD, Biostir, Kobe, Japan) at a concentration of 5 mg Dfb extract/1 g hydrophilic petrolatum and Dfb ophthalmic ointment containing 5 mg Dfb extract/1 g or 2.5 mg Dfb extract/1 g white petrolatum (PROPETO[®], Maruishi Pharmaceutical, Tokyo, Japan) were used in this study.

AKC mouse model

NC/Nga mice were sensitized by repeated percutaneous applications of the Dfb dermatologic ointment (percutaneous sensitization) as shown in Table 1. On the first sensitization (day 1), we applied 100 mg of Dfb dermatologic ointment onto the denuded dorsal skin and on the surface of both ears of the mice. This topical application was repeated twice weekly. By day 17, we confirmed that the development of AD-like skin lesions was confirmed. On days 18–20, NC/Nga mice were challenged by the application of Dfb ophthalmic ointment (ophthalmic challenge), once daily for 3 consecutive days. At 24 h after the final application, clinical findings were recorded, and then the mice were euthanized via an overdose of pentobarbital; thereafter, the eyeballs and eyelids were harvested from the mice (Table 1).

Study 1: Evaluation of dose-dependent response to house-dust-mite antigen

To evaluate dose-dependent response, an ophthalmic challenge on days 18–20 was performed once daily by applying either 5 (high-dose HDM antigen; High-HDM group, n = 6) or 2.5 mg/g (low-dose HDM antigen; Low-HDM group, n = 5) Dfb ophthalmic ointment. Moreover, five mg of Dfb ophthalmic ointment was used for one eye application. The control mice (control group, n = 7) received percutaneous

sensitization without any ophthalmic ointment. Clinical and histological findings and the mRNA expression levels were compared among the three groups.

Study 2: Evaluation of protective effects of anti-IL-1 α antibody subcutaneous injection

To evaluate the protective effects of the anti-IL-1 α antibody, the anti-mouse IL-1 α rabbit polyclonal antibody solution (20 μ L in each lid of 25 ng/ μ L anti-IL-1 α ; arigo Biolaboratories, Taiwan, ROC) (IL-1 α group, n = 5) or saline (vehicle group, n = 5) was subcutaneously injected into the upper and lower lids on day 17. Ophthalmic challenge on days 18–20 was performed once daily by applying 5 mg/g Dfb ophthalmic ointment. Clinical and histological findings and the mRNA expression levels were compared between the IL-1 α and vehicle groups.

Histological and immunohistochemical examination

Eosinophilic infiltration in the conjunctival tissues

The entire conjunctiva and cornea of mice in all groups were embedded in paraffin after fixing with 4% paraformaldehyde (PFA) solution (WAKO, Tokyo, Japan). The paraffin block was cut into approximately 4- μ m-thick sections using a microtome (HM505E; Microm International GmbH, Walldorf, Germany). The paraffin sections were stained using the direct fast scarlet (DFS) staining method to detect eosinophils¹³.

After staining the conjunctiva sections from each group, photographs were taken under a light microscope (BZ-9000; KEYENCE Japan; Osaka, Japan). The total area of the subconjunctival tissue was calculated in each digital image using a semi-automated image analysis method with Adobe Photoshop Elements 14 software (Adobe Systems Inc., San Jose, CA, USA). The density of eosinophils (mm^2) stained by DFS staining was calculated in the subconjunctival tissue. The cell densities were determined and averaged in 15 thin sections for each group.

Immunofluorescence staining for Gr-1, IL-13, and CD4

For Gr-1 expression analysis by immunofluorescence, unfixed frozen sections used in laser microdissection (described below) were used by post-fixing with 4% PFA for 3 min. The frozen sections were then incubated in blocking buffer (Antibody Diluent/Block; PerkinElmer, MA, USA) for 30 min, and then with purified rat anti-mouse Ly-G6 and Ly-C6 (Gr-1) antibody (BD Pharmingen, CA, USA), which was detected with Alexa fluor[®]-488-labeled donkey anti-rat IgG (Life Technologies; Tokyo, Japan), overnight at 4°C. Finally, the slides were counterstained with 4'-6-diaminido-2-phenylindole (DAPI)-fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

For IL-13 expression analysis by immunofluorescence, the paraffin-embedded sections were incubated with 5% normal donkey serum (Vector Laboratories, Burlingame, CA, USA) for 30 min to block non-specific reactions. The slides were then incubated overnight at 4 °C with an anti-IL-13 rabbit polyclonal antibody (ab 106732, 1:200; Abcam, Tokyo, Japan), which was detected with Alexa Fluor[®]-488-labeled donkey anti-rabbit IgG (Life Technologies). Finally, the slides were counterstained with DAPI-fluoromount-G (SouthernBiotech).

For double-immunofluorescence staining of IL-13 and CD4, unfixed frozen sections were used by post-fixing with 4% PFA for 3 min. The frozen sections were blocked with blocking buffer (Antibody Diluent/Block, PerkinElmer) for 30 min at room temperature. Thereafter, the slides were incubated with a cocktail of anti-mouse IL-13 rabbit polyclonal antibody (Abcam) and anti-mouse CD4 rat monoclonal antibody (Invitrogen, CA, USA) overnight at 4°C. The anti-mouse IL-13 rabbit polyclonal antibody (Abcam) was detected with Alexa Fluor[®]-647-labeled donkey anti-rabbit IgG (Life Technologies Japan) and the anti-mouse CD4 rat monoclonal antibody was detected with Alexa Fluor[®]-488-labeled donkey anti-rat IgG (Life Technologies Japan). Finally, the slides were counterstained with DAPI-fluoromount-G (SouthernBiotech).

Images were captured using a BIOREVO digital camera (BZ-9000; KEYENCE Japan, Osaka, Japan).

RNA isolation, and PCR array and quantitative PCR analyses

mRNA isolation via laser-assisted microdissection

The conjunctival tissues from mice were embedded in OCT compound (Sakura Seiki, Tokyo, Japan) without prior fixation. Frozen blocks of the conjunctival tissues were sectioned (20 μ m thick) on RNase-free polyethylene naphthalate-coated glass slides (Leica Microsystems, Tokyo, Japan). The tissue sections were subsequently incubated for 30 s in cold 100% methanol, and then immersed in distilled H₂O and 0.05% toluidine blue, and air-dried. Laser-assisted microdissection was immediately performed using the LMD7000 system (Leica Microsystems, Wetzlar, Germany). The dissected samples were collected into Thermo-Tube caps (Thermo Scientific Japan, Yokohama, Japan) containing 10 μ L of mineral oil (Life Technologies). The total RNA was extracted as described previously ¹⁴, and the samples were stored at -80°C until further analysis.

PCR array analysis

To identify the differences in adaptive and innate immunity-associated gene expression between the Control (n = 1) and High-HDM (n = 1) groups, PCR array analysis was performed to screen for alterations in the adaptive and innate immunity-associated gene profiles. cDNA was synthesized from the total RNA isolated above using the High-Capacity cDNA Reverse Transcription kit (Life Technologies). The samples were used to analyze the expression of 84 genes encoding adaptive and innate immunity-associated factors, using the RT² Profiler™ PCR Array Mouse Innate & Adaptive Immune Responses (PAMM-052ZA; Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Data were analyzed using RT² Profiler™ PCR Array software and $\Delta\Delta C_t$ -based fold changes were calculated.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

For real-time RT-PCR, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit. To assess the mRNA expression levels of cytokines and chemokines in the conjunctiva among the High-HDM, Low-HDM, and control groups, real-time RT-PCR was performed using TaqMan Gene Expression Assays (Life Technologies Japan) for mouse IL-13 (*Il13*; Mm00434204_m1), IL-17A (*Il17a*; Mm00439618_m1), CCL11/eotaxin-1 (*Ccl11*; Mm00441238_m1), and

CCL24/eotaxin-2 (*Ccl24*; Mm00444701_m1). The data were analyzed using the $\Delta\Delta C_t$ method and normalized to a housekeeping gene (*actin*; Mm01184552_m1).

Statistical analysis

The differences in eosinophil density and real-time RT-PCR results among the High-HDM, Low-HDM, and control groups were evaluated using the nonparametric Steel–Dwass test, and those between the IL-1 α and vehicle groups were analyzed using Mann–Whitney U test. *P* values of <0.05 were considered to indicate statistical significance. Statistical analyses were carried out using MAC Toukei–Kaiseki v.2 software (Esumi, Tokyo, Japan).

Results

Clinical features of the AKC mouse model

The mice in the High-HDM, Low-HDM, and control groups were visually observed for clinical phenotypes. All mice in the Low-HDM and High-HDM groups developed atopic dermatitis-like dermatitis (Figure 1a) and conjunctival hyperemia/blepharitis accompanied by AKC (Figure 1b), whereas those in the control group developed only atopic dermatitis-like dermatitis. Five of the six mice in the

High-HDM group exhibited shield ulcer-like keratitis (Figure 1b). Histologically, the shield ulcer-like keratitis showed epithelial damage or defects with severe inflammatory cell infiltration including eosinophils (Figure 1c).

[Figure 1 near here]

Alterations in neutrophilic and eosinophilic inflammation in the conjunctiva by dose-dependent induction with the HDM antigen

Histological findings for neutrophils in the cornea and conjunctiva

Gr-1 immunofluorescence staining showed only a few Gr-1-positive cells in the conjunctival tissue, but many Gr-1-positive cells in the corneal ulceration area in the High-HDM group (Figure 1d).

Eosinophilic infiltration in the conjunctival tissue

A histological assessment of eosinophilic infiltration was performed to ascertain the alterations in allergic inflammation in the conjunctiva. In paraffin-embedded conjunctival specimens subjected to DFS staining, no (Figure 2a), mild (Figure 2b), and severe (Figure 2c) eosinophil infiltration was observed in the control, Low-HDM, and High-HDM groups, respectively. The density of eosinophils in the subconjunctival tissue was 1.8 (0.3–4.1), 1.3 (0.3–3.1), and 0 (0–0.4) [median (range)] $\times 10^3$ cells/mm² in

the High-HDM, Low-HDM, and control groups, respectively. The density of eosinophils in the subconjunctival tissue in the High- and Low-HDM groups was significantly higher than that in the control group ($P < 0.01$ and $P < 0.01$ in High-HDM vs. control and Low-HDM vs. control, respectively, Steel–Dwass test, Figure 2d)

[Figure 2 near here]

PCR array analysis for adaptive and innate immunity-associated factors in the conjunctiva

Initially, adaptive and innate immunity-associated factors were considered as differentially expressed (increased) based on 5-fold increase in mRNA expression in the High-HDM group compared with that in the control group. The selected factors, shown in Table 2, included the Th2 cytokine *Il13* and *Ccr6* mRNAs expressed by Th17 cells.

Quantitative PCR analysis of mRNA expression of IL-13, IL-17A, CCL11, and CCL24 in the conjunctiva

We assessed the expression level of *Il13* and *Il17A* in addition to *Ccl11*/eotaxin-1 and *Ccl24*/eotaxin-2 encoding eosinophil-associated chemokines using the quantitative real-time PCR method, because PCR array indicated that Th2- and Th17-associated gene expression was upregulated.

The quantitative PCR analysis revealed that the *Il13* mRNA expression in the High-HDM group was significantly higher than that in the control and Low-HDM groups ($P < 0.05$ and $P < 0.05$, respectively, Steel–Dwass test, Figure 3a).

Ccl11/eotaxin-1 mRNA expression in the High-HDM group was significantly higher than that in the control group (Figure 3c). There were no significant differences in the mRNA expression of *Il17A* and *Ccl24*/eotaxin-2 among the High-HDM, Low-HDM, and control groups (Figure 3b and 3d).

[Figure 3 near here]

Histological immunofluorescence staining for IL-13

In the High-HDM group, IL-13-positive cells were observed in the subconjunctival and subepithelial areas of the conjunctival tissues (Figure 4a). The infiltration of IL-13-positive cells in the conjunctival tissues was more frequent in the fornix than in the palpebral and bulbal conjunctivae (Figure 4a). IL-13-positive cell infiltration was not observed in the negative control (Figure 4b).

[Figure 4 near here]

Alterations in eosinophilic inflammation in the conjunctiva by eyelid subcutaneous injection of the anti-IL-1 α antibody

We assessed the anti-eosinophilic inflammatory effect of eyelid subcutaneous injection of anti-IL-1 α antibody in the AKC mouse model because IL-1 is both an innate immunity- and Th17 response-associated cytokine.

Eosinophilic infiltration in the conjunctival tissue

DFS staining showed that eosinophil infiltration in the IL-1 α group was milder than that in the vehicle group (Figure 5a and 5b). The density of eosinophils in the subconjunctival tissue in the IL-1 α and vehicle groups was 1.2 (0–5.2) and 3.1 (0.6–9.0) [median (range)] $\times 10^3$ cells/ mm², respectively. Eosinophil density in the subconjunctival tissues in the IL-1 α group was significantly reduced compared with that in the vehicle group ($P < 0.01$, Mann–Whitney U-test, Figure 5c).

[Figure 5 near here]

Quantitative PCR analysis of mRNA expression of IL-13, IL-17A, CCL11, and CCL24 in the conjunctiva

The levels of *Il13*, *Il17A*, and *Ccl24* mRNA in the IL-1 α group were significantly lower than those in the vehicle group (Figure 6a, 6b, and 6d). No significant difference was observed in *Ccl11* mRNA expression levels between the IL-1 α and vehicle groups (Figure 6c).

[Figure 6 near here]

Histological immunofluorescence staining for IL-13

In the vehicle group, IL-13-positive cells were observed in the subconjunctival and subepithelial areas of the conjunctival tissues, as in the High-HDM group (Figure 4c).

The proportion of IL-13-positive cells in the conjunctiva of the IL-1 α group was lower than that in the vehicle group (Figure 4d). Double staining of CD4 and IL-13 revealed that most of the IL-13-positive cells were negative for CD4 (Figure 4e).

Discussion

We investigated eosinophilic inflammation caused by crude HDM antigen on the ocular surface using an AKC mouse model. Our study clarified the following. (1) In the AKC mouse model, eosinophilic inflammation in the conjunctiva successfully developed, and it was exacerbated in a dose-dependent manner by crude HDM antigen application. (2) IL-13 constitutes a major cytokine expressed in the conjunctival tissue in conjunction with eosinophilic inflammation. (3) Subcutaneous injection of the anti-IL-1 α antibody to the eyelids before crude HDM antigen application partially attenuated eosinophilic inflammation and reduced IL-13 expression in the conjunctiva.

Der p 1, a main component of HDM, exhibits protease activity and induces barrier dysfunction in the skin and mucosal epithelium¹⁵. Consequently, innate immunity-associated cytokines including TSLP, IL-25, and IL-33 are released from the barrier-compromised epithelium. Moreover, Der p 2, another component of HDM, exhibits structural homology with myeloid differentiation 2 and acts as a lipopolysaccharide-binding component of the toll-like receptor-4 (TLR-4) signaling complex¹⁶. TLR-4 signaling also increases the secretion of innate immunity-associated cytokines including IL-1 α , IL-25, and IL-33 from epithelial cells⁸. Therefore, in addition to HDM-specific IgE antibody-mediated immediate hypersensitivity, the crude HDM antigen may induce HDM-associated innate immune responses to simultaneously mediate various induction processes.

Clinically, HDM is considered an exacerbation factor of atopic dermatitis, asthma, allergic rhinitis, and AKC. In atopic dermatitis, the inhalation of HDM exacerbates the skin lesions of atopic dermatitis¹⁷. In contrast, sublingual allergen immunotherapy with the HDM antigen is used for allergic rhinitis and asthma, with demonstrated efficacy and safety¹⁸. Thus, the pathogenic role of the HDM antigen in allergic diseases remains controversial. However, it is possible that the dose and invasive route of HDM antigen constitute critical factors in the allergic inflammation of allergic diseases. In particular,

in the present study, the transcutaneous sensitized AKC mouse model showed dose-dependent exacerbation of eosinophilic inflammation in the conjunctiva upon HDM antigen application. In addition, the expression of *IL-13* and *CCL11* mRNA in the conjunctival tissues showed an increasing trend similar to that of eosinophil counts. Furthermore, eyelid injection of the anti-IL-1 α antibody improved eosinophilic inflammation in the conjunctiva. A previous study reported that IL-13 and IL-4 synergistically increase the production of CCL11¹⁹. These findings suggest that eosinophilic inflammation of the conjunctiva caused by AKC may involve IL-13, which is induced by IL-1 α . Furthermore, these results indicated that the development threshold and exacerbation dose of the HDM antigen should be considered in the medical evaluation and treatment of AKC.

The major pathologic condition of allergic inflammation in the target organs resulting from HDM antigen exposure is type 2 inflammation. Th2 cytokines IL-4, IL-5, and IL-13 secreted by Th2 cells, ILC2, mast cells, and other allergy-associated immune cells exacerbate such type 2 inflammation. In the present study, the increase in eosinophilic infiltration and *Il13* mRNA expression in the conjunctiva of AKC mouse upon crude HDM application may reflect the exacerbation of type 2 inflammation on the ocular surface. Imai et al.²⁰ reported that hK14mIL33tg transgenic mouse line

developed keratoconjunctivitis resembling human AKC, along with substantially increased ILC2 production of IL-5 and IL-13 in the cornea. Similarly, the PCR array and quantitative PCR analyses revealed that *Il13* mRNA expression in the conjunctiva of our AKC mouse model increased in a dose-dependent manner along with the eosinophil count in the conjunctiva. *Il17a* mRNA expression also exhibited a tendency to increase in a dose-dependent manner, although this trend did not reach statistical significance. Moreover, the histological analysis by immunofluorescence staining revealed that IL-13-positive and CD4-negative cells infiltrated the conjunctival tissues of the AKC mouse model. These findings further indicated that IL-13, produced by inflammatory cells other than Th2 cells, is a major inflammatory cytokine associated with eosinophilic inflammation in our AKC mouse model following HDM antigen exposure. Therefore, it is possible that the main IL-13-producing cell in AKC may be ILC2.

Medical treatment to counter IL-13 expressed on the ocular surface concomitant with allergic inflammation has not been fully established. In atopic diseases, the role of eosinophilic inflammation in immunotherapy has been investigated ²¹. Dupilumab, a fully human monoclonal antibody for IL-4 receptor α (IL-4R α), inhibits the signaling of both IL-4 and IL-13, and it has shown promising efficacy in the treatment of

moderate-to-severe atopic dermatitis²². Benralizumab, a humanized monoclonal antibody for IL-5R α , can reduce asthma exacerbation rate and blood eosinophil count in patients with eosinophilic asthma²³. Additionally, corticosteroid or immunosuppressant application constitutes a major medical approach for the treatment of AKC and VKC with severe eosinophilic inflammation in the conjunctiva. However, because steroid- or immunosuppressant-resistant VKC and AKC have been identified²⁴, the development of additional medical treatment options for refractory cases is necessary.

IL-1 α , a member of the IL-1 family, is produced by damaged epithelial cells as an alarmin and by macrophages as a proinflammatory cytokine²⁵. Furthermore, IL-1 α induces IL-17 production from Th17 and group 3 innate lymphoid cell (ILC3) cells^{26,27}.

Therefore, the monoclonal antibody against IL-1 α is being tested in the management of type 2 diabetes, cancer, cancer cachexia, leukemia, psoriasis, vascular diseases, and scarring acne vulgaris²⁸. In allergic diseases, the Th2 cytokine produced by Th2 and

ILC2 cells is associated with eosinophil inflammation, whereas the Th17 cytokine IL-17 is associated with neutrophilic inflammation. In our previous study¹², we showed that the mRNA expression of *eotaxin-2-IL-16*, which may be associated with eosinophilic inflammation, and that of *IL-1 α -IL-8*, which may be associated with neutrophilic inflammation, were detected on the ocular surface of patients with severe VKC or AKC.

In contrast, Willart et al.⁸ reported that IL-1 α functions upstream of the cytokine cascade leading to epithelial and dendritic cell activation in response to inhaled HDM antigen. In the present study, decreased *Il13* and *Il17* mRNA expression in the conjunctiva was observed in addition to decreased eosinophil count following the subcutaneous injection of the anti-IL-1 α antibody to the eyelids. These results demonstrated that eyelid subcutaneous injection of the anti-IL-1 α antibody may alleviate conjunctival allergic inflammation. In comparison, supratarsal injection of steroid has demonstrated therapeutic efficacy against refractory allergic keratoconjunctivitis²⁹, however, steroid therapy is associated with the risk of elevation of intraocular pressure. Thus, clinical assessment of eyelid subcutaneous injection as an antibody therapy for refractory AKC is warranted. The subcutaneous injection of the anti-IL-1 α antibody to the eyelids may improve the efficacy of topical treatment of refractory AKC when used in combination with conventional therapies including immunosuppressive eye drops for eosinophilic inflammation caused by type 2 inflammation of the conjunctiva.

Our study had the following limitations. (1) We were unable to identify IL-13-positive cells as ILC2; thus, quantitative investigation of IL-13 production from ILC2 cells in the conjunctiva of the AKC mouse model requires further evaluation. (2)

In antibody therapy for epithelial cytokines associated with innate immunity including IL-1 α (tested herein), TSLP, IL-25, and IL-33, the therapeutic effect of individual antibodies for each factor against eosinophil inflammation should be compared. The antibody that is optimal for eyelid subcutaneous injection should be chosen.

In conclusion, we found that crude HDM antigen induced a dose-dependent increase in eosinophilic infiltration and *Il13* mRNA expression in the conjunctiva of an AKC mouse model. These inflammatory alterations were partially improved by eyelid injection of the anti-IL-1 α antibody. These findings suggest that IL-1 α -induced IL-13 production constitutes a major exacerbating factor for HDM-induced AKC in addition to a potential therapeutic target.

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Disclosure Statement

None of authors have any financial interest or conflict of interest to disclose.

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Figure Legends

Figure 1. Clinical and histological findings of the AKC mouse model in the High-HDM group. (a) Development of atopic dermatitis-like dermatitis in the back and pinna. (b) Development of conjunctival hyperemia, and shield ulcer-like corneal epithelial damage on the ocular surface. (c) Invasion of numerous inflammatory cells including eosinophils into the corneal epithelium (black arrow), with apparent epithelial deficiency. (d) In immunofluorescence staining for Gr-1 in the High-HDM group, many Gr-1-positive cells were observed in the corneal ulceration area and only a few Gr-1-positive cells were found around the vessels in the conjunctival tissue.

Figure 2. Eosinophilic infiltration in the conjunctiva of the High-HDM, Low-HDM, and control groups. Histological examination of eosinophilic infiltration in the conjunctiva of the control (a), Low-HDM (b), and High-HDM (c) groups (DFS staining). The blue arrowheads indicate eosinophils. Bar = 100 μ m. The box-and-whisker plot shows that the eosinophil density in the conjunctiva of the High-HDM (n = 6) and Low-HDM (n = 5) groups significantly increased compared with that in the control (n = 7) group (d). * P < 0.05 (Steel–Dwass test).

Figure 3. Messenger RNA levels of cytokines and chemokines in the conjunctiva. mRNA levels of IL-13 (a), IL-17A (b), CCL11 (c), and CCL24 (d) in the High-HDM (n = 6), Low-HDM (n = 5), and control (n = 7) groups as measured by quantitative polymerase chain reaction analysis. * P < 0.05 (Steel–Dwass test); NS, not significant.

Figure 4. Immunofluorescence staining of conjunctival tissues for IL-13. In the High-HDM group, IL-13-positive cells (green) were observed in the subconjunctival

tissues and the subepithelial area of conjunctival tissues. (a) High-HDM group; (b) negative control of the High-HDM group. Bar = 100 μ m. In the IL-1 α and vehicle groups, the density of IL-13-positive cells (pink) in the conjunctiva of the IL-1 α group was lower than that of the vehicle group. (c) Vehicle group and (d) IL-1 α group. Bar = 100 μ m. In double-staining for CD4 and IL-13, most of the IL-13-positive cells (pink) were negative for CD4 (green). (e) Vehicle group. Bar = 100 μ m.

Figure 5. Eosinophilic infiltration in the conjunctiva of the IL-1 α and vehicle groups. Histological examination of eosinophilic infiltration in the conjunctiva of the vehicle (a) and IL-1 α (b) groups (DFS staining). The blue arrowheads indicate eosinophils. Bar = 100 μ m. Eosinophil density in the conjunctiva of the IL-1 α group (n = 5) significantly decreased compared with that in the vehicle (n = 5) group. $**P < 0.01$ (Mann–Whitney U-test).

Figure 6. mRNA levels of cytokines and chemokines in the conjunctiva. mRNA levels of IL-13 (a), IL-17A (b), CCL11 (c), and CCL24 (d) in the IL-1 α (n = 5) and vehicle (n = 5) groups as measured by quantitative polymerase chain reaction. $**P < 0.05$ (Mann–Whitney U-test); NS, not significant; Ab, antibody.

Table 1. Experimental design of the atopic keratoconjunctivitis mouse model

Table 2. Comparison of adaptive and innate immunity-associated gene expression

between the High-HDM and control groups using the PCR array

Table 1. Experimental design of the atopic keratoconjunctivitis mouse model

	Study 1			Study 2	
Day	Low-HDM group	High-HDM group	Control group	IL-1 α group	Vehicle group
1	←	←	←	←	←
4	←	←	←	←	←
7	←	←	←	←	←
10	←	←	←	←	←
14	←	←	←	←	←
17	←	←	←	←	←
	AD-like dermatitis				
				■	▲
18	●	●		●	●

Table 2. Comparison of adaptive and innate immunity-associated gene expression between the High-HDM and control groups using the PCR array

Messenger RNA	Fold increase (vs. control group) *	
	Low-HDM group	High-HDM group
C3	2.90	6.73
C5ar1	4.91	3.82
CCL12	9.22	2.69
CCL5	-3.53	2.45
CCR4	1.06	2.91
CCR6	1.66	7.28
CD4	4.77	24.31
CXCL10	5.41	11.47
CXCR3	7.57	1.55
IFNG	1.06	4.16
IL13	2.97	6.32
IL1B	1.30	2.68
IL23A	1.59	4.42
IL5	1.06	2.51
MPO	1.06	3.20
NLRP3	2.46	1.09
STAT4	1.06	4.57
TLR6	4.75	-1.04
TLR8	1.40	4.92
TRAF6	1.08	2.98

*, mRNAs with a 2-fold or greater increase are shown in the table, and mRNAs with a 5-fold or greater increase are in **bold** font.

List of mRNAs measured by PCR array

Serum amyloid P-component; Complement component 3 (C3); Complement component 5a receptor 1 (C5ar1); Caspase 1; Chemokine (C-C motif) ligand 12 (CCL12); Chemokine (C-C motif) ligand 5 (CCL5); Chemokine (C-C motif) receptor 4 (CCR4); Chemokine (C-C motif) receptor 5; Chemokine (C-C motif) receptor 6 (CCR6); Chemokine (C-C motif) receptor 8; CD14 antigen; CD4 antigen (CD4); CD40 antigen; CD40 ligand; CD80 antigen; CD86 antigen; CD8 antigen, alpha chain; C-reactive

Figure 1

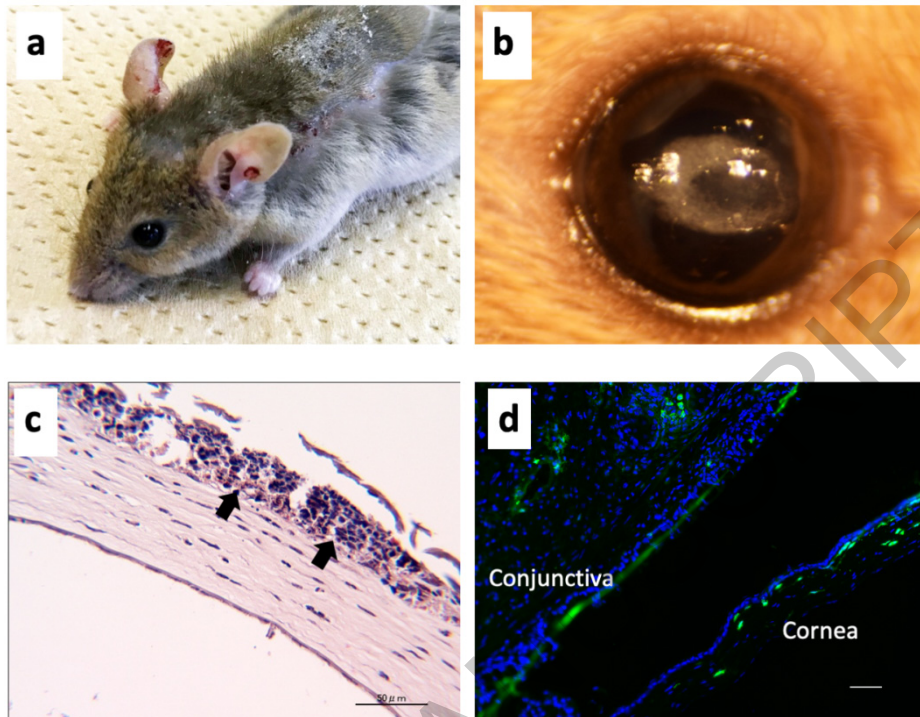


Figure 2

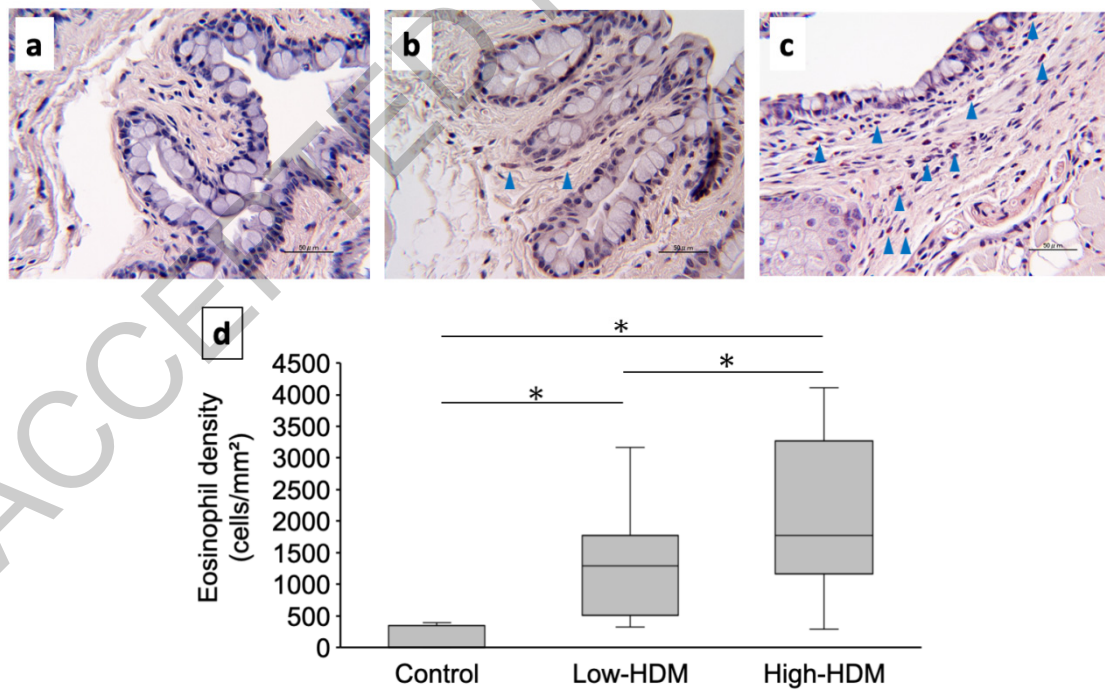


Figure 3

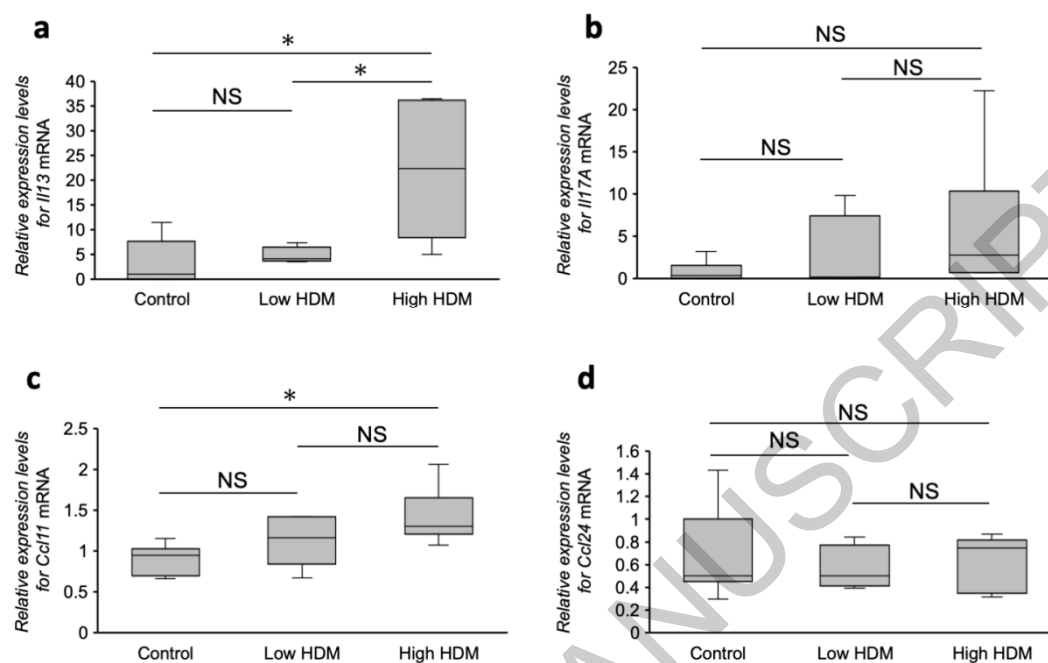


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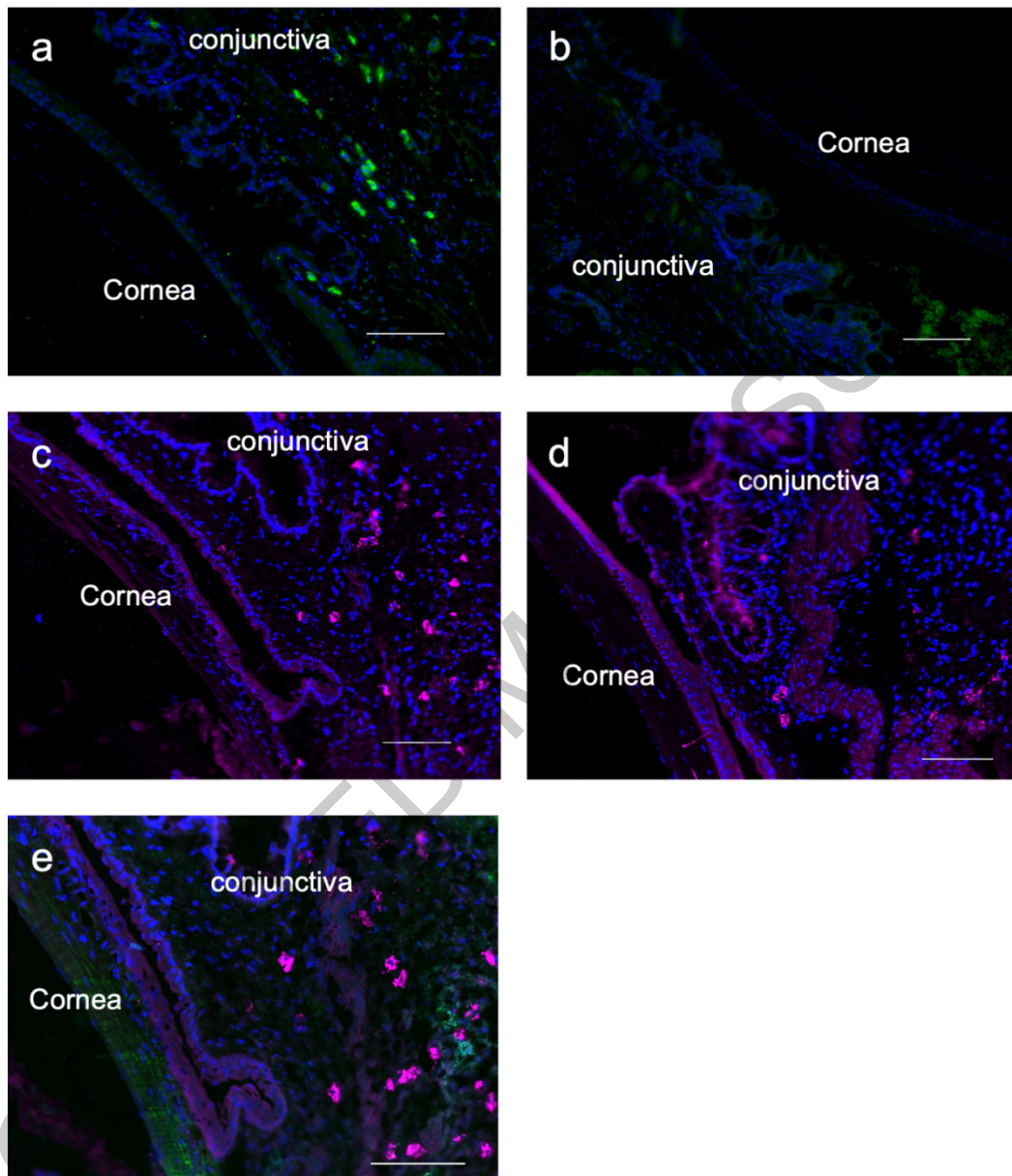


Figure 5

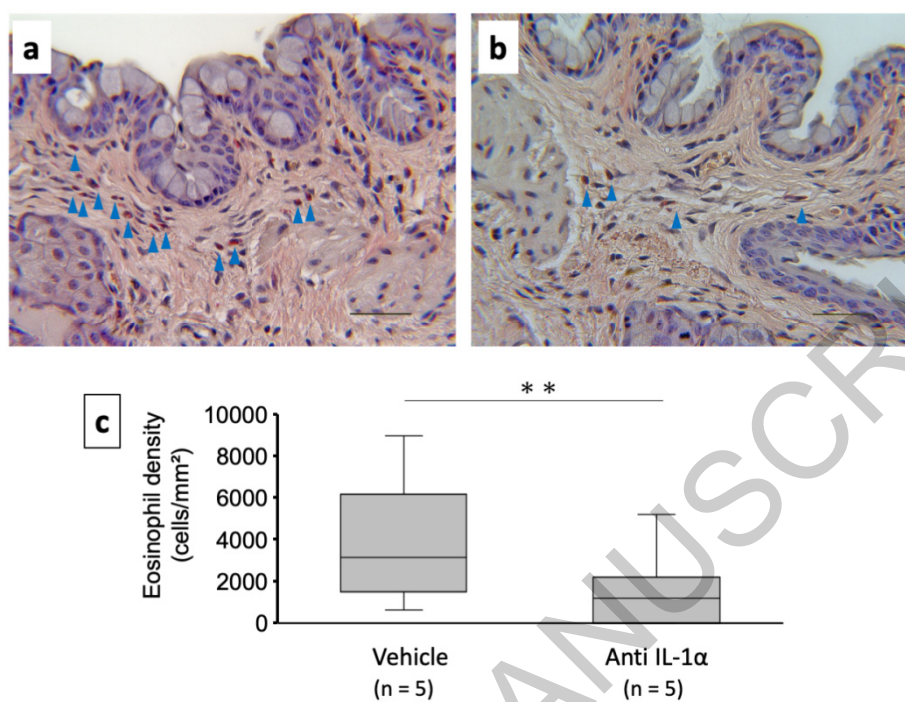


Figure 6

