



Pollen shells and soluble factors play non-redundant roles in the development of allergic conjunctivitis in mice

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ABSTRACT

Purpose: We aimed to clarify the role of particulate allergen exposure to the conjunctiva in the development of allergic conjunctivitis.

Methods: We administered ragweed pollen suspension, pollen extract, pollen shell, particulate air pollutants, and their combinations to the mouse conjunctiva five days a week without prior sensitization. Clinical signs were scored. Histological changes, cellular infiltrations, mRNA expressions, lymph node cell recall responses, and serum immunoglobulin levels were assessed. Immune cell-depleting antibodies and ST2 knockout mice were used to investigate the cellular and molecular requirements.

Results: Pollen suspension, but not the extract or shell alone, induced robust eosinophilic conjunctivitis, accompanied by a proliferative response of epithelial cells. A combination of pollen extract and shell completely restored eosinophil accumulation. In addition, eosinophilic conjunctivitis was induced by a mixture of particulate air pollutants and pollen extract. Mechanistically, eosinophil accumulation was ameliorated by deficiency of the IL-33 receptor ST2 and abolished by depleting CD4⁺ T cells. Pollen shells, but not the extract, induced IL-33 release from conjunctival epithelial cells in vivo.

Conclusions: Our results indicate the non-redundant roles for the allergens' particulate properties and soluble factors in the development of allergic conjunctivitis.

1. Introduction

Ocular allergy is a common allergic manifestation [1–4]. Its prevalence has increased in the past decades [3,5,6] and is estimated to have reached approximately 50% in Japan [1] and 40% in the US [3]. Seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) are the most common but least severe forms, while vernal

keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) are relatively rare but severe forms that potentially lead to corneal involvement and ultimately to vision loss [1,2,4,7–10]. The proliferative lesions such as giant papillae are observed in VKC, and in some cases of AKC [2,7–10]. In addition, giant papillary conjunctivitis (GPC) may develop as a result of mechanical irritation such as contact lenses [2,7,11].

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; DAPI, 4',6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; PBS, phosphate buffered saline; Th, helper T; Tpath2, pathogenic type 2 helper T; TSLP, thymic stromal lymphopoietin.

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The ocular allergy often accompanies other allergic conditions such as allergic rhinitis, atopic eczema [1,12], asthma [13], and food allergy [2,14]. Among them, the most popular comorbidity is the allergic rhinitis; however, isolated ocular symptoms have also been documented in 6% of the general population [3]. Importantly, the sensitized allergen patterns confirmed by skin test were skewed to the weed pollens in patients with ocular symptoms, compared to the grass, mold, or indoor allergens [3]. However, it remains obscure what properties of weed allergens are essential to sensitize patients to cause solely ocular symptoms.

Epidemiological studies have been performed to elucidate the association between particulate air pollutants and conjunctivitis [15,16]. In addition, their association with allergic conjunctival diseases has been reported [1,17,18]. For instance, particulate matter (PM) exposure increases outpatient visits of allergic conjunctivitis [17]. The levels of PM₁₀, which is a PM with an aerodynamic diameter less than 10 µm, are associated with the prevalence of VKC [1]. Furthermore, the skin prick test response to Asian dust has been found to correlate with the mean wheal diameters for pollens and fungi, implying its adjuvant effect [18]. Although the mechanistic studies on the effect of particulate air pollutants on the ocular surface are relatively limited [19–23], their biological and immunomodulatory effects on the airways have been extensively studied [24]. However, since the lower respiratory tract is accessible to only small particles with a median aerodynamic diameter under 2.5 µm, the effect of larger particles such as pollen grains onto the mucosal surface has been less characterized.

Interleukin (IL)-33 is an epithelial cell-derived cytokine, which is considered as one of the key regulators of type 2 immunity [25,26]. It is stored in the nuclei in the steady state, and actively released upon detection of external damage, potential pathogens, or allergens [25–29]. Necrotic cells also release IL-33 slowly from their chromatin [30]. Thereafter, the protease activity from various sources including allergen proteases from various pollens, fungi, mites, and cockroaches act as amplifiers of IL-33 activity [25,31,32].

IL-33 has been implicated in allergic conjunctival diseases [33]. The serum level of IL-33 was found to be elevated in the Cedar pollen allergic rhinoconjunctivitis patients [34]. In addition, local expression of IL-33 in giant papillae of AKC patients was confirmed in the epithelial cells as well as in the vascular endothelial cells [35]. Functionally, in a sensitization-elicitation type of murine allergic conjunctivitis model, exogenous IL-33 augmented eosinophil accumulation [36]. On the other hand, IL-33 deficiency, but not thymic stromal lymphopoietin (TSLP) or IL-25 deficiency, ameliorated conjunctivitis in the elicitation phase [37]. Furthermore, transgenic expression of IL-33 in epithelial cells in vivo provoked spontaneous keratoconjunctivitis resembling phenotypes of AKC, with marked increase of type 2 innate lymphoid cell (ILC2) population in the cornea [38]. Instillation of papain-soaked contact lenses to murine eyes induced IL-33 expression, leading to eosinophil accumulation dependently on TSLP, basophils, and ILC2, but not on T cells [39]. These observations suggest that IL-33 plays a role both in the innate type of conjunctival inflammation and in the elicitation phase of allergic conjunctivitis. However, the role of allergens' particulate properties on the release of IL-33 remains unclear.

In addition to the above-mentioned observation that a substantial percentage of the population develops ocular allergic symptoms without concomitant rhinitis or other allergies [3], it has been reported that up to 60% of ocular allergy patients consulting ophthalmologists had solely ocular symptoms [40]. Furthermore, the patients with elevated levels of tear IgE, without detectable serum allergen specific IgE have been reported [41–43]. Thus, we hypothesized that these patients might have been sensitized to allergens through their eyes. To address this hypothesis, we chose ragweed (RW) pollens as allergens, and repeatedly applied them to the mouse conjunctiva. Intriguingly, RW pollen suspension, but not its extract, induced allergic symptoms, accompanied by robust accumulation of eosinophils. By generating virtually protein-free pollen shells, we attempted to dissect the mechanisms underlying this

novel particulate pollen-induced mucosal sensitization model of allergic conjunctivitis.

2. Methods

For more information, see the Methods section in this article's Supporting Information.

2.1. Mice

C57BL/6 (B6) mice were bred in the animal facility of Juntendo University or purchased from Sankyo Labo Service Corporation (Tokyo, Japan). ST2 knockout mice were generated at TransGenic Inc (Fukuoka, Japan) using CRISPR/Cas9 method. All the animal experiments were approved by the ethical committee of Juntendo University (approval number 2020136) and performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the animal use in Ophthalmic and Vision Research.

2.2. Conjunctival sensitization model of allergic conjunctivitis

Allergic conjunctivitis was induced by repeated topical application of pollen suspension five days a week without prior sensitization. Each mouse conjunctival sac received 0.5 mg of pollen grains suspended in 2.5 µL of PBS. Pollen grains of RW (*Ambrosia artemisiifolia*, from Polysciences, PA, USA), Asian dust, or PM (urban dust; both from the National Institute for Environmental Studies, Japan) were used at the same concentration. PBS eye drops were used as controls. Mouse eyes were scored for the signs of immediate hypersensitivity responses 30 min after topical challenges, according to previously published criteria with slight modification [37,44]. Briefly, conjunctival redness, chemosis, tearing, and eyelid edema were graded as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Signs that appeared to be in the middle of the grades were scored in increments of 0.5. Scores of the left and right eyes were averaged to calculate the final clinical score assigned to each mouse. Mice were sacrificed 24 h or indicated time after the last challenge and subjected to analysis.

2.3. Preparation of ragweed pollen extract, shells, defatted, crushed, or heated pollens

To prepare RW pollen extract, the pollen grains were homogenized at a final concentration of 200 mg pollen per mL of PBS, and soluble fraction was extracted at 4 °C overnight. (See supplementary methods for details.)

The shells of RW pollen were produced following a previously published protocol [45]. Briefly, the pollens were defatted with acetone under reflux at 60 °C overnight, followed by an incubation in phosphoric acid at 60 °C for 7 d. After washing, the pollens were incubated in 6% w/v KOH solution at 80 °C for 6 h, twice. Washed pollen shells were dried overnight. Pollen shells were weighed and suspended in PBS or RW extract at the same particle concentrations as RW pollen preparations. (See supplementary methods for details.)

To prepare the crushed pollens, raw pollen grains were bead-homogenized using TissueLyser II (Qiagen, Germany). Crushed pollens were resuspended in PBS and broken morphology was confirmed under the microscope.

Heated pollen was prepared by incubating the raw pollen suspension in PBS at 72 °C for 10 min. After cooling, the suspension was administered to the mice.

3. Results

3.1. Repeated topical application of pollen suspension induces allergic conjunctivitis

We asked whether conjunctival exposure to pollen alone can induce sensitization and allergic conjunctivitis by repeated application of RW pollen suspension or its extract to the conjunctival sac (Fig. 1A). Intriguingly, pollen suspension, but not its extract, induced signs of acute allergic conjunctivitis in three weeks (Fig. 1B). Histological examination of the pollen suspension-treated mouse conjunctiva revealed accumulation of eosinophils and proliferative responses of epithelial cells (Fig. 1C and Supplementary Figure E1). Eosinophilic inflammation was readily observed in two weeks, and it further increased upon

repeated challenges (Fig. 1C–E). Conjunctival expression of Muc5ac was decreased reflecting the decrease of filled goblet cells, while the expression of periostin was increased indicating the development of Th2 type inflammation (Fig. 1C and F). Lymph node cells stimulated with the pollen extract produced mainly Th2 type cytokines, although interferon gamma, which also has a role in the development of allergic conjunctivitis [46], was also detected (Fig. 1G). Although local tissue IgE content started to increase in two weeks (Supplementary Figure E2), the systemic RW-specific humoral responses were delayed; RW-specific IgG1 was detectable in two weeks, and RW-specific IgE and IgG2a followed in five weeks (Fig. 1H). These results illustrate that the repeated conjunctival exposure to pollen suspension can effectively sensitize the mice and induce allergic conjunctivitis.

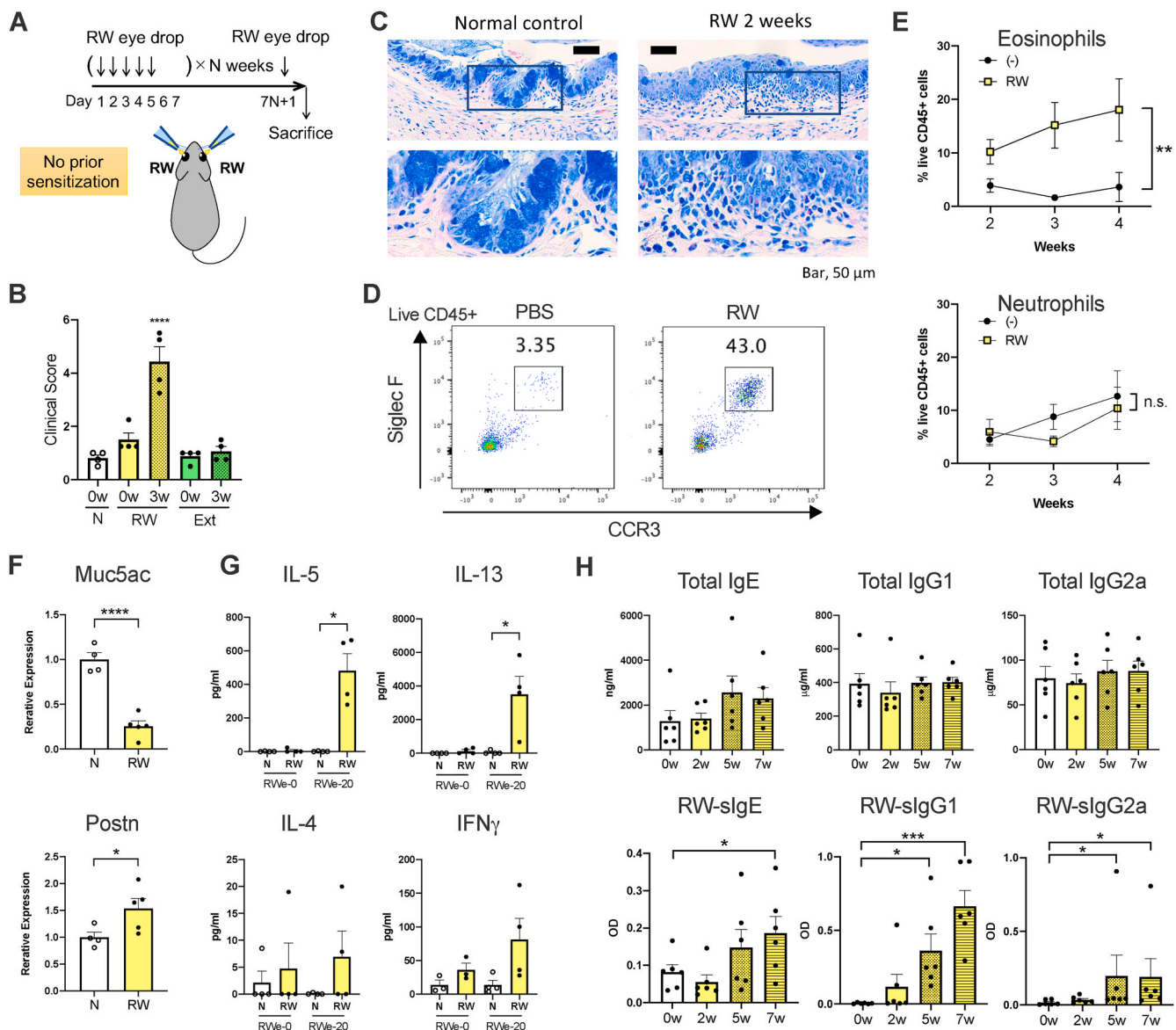


Fig. 1. Topical challenges to the conjunctiva sensitize mice to develop allergic conjunctivitis. (A) Diagram for inducing allergic conjunctivitis. (B) The clinical scores on the first and the last challenges. N, normal control; Ext, ragweed pollen extract. (C) Giemsa staining of the conjunctival tissue at 200x magnification. Bar, 50 µm. (D) Representative eosinophil frequency in the conjunctiva after three weeks of challenges. (E) Time-course of eosinophil and neutrophil frequency analyzed by flow cytometry. ** $p < 0.01$ by two-way ANOVA. (F) mRNA expressions in conjunctiva were quantified by qPCR using 18S as an internal control. Expressions levels in the normal conjunctiva were normalized to one. * $p < 0.05$, **** $p < 0.0001$ by Student's t-test. (G) Cytokine production from 0 or 20 µg/mL of the ragweed extract (RWe)-stimulated draining lymph node cells were quantified by ELISA. * $p < 0.05$ by Student's test with Welch's correction. (H) Serum immunoglobulins were quantified by ELISA. sIgE, specific IgE. * $p < 0.05$, *** $p < 0.001$ by repeated measure ANOVA with Geisser-Greenhouse's correction and Holm-Sidak's multiple comparison (for IgE) or Friedman test with Dunn's multiple comparison test (for IgG1 and IgG2a). Data represents mean $\pm$ S.E.M.

3.2. Shells and soluble factors are necessary and sufficient to induce allergic conjunctivitis

Next, we asked which pollen factors are required to induce eosinophilic conjunctivitis. To address this question, we produced RW pollen shells, essentially lacking protein and lipid content (Fig. 2A–C) [45]. In addition to the pollen shell, extract, and the mixture of shell and extract, the crushed, defatted, and heat inactivated RW pollens were used. Clinical signs were ameliorated in the absence of soluble factors or shells, and fully restored by the combination (Fig. 2D). Furthermore, the combination of pollen shells and RW extract, but not the shell or extract alone, induced robust accumulation of eosinophils. Defatted, crushed, or heat-inactivated pollens showed intermediate activity in inducing eosinophilic inflammation (Fig. 2E and F). Flow cytometric analysis of accumulating cells in the conjunctiva further revealed a marked increase of ST2⁺CD4⁺ T (Tpath2) cells, which are known to produce a large amount of IL-5 in response to antigen presentation [47] (Fig. 2G). The number of ST2⁺CD4⁺ T cells well correlated with the eosinophil numbers in this setting, suggesting that the magnitude of type 2 inflammation is influenced by both the pollen shell and the extract (Fig. 2H). Accordingly, the IL-5 production of extract-stimulated draining lymph node cells showed a similar pattern to the eosinophil numbers (Fig. 2I and J). These results demonstrate that the pollen shells and soluble factors play non-redundant roles in inducing Th2 type allergic inflammation.

In order to test the pollen shell-dependency in the elicitation phase, we further performed the conventional allergic conjunctivitis model, which is induced by systemic sensitization followed by topical challenges. Interestingly, the pollen shells were also required for the elicitation phase of the conventional allergic conjunctivitis (Supplementary Figure E3A–D). In addition, the eosinophil number showed a strong correlation with the number of CD4⁺ST2⁺ T cells (Supplementary Figure E3E), suggesting that the similar mechanisms are operating in both models.

3.3. ST2 is required for full expression of the pollen suspension-induced allergic conjunctivitis

Epithelial cells are known to release pro-allergic cytokines such as IL-33, IL-25, and TSLP upon detection of mucosal damage or the invasion by pathogens [25,26]. In addition, RW extract has been reported to induce IL-33 expression in epithelial cells through toll-like receptor 4 (TLR4) stimulation [48]. Since IL-33 is known to induce Th2 cells and enhance production of IL-5 from ST2⁺CD4⁺ T cells in the lung [49,50], we investigated the possible roles of the IL-33/ST2 pathway in the development of conjunctivitis. The total IL-33 tissue content was not significantly altered by the induction of conjunctivitis (Supplementary Figure E4A). However, the nuclear IL-33 intensity was decreased, suggesting that the IL-33 is released from the nuclear storage (Supplementary Figure E4B, C). To directly examine the role of IL-33/ST2 pathway, we generated ST2 deficient mice by genome editing (Supplementary Figure E5) and applied RW pollen suspension to them. Deficiency of ST2 ameliorated the acute clinical signs as well as eosinophil accumulation in the conjunctiva (Fig. 3A–E). When lymph node cells were stimulated with the RW extract, the production of IL-5 was significantly decreased in ST2 knockout mice (Fig. 3D). These results indicate that the IL-33/ST2 pathway enhances acquired immune responses during the development of allergic conjunctivitis.

3.4. Depletion of CD4⁺ T cells abrogates the development of eosinophilic conjunctivitis

Next, we set out to explore the cellular requirement for the conjunctival allergic inflammation. The cells that express ST2 on their surface in the conjunctival tissue include mast cells, ILC2s, and a subpopulation of CD4⁺ T cells (Supplementary Fig. E5A and B). To test the

involvement of CD4⁺ T cells and/or ILC2s, we injected anti-CD4 depleting antibody or anti-Thy1.2 antibody [51] to the conjunctiva one day before starting RW pollen exposure (Fig. 4A). After two weeks of induction, the acute clinical signs were suppressed in both the groups (Fig. 4B). Moreover, a single injection abrogated eosinophil accumulation completely (Fig. 4C–F). Although the final numbers of the lymph node cells were not decreased (Fig. 4G), their recall response was reduced (Fig. 4H), suggesting that the Th2 type inflammation against RW is specifically suppressed in these mice. These results indicate that the acquired immunity involving CD4⁺ T cells is operative in the development of allergic conjunctivitis.

3.5. Particulate pollutant promotes RW extract-induced allergic conjunctivitis

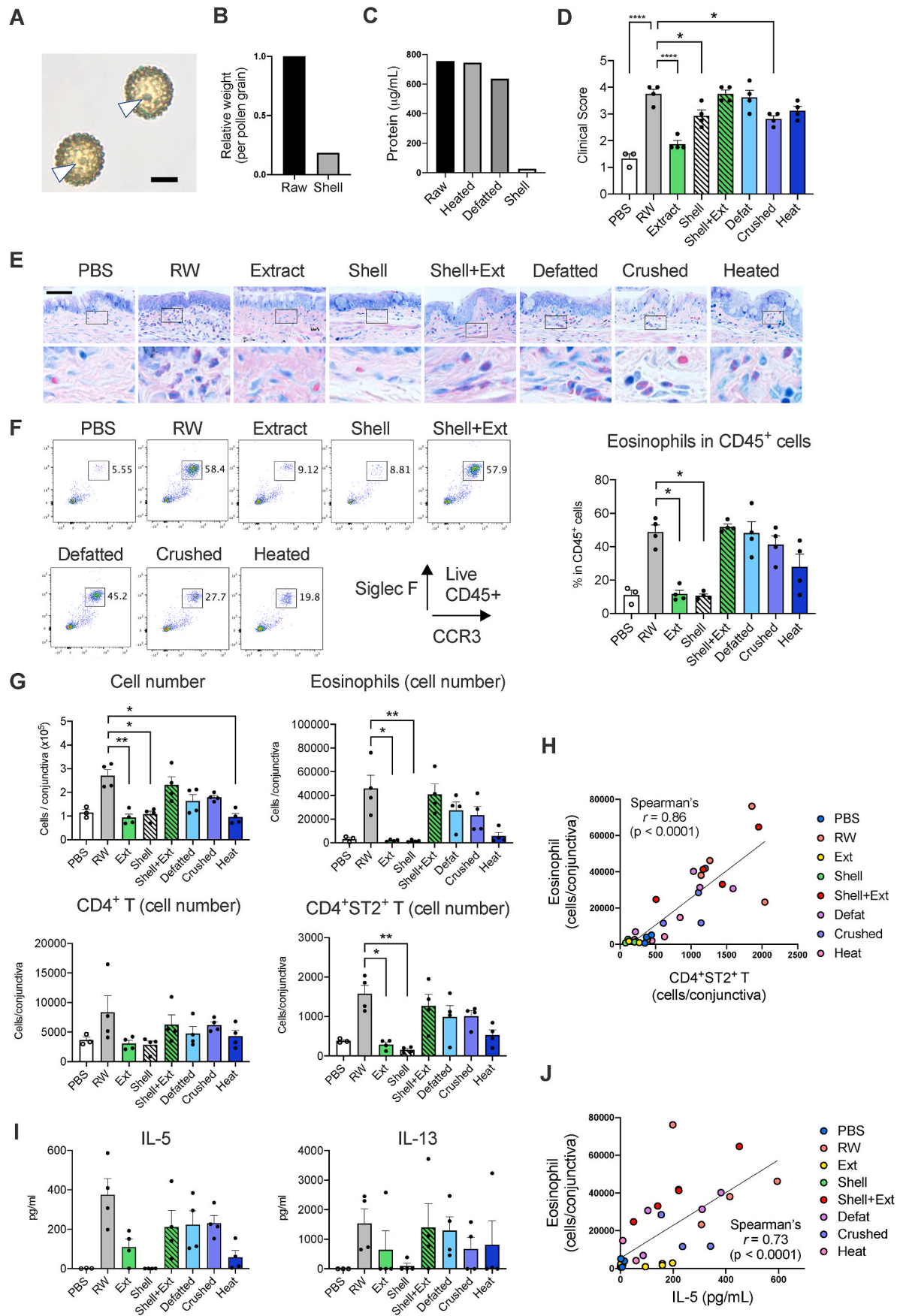
Particulate pollutants such as Asian dust and the PM from the diesel exhaust are implicated in the exacerbation of allergic rhinoconjunctivitis. We hypothesized that the particulate pollutants might mimic the effect of pollen shells during allergen exposure to the conjunctiva. To test this possibility, we mixed RW extract with Asian dust or PM and applied it to the mouse conjunctiva (Fig. 5A). Acute clinical signs were more severe when the extract was mixed with particulate pollutants (Fig. 5B). However, the signs were observed with the particulate pollutants alone as well (Fig. 5B), as reported in the case of PM2.5 [19,23] in mice. Eosinophil infiltration was significantly increased when the pollen extract was mixed with Asian dust and tended to increase when mixed with the PM (Fig. 5C–F). Although Asian dust or PM might cause injury to the epithelial layers, increase of the neutrophil infiltration was not observed (Fig. 5D–F), indicating that the caused injury was minor if any. RW extract-stimulated lymph node cells produced Th2 type cytokines, which correlated with eosinophil accumulation (Fig. 5G and H). Collectively, these results suggest that the particulate air pollutants act like adjuvants and promote sensitization via the conjunctival mucosa and development of allergic conjunctivitis.

3.6. Pollen shell induces IL-33 release from conjunctival epithelial cells

Finally, to explore the possible role of the pollen shells in the induction of the allergic conjunctivitis, we examined IL-33 release in the conjunctiva. In the steady state, IL-33 was detected in the nuclei of the epithelial cells and some stromal cells (Fig. 6A, B, and F). Upon RW pollen administration, IL-33 was released from epithelial cells in 1 h and began to be restored in 2 h (Fig. 6B–E). However, the release was not observed in stromal cells (Fig. 6B). Most importantly, only the preparations containing shells, but not extract alone, were able to induce significant IL-33 release from the epithelial cells (Fig. 6E and F), suggesting a possible role for pollen shells in driving the IL-33-ST2 axis in the development of allergic conjunctivitis.

4. Discussion

Previous studies on pollen-induced allergic conjunctivitis have utilized sensitized models to investigate the mechanisms underlying conjunctivitis development in the elicitation phase. In the present study, we asked whether conjunctival exposure to particulate allergens induces sensitization and develops local ocular allergic diseases. Repeated topical application of RW pollen suspension to the conjunctiva induced acute clinical signs as well as histological changes, including eosinophil infiltration and a decrease in goblet cell density. Depletion of CD4⁺ T cells in conjunctiva abrogated eosinophilic inflammation, demonstrating the essential role of acquired immunity. Mucosal sensitization required both pollen shells and soluble factors to recruit ST2⁺CD4⁺ T cells and eosinophils to the conjunctiva. In fact, genetic deletion of the IL-33 receptor ST2 reduced the magnitude of the conjunctivitis, as well as sensitization. Furthermore, the mixture of RW extract and particulate air pollutant induced similar eosinophilic conjunctivitis. Finally, pollen



(caption on next page)

Fig. 2. Pollen shells and soluble factors cooperate to induce allergic conjunctivitis (A) Microscopic observation of the pollen shells. Arrowheads indicate holes made during shell preparation. Bar, 10 μ m. (B) Relative weight of the shell compared to raw whole pollen grain. (C) Protein concentrations of 2 mg/mL pollen suspension and the same grain concentrations of heated (72 °C for 10 min), defatted (acetone reflux overnight), and shell particles. (D) Clinical score. * $p < 0.05$, **** $p < 0.0001$ by one-way ANOVA with Holm-Sidak's multiple comparisons. (E) Giemsa staining of the conjunctiva. Bar, 50 μ m. (F) Conjunctival eosinophil populations among CD45⁺ cells. (G) Cell numbers of indicated populations in the conjunctiva. (F, G) * $p < 0.05$, ** $p < 0.01$ by Kruskal-Wallis test with Dunn's multiple comparison. (H) Correlation of CD4⁺ST2⁺ T cell and eosinophil numbers in the conjunctiva. (I) Cytokine production from the RW extract-stimulated draining lymph node cells. (J) Correlation of the IL-5 production from lymph node cells and eosinophil numbers in the conjunctiva. Data represents mean $\pm$ S.E.M.

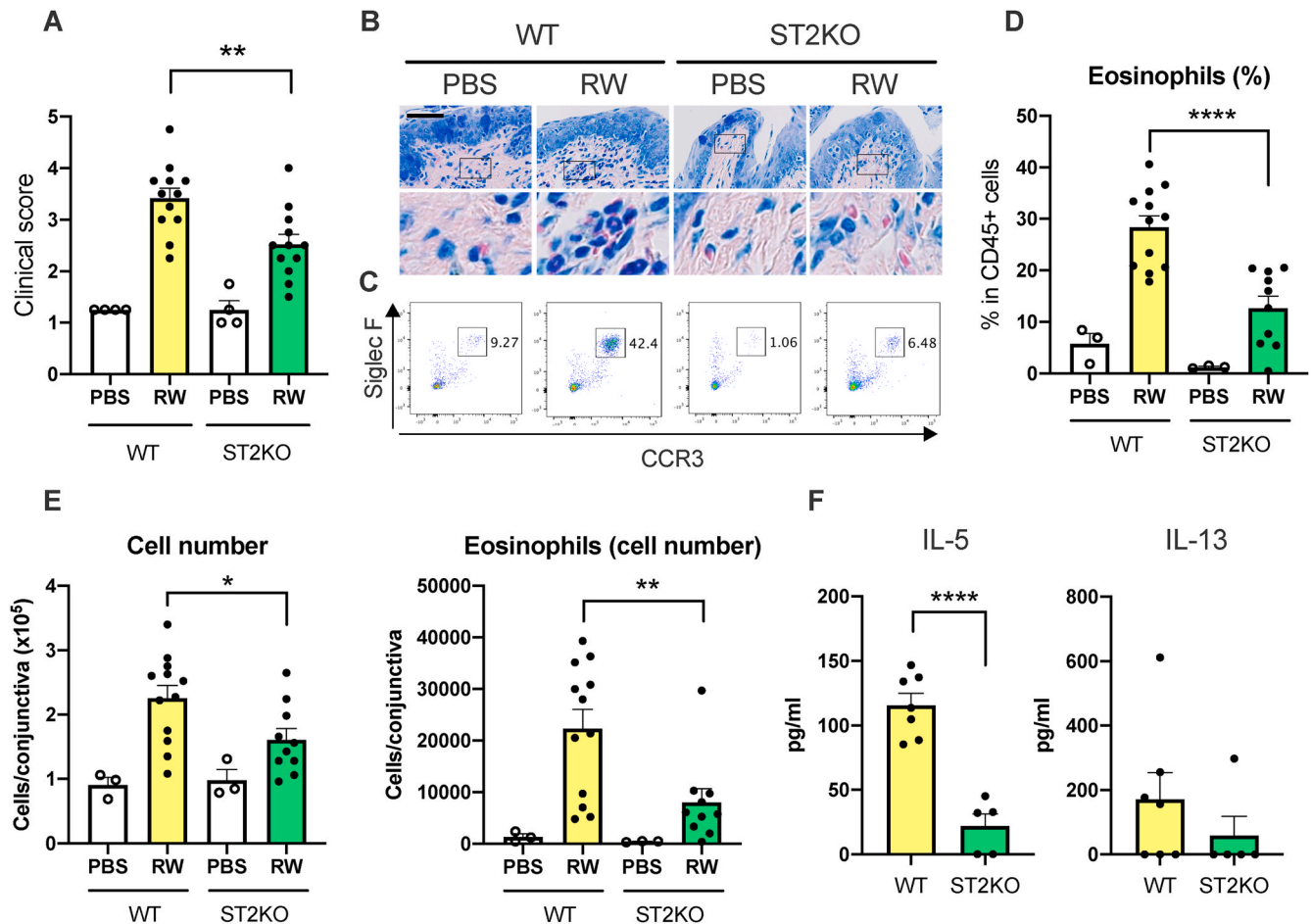


Fig. 3. The IL-33/ST2 pathway contributes to the mucosal sensitization and eosinophil accumulation (A) Clinical scores. (B) Giemsa staining of the conjunctiva. Bar, 50 μ m. (C, D) Eosinophil populations among CD45⁺ cells in the conjunctiva. (E) Whole and eosinophil cell numbers were enumerated. (F) Cytokine production of the RW extract-stimulated draining lymph node cells. Data represents mean $\pm$ S.E.M. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ by Student's t-test with Welch's correction.

shells stimulated conjunctival epithelial cells to release IL-33 in vivo. These results suggest that the particulate property shared by some airborne allergens cooperates with soluble factors to induce mucosal sensitization leading to allergic conjunctivitis.

The immunomodulatory effect of antigen exposure to the conjunctival mucosal surface has been investigated in the context of tolerogenic immunoregulation [52–56]. In these studies, the application of pure protein antigen ovalbumin (OVA) to the healthy conjunctiva induced anergic T cells or regulatory T cells and suppressed delayed-type hypersensitivity induced at the ear pinna [53,54]. This tolerogenic response was mediated by the goblet cells, and retinoic acid was found to be a factor suppressing Th1 responses [55,56]. However, benzalkonium chloride (BAK)-induced epithelial damage abolished the tolerogenic activity [53]. Tolerance was restored when an NF- κ B inhibitor was applied together [52]. These observations clearly demonstrate that the inert pure proteins such as OVA or self-antigens are recognized as safe substances by the immune system. However, natural antigens such as pollens and house dust mite allergens have a wide range of protease

activities [57], in addition to the innate immune receptor agonists [48]. Furthermore, pollens have been shown to possess reactive oxygen species (ROS)-generating activity, which exacerbates the sensitized model of allergic conjunctivitis [58]. Our result that the heated pollens have diminished activity imply that such ROS-generating activity, LPS-like activity that is potentially labile to heat [59], and/or the protease activity are the important elements on the soluble factor side. However, the pollen shells were required to induce IL-33 release from epithelial cells and to cause eosinophilic inflammation. One possible mechanism is that the shell wall component that is durable to the heat, acid, and base treatment can be sensed by the yet unknown receptor expressed on the epithelial cells. In fact, it has been reported that the conjunctival cells in VKC patients overexpress multiple pattern recognition receptors [60]. In line with this, the crushed pollens retained some activity to induce eosinophilic conjunctivitis. Another possibility is that the mechanical sensing by either the epithelial cells or the peripheral nerves induces the IL-33 release. However, the exact mechanism remains to be explored.

In our mucosal sensitization model, RW-specific IgE production was

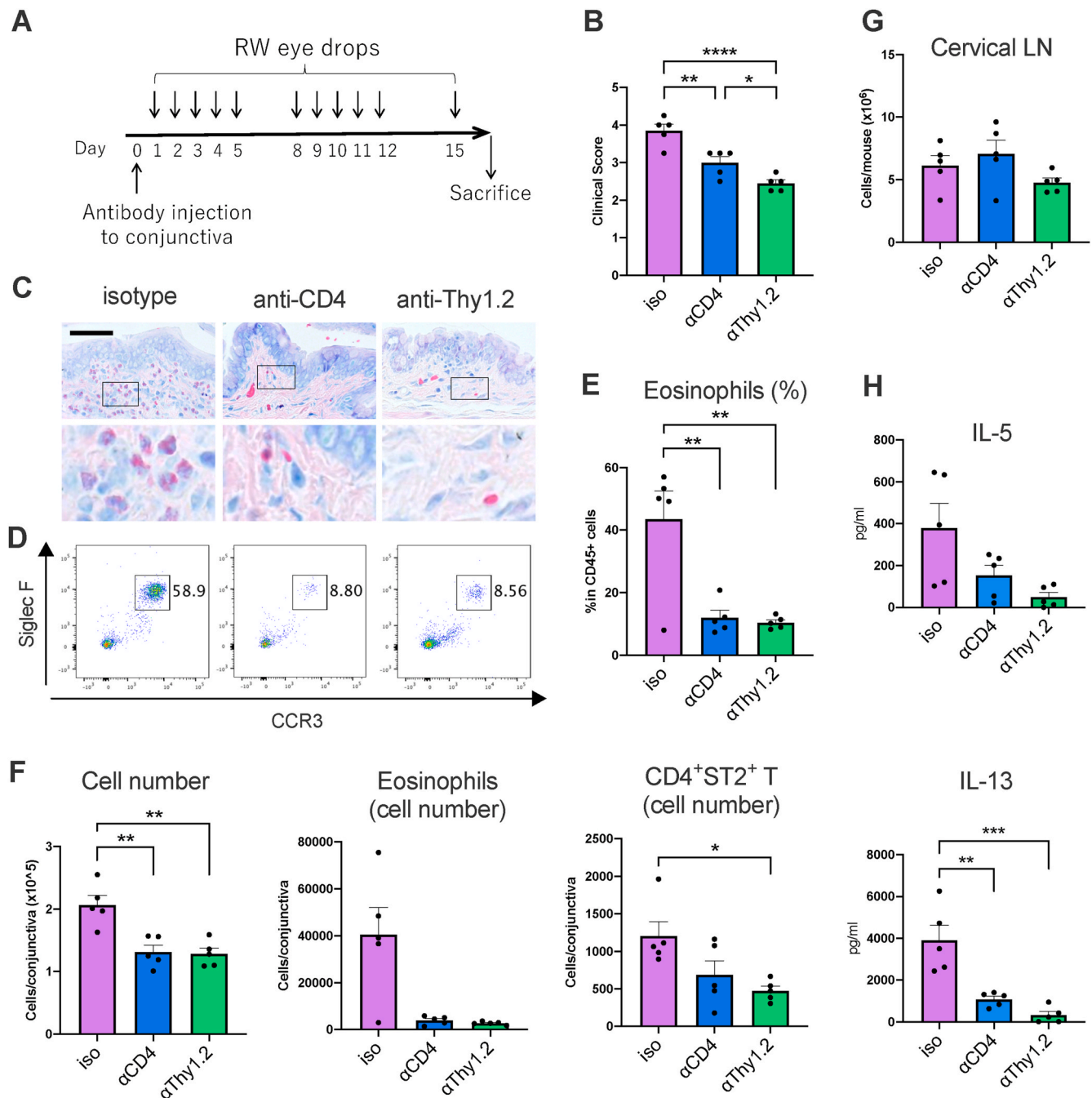


Fig. 4. Topical T cell depletion abolishes eosinophil accumulation and mitigates clinical signs (A) Diagram for antibody and RW pollen administration. (B) Clinical scores. (C) Giemsa staining of the conjunctiva. Bar, 50 μm. (D, E) Eosinophil populations among CD45⁺ cells in the conjunctiva. (F) Absolute numbers of indicated cell populations. (G) Absolute numbers of the draining lymph node (LN) cells. (H) Cytokines produced by RW extract-stimulated draining lymph node cells. Data represents mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by ANOVA with Tukey's multiple comparison.

somewhat delayed compared to the eosinophil infiltration. In light of the report that the T cell response recruits eosinophils more efficiently than IgE-mediated mast cell activation does [61], Th2 cell-dependent responses may be predominant in our model. As such, infiltrating ST2⁺CD4⁺ T cell numbers significantly correlated with the eosinophil numbers. Moreover, depletion of CD4⁺ T cells completely abrogated the eosinophil infiltration. Although the antigens with protease activities were used in this study, this aspect is in sharp contrast with the papain-soaked contact lens model of eosinophilic ocular inflammation, in which ILC2 plays a role, but T cells are dispensable [39]. However, this does not exclude the contributions of ILC2 and mast cells in our model.

The concept of “local ocular allergy” and the local sensitization have been proposed for VKC and allergic conjunctivitis patients for more than three decades [41–43,62]. In those cases, the suspected allergen-specific IgE could not be detected in the sera, but the tear IgE or the conjunctival provocation test was found to be positive. In addition, the patients with no detectable tear IgE or serum allergen-specific IgE have been described [41,43]. Although we have not measured IgEs locally produced in tears, we found elevated levels of IgE in the conjunctival tissue before the serum IgE levels increase. The model presented here may serve as a useful tool to further investigate the mechanisms underlying the development of local ocular allergy.

We observed thickening of squamous cell layers and a decrease in

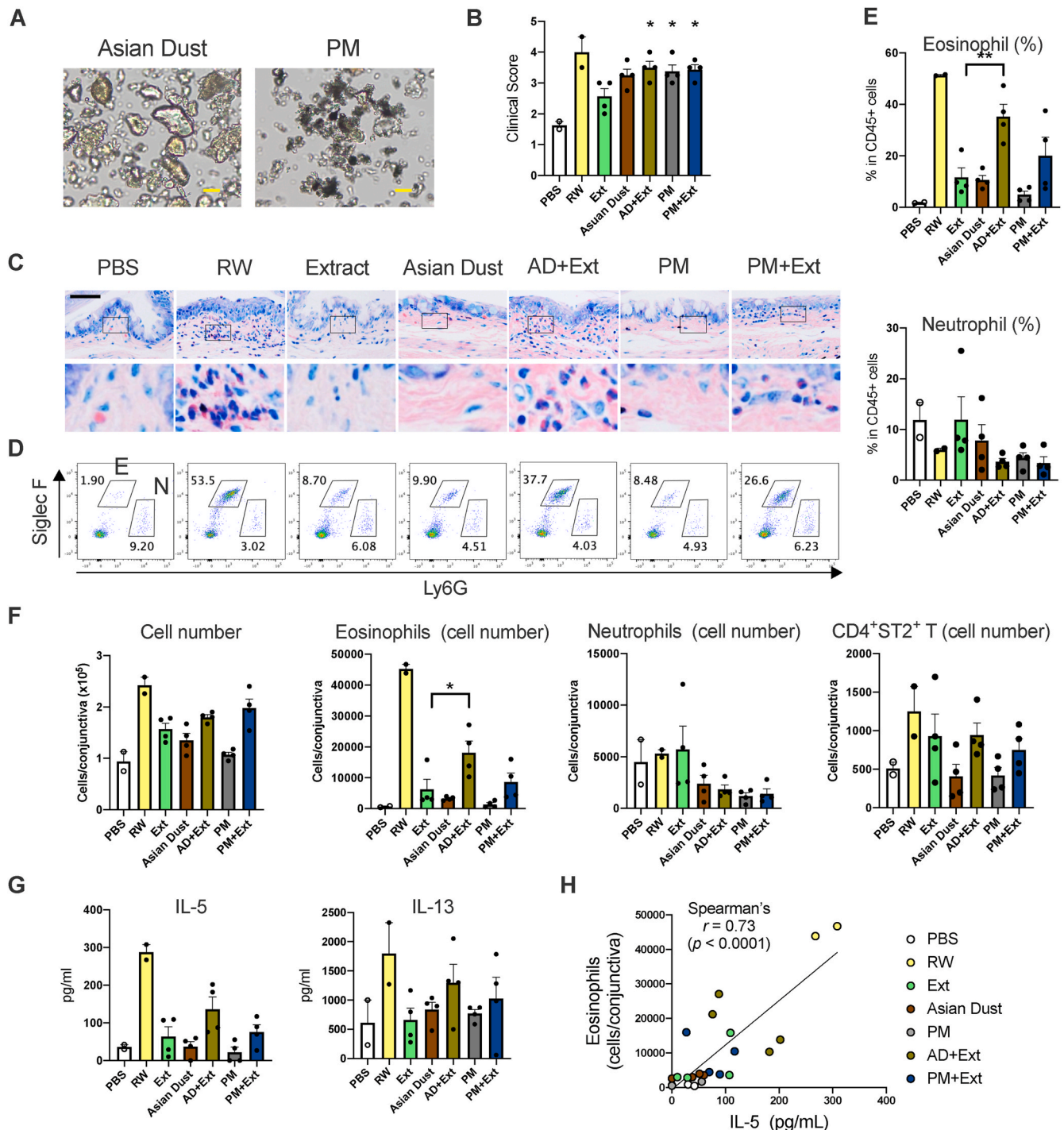


Fig. 5. Air pollutant particles enhances mucosal sensitization and allergic conjunctivitis. (A) Microscopic observation of Asian dust and particulate matter (PM) used in the experiment. Bar, 10 μ m. (B) Clinical scores. (C) Giemsa staining of the conjunctiva. Bar, 50 μ m. (D, E) Eosinophil and neutrophil population in CD45⁺ cells. Representative gating (D) and statistics (E) are shown. E, eosinophil; N, neutrophil. (F) Absolute cell numbers of the indicated populations. (G) Cytokine production from extract-stimulated draining lymph node cells. (H) Correlation between IL-5 production from extract-stimulated lymph node cells and eosinophil numbers. Data represents mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by ANOVA with Dunnett's multiple comparisons against extract group. PBS and RW groups were excluded from the ANOVA analysis.

filled goblet cells, irrespective of the kind of particulate components. Apparently, the proliferation of epithelial layers seems to be correlated with the extent of eosinophil infiltration. The proliferative response of epithelial layer has been documented in VKC papillae [63]. On the contrary, there was a decrease in Muc5ac expression in our model. However, the decrease of filled goblet cells after allergen challenges have been documented in the conventional systemically sensitized

models of allergic conjunctivitis [64]. In addition, both the increase and decrease of Muc5ac levels have been reported depending on the time points after the allergen challenge [64]. Therefore, it requires careful investigation to understand the pathological significance of filled goblet cell number decrease observed in our model.

The single ocular symptoms found in 6% of the general population [3] support the notion of local ocular sensitization. At the clinics of

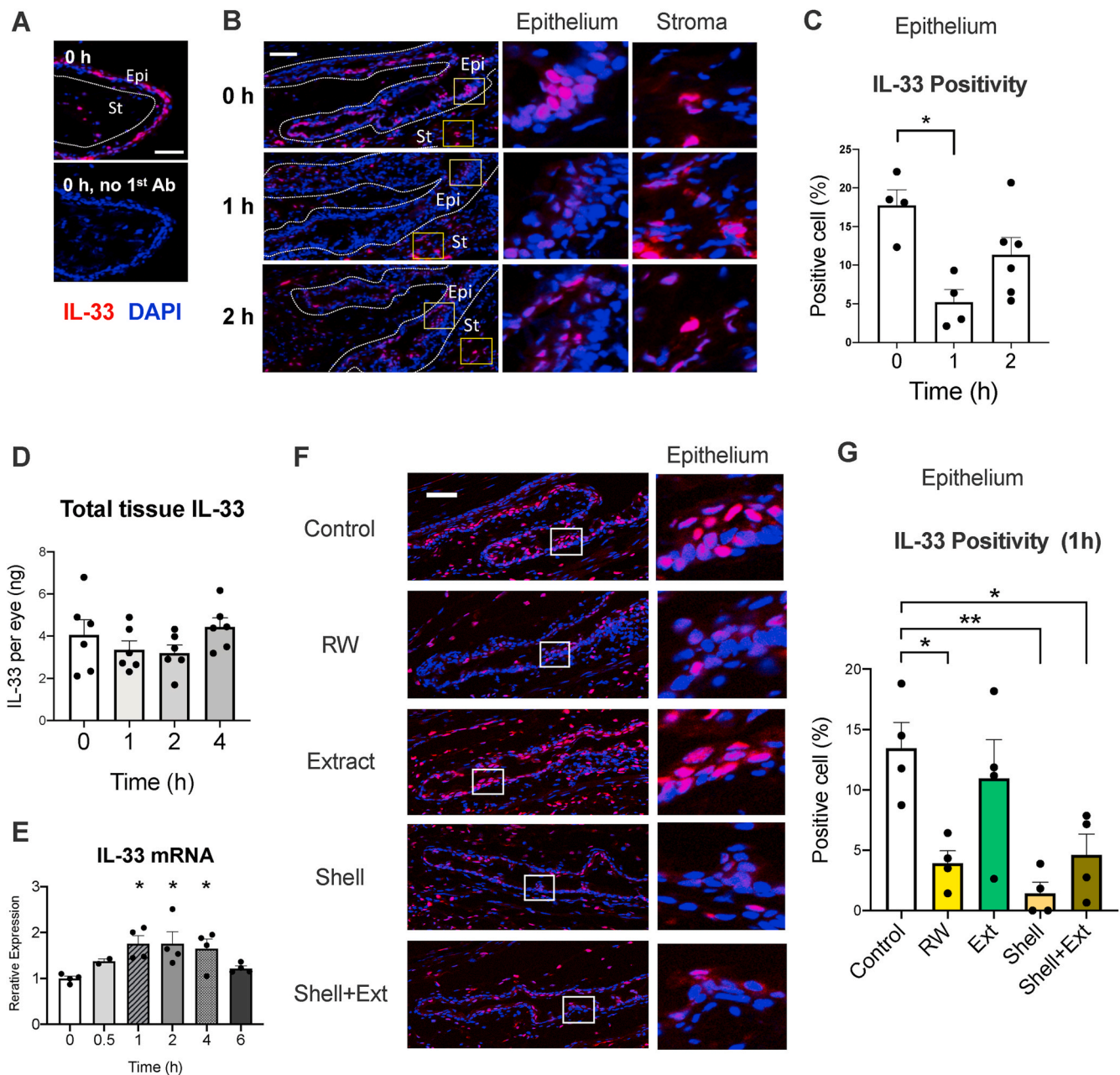


Fig. 6. RW pollen shell is required for the IL-33 release from conjunctival epithelial cells. (A, B, F) Localization of IL-33 by immunofluorescence after RW pollen administration. (A) Staining specificity, (B) time course, and (F) the effect of indicated preparations after 1 h are shown. Ab, antibody; Epi, epithelium; St, stroma of conjunctiva. (C, G) Enumeration of IL-33 positivity in the epithelium is shown. (D) Total IL-33 content determined by ELISA for tissue homogenate. (E) mRNA expression of IL-33 determined by qPCR. Data represents mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by ANOVA with Dunnett's multiple comparisons against 0 h or control group.

pediatricians, the comorbidity rate of allergic conjunctivitis and rhinitis has been estimated to be 92–97% [12]. On the other hand, 60% of ocular allergy patients visiting ophthalmologists have been reported to display solely ocular symptoms [40]. This discrepancy may not necessarily be surprising. However, considering the fact that ocular allergy is often underdiagnosed and untreated [2,65], more attention should be paid to the local ocular sensitization to improve the daily life of the patients [66].

In conclusion, we demonstrated that the conjunctival mucosa functions as a stage of sensitization, where the allergens' particulate properties and soluble factors play non-redundant roles. Further investigation is required to elucidate their specific roles in the development of local allergic conjunctivitis.

Author contributions

T.A. and J.K. conceived the project; S.F. and T.A. designed the study; S.F., T.A., M.M., M.K., K.I., A.Kaitani, A.Kamei, M.K. performed experiments; Y.S. and M.N. contributed to the preparation of samples; S.F. and T.A. analyzed and interpreted the results, and prepared figures; N. N., K.M., T.S., H.O., K.O., M.N., A.M., and N.E. contributed to the interpretation of data; T.A. and S.F. wrote the manuscript; J.K. and K.O. supervised the entire project; All authors provided critical feedback and helped shape the research, analysis and manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtos.2021.08.009>.

References

- Miyazaki D, Fukagawa K, Fukushima A, Fujishima H, Uchio E, Ebihara N, et al. Air pollution significantly associated with severe ocular allergic inflammatory diseases. *Sci Rep* 2019;9:18205.
- Dupuis P, Prokopic CL, Hynes A, Kim H. A contemporary look at allergic conjunctivitis. *Allergy Asthma Clin Immunol* 2020;16:5.
- Singh K, Axelrod S, Bielory L. The epidemiology of ocular and nasal allergy in the United States, 1988–1994. *J Allergy Clin Immunol* 2010;126:778–783 e6.
- Rodrigues J, Kuruvilla ME, Vanijchareonkarn K, Patel N, Hom MM, Wallace DV. The spectrum of allergic ocular diseases. *Ann Allergy Asthma Immunol* 2021;126:240–54.
- Brozek G, Lawson J, Szumilas D, Zejda J. Increasing prevalence of asthma, respiratory symptoms, and allergic diseases: four repeated surveys from 1993–2014. *Respir Med* 2015;109:982–90.
- Rosario N, Bielory L. Epidemiology of allergic conjunctivitis. *Curr Opin Allergy Clin Immunol* 2011;11:471–6.
- Miyazaki D, Takamura E, Uchio E, Ebihara N, Ohno S, Ohashi Y, et al. Japanese guidelines for allergic conjunctival diseases 2020. *Allergol Int* 2020;69:346–55.
- Chen JJ, Applebaum DS, Sun GS, Pflugfelder SC. Atopic keratoconjunctivitis: a review. *J Am Acad Dermatol* 2014;70:569–75.
- Jabbardari S, Starnes TW, Kurji KH, Eslani M, Cortina MS, Holland EJ, et al. Management of advanced ocular surface disease in patients with severe atopic keratoconjunctivitis. *Ocul Surf* 2019;17:303–9.
- Feizi S, Javadi MA, Alemzadeh-Ansari M, Arabi A, Shahraki T, Khairkhan A. Management of corneal complications in vernal keratoconjunctivitis: a review. *Ocul Surf* 2021;19:282–9.
- Kenny SE, Tye CB, Johnson DA, Khairkhan A. Giant papillary conjunctivitis: a review. *Ocul Surf* 2020;18:396–402.
- Gradman J, Wolthers OD. Allergic conjunctivitis in children with asthma, rhinitis and eczema in a secondary outpatient clinic. *Pediatr Allergy Immunol* 2006;17:524–6.
- Neto HJ, Rosario NA, Westphal GL, Riedi CA, Santos HL. Allergic conjunctivitis in asthmatic children: as common as underreported. *Ann Allergy Asthma Immunol* 2010;105:399–400.
- Brown-Whitehorn TF, Spergel JM. The link between allergies and eosinophilic esophagitis: implications for management strategies. *Expet Rev Clin Immunol* 2010;6:101–9.
- Chen R, Yang J, Zhang C, Li B, Bergmann S, Zeng F, et al. Global associations of air pollution and conjunctivitis diseases: a systematic review and meta-analysis. *Int J Environ Res Publ Health* 2019;16.
- Ko R, Hayashi M, Hayashi H, Hayashi K, Kato H, Kurata Y, et al. Correlation between acute conjunctivitis and Asian dust on ocular surfaces. *J Toxicol Environ Health* 2016;79:367–75.
- Mimura T, Ichinose T, Yamagami S, Fujishima H, Kamei Y, Goto M, et al. Airborne particulate matter (PM_{2.5}) and the prevalence of allergic conjunctivitis in Japan. *Sci Total Environ* 2014;487:493–9.
- Mimura T, Yamagami S, Fujishima H, Noma H, Kamei Y, Goto M, et al. Sensitization to Asian dust and allergic rhinoconjunctivitis. *Environ Res* 2014;132:220–5.
- Tang YJ, Chang HH, Chiang CY, Lai CY, Hsu MY, Wang KR, et al. A murine model of acute allergic conjunctivitis induced by continuous exposure to particulate matter 2.5. *Invest Ophthalmol Vis Sci* 2019;60:2118–26.
- Fu Q, Lyu D, Zhang L, Qin Z, Tang Q, Yin H, et al. Airborne particulate matter (PM_{2.5}) triggers autophagy in human corneal epithelial cell line. *Environ Pollut* 2017;227:314–22.
- Li J, Tan G, Ding X, Wang Y, Wu A, Yang Q, et al. A mouse dry eye model induced by topical administration of the air pollutant particulate matter 10. *Biomed Pharmacother* 2017;96:524–34.
- Lyu D, Chen Z, Almansoor S, Chen H, Ye Y, Song F, et al. Transcriptomic profiling of human corneal epithelial cells exposed to airborne fine particulate matter (PM_{2.5}). *Ocul Surf* 2020;18:554–64.
- Lee HS, Han S, Seo JW, Jeon KJ. Exposure to traffic-related particulate matter 2.5 triggers Th2-dominant ocular immune response in a murine model. *Int J Environ Res Publ Health* 2020;17.
- Wei T, Tang M. Biological effects of airborne fine particulate matter (PM_{2.5}) exposure on pulmonary immune system. *Environ Toxicol Pharmacol* 2018;60:195–201.
- Liew FY, Girard JP, Turnquist HR. Interleukin-33 in health and disease. *Nat Rev Immunol* 2016;16:676–89.
- Roan F, Obata-Ninomiya K, Ziegler SF. Epithelial cell-derived cytokines: more than just signaling the alarm. *J Clin Invest* 2019;129:1441–51.
- Cho W, Mittal SK, Elbasiony E, Chauhan SK. Activation of ocular surface mast cells promotes corneal neovascularization. *Ocul Surf* 2020;18:857–64.
- Elbasiony E, Mittal SK, Foulsham W, Cho W, Chauhan SK. Epithelium-derived IL-33 activates mast cells to initiate neutrophil recruitment following corneal injury. *Ocul Surf* 2020;18:633–40.
- Zhang M, Liu L, Lin X, Wang Y, Li Y, Guo Q, et al. A translocation pathway for vesicle-mediated unconventional protein secretion. *Cell* 2020;181:637–652 e15.
- Travers J, Rochman M, Miracle CE, Habel JE, Brusilovsky M, Caldwell JM, et al. Chromatin regulates IL-33 release and extracellular cytokine activity. *Nat Commun* 2018;9:3244.
- Cayrol C, Duval A, Schmitt P, Roga S, Camus M, Stella A, et al. Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nat Immunol* 2018;19:375–85.
- Hiraishi Y, Yamaguchi S, Yoshizaki T, Nambu A, Shimura E, Takamori A, et al. IL-33, IL-25 and TSLP contribute to development of fungal-associated protease-induced innate-type airway inflammation. *Sci Rep* 2018;8:18052.
- Qian Y, Zhang M. The functional roles of IL-33/ST2 Axis in ocular diseases. *Mediat Inflamm* 2020;2020:5230716.
- Sakashita M, Yoshimoto T, Hirota T, Harada M, Okubo K, Osawa Y, et al. Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin Exp Allergy* 2008;38:1875–81.
- Matsuda A, Okayama Y, Terai N, Yokoi N, Ebihara N, Tanioka H, et al. The role of interleukin-33 in chronic allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2009;50:4646–52.
- Matsuba-Kitamura S, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Taki Y, Muto T, et al. Contribution of IL-33 to induction and augmentation of experimental allergic conjunctivitis. *Int Immunol* 2010;22:479–89.
- Asada Y, Nakae S, Ishida W, Hori K, Sugita J, Sudo K, et al. Roles of epithelial cell-derived type 2-initiating cytokines in experimental allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2015;56:5194–202.
- Imai Y, Hosotani Y, Ishikawa H, Yasuda K, Nagai M, Jitsukawa O, et al. Expression of IL-33 in ocular surface epithelium induces atopic keratoconjunctivitis with activation of group 2 innate lymphoid cells in mice. *Sci Rep* 2017;7:10053.
- Sugita J, Asada Y, Ishida W, Iwamoto S, Sudo K, Suto H, et al. Contributions of Interleukin-33 and TSLP in a papain-soaked contact lens-induced mouse conjunctival inflammation model. *Immun Inflamm Dis* 2017;5:515–25.
- Leonardi A, Pillego F, Castegnaro A, Lazzarini D, La Gloria Valerio A, Mattana P, et al. Allergic conjunctivitis: a cross-sectional study. *Clin Exp Allergy* 2015;45:1118–25.
- Leonardi A, Battista MC, Gismondi M, Fregona IA, Secchi AG. Antigen sensitivity evaluated by tear-specific and serum-specific IgE, skin tests, and conjunctival and nasal provocation tests in patients with ocular allergic disease. *Eye* 1993;7(Pt 3):461–4.
- Leonardi A, Borghesan F, Faggian D, Plebani M. Microarray-based IgE detection in tears of patients with vernal keratoconjunctivitis. *Pediatr Allergy Immunol* 2015;26:641–5.
- Yamana Y, Fukuda K, Ko R, Uchio E. Local allergic conjunctivitis: a phenotype of allergic conjunctivitis. *Int Ophthalmol* 2019;39:2539–44.
- Magone MT, Chan CC, Rizzo LV, Kozhich AT, Whitcup SM. A novel murine model of allergic conjunctivitis. *Clin Immunol Immunopathol* 1998;87:75–84.
- Gonzalez-Cruz P, Uddin MJ, Atwe SU, Abidi N, Gill HS. A chemical treatment method for obtaining clean and intact pollen shells of different species. *ACS Biomater Sci Eng* 2018;4:2319–29.
- Stern ME, Siemasko K, Gao J, Duong A, Beauregard C, Calder V, et al. Role of interferon-gamma in a mouse model of allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2005;46:3239–46.
- Yamamoto T, Endo Y, Onodera A, Hirahara K, Asou HK, Nakajima T, et al. DUSP10 constrains innate IL-33-mediated cytokine production in ST2(hi) memory-type pathogenic Th2 cells. *Nat Commun* 2018;9:4231.
- Li J, Zhang L, Chen X, Chen D, Hua X, Bian F, et al. Pollen/TLR4 innate immunity signaling initiates IL-33/ST2/Th2 pathways in allergic inflammation. *Sci Rep* 2016;6:36150.
- Nakayama T, Hirahara K, Onodera A, Endo Y, Hosokawa H, Shinoda K, et al. Th2 cells in health and disease. *Annu Rev Immunol* 2017;35:53–84.
- Endo Y, Hirahara K, Iinuma T, Shinoda K, Tumes DJ, Asou HK, et al. The interleukin-33-p38 kinase axis confers memory T helper 2 cell pathogenicity in the airway. *Immunity* 2015;42:294–308.
- Finkin S, Yuan D, Stein I, Taniguchi K, Weber A, Unger K, et al. Ectopic lymphoid structures function as microniches for tumor progenitor cells in hepatocellular carcinoma. *Nat Immunol* 2015;16:1235–44.

- [52] Guzman M, Sabbione F, Gabelloni ML, Vanzulli S, Trevani AS, Giordano MN, et al. Restoring conjunctival tolerance by topical nuclear factor-kappaB inhibitors reduces preservative-facilitated allergic conjunctivitis in mice. *Invest Ophthalmol Vis Sci* 2014;55:6116–26.
- [53] Galletti JG, Gabelloni ML, Morande PE, Sabbione F, Vermeulen ME, Trevani AS, et al. Benzalkonium chloride breaks down conjunctival immunological tolerance in a murine model. *Mucosal Immunol* 2013;6:24–34.
- [54] Egan RM, Yorkey C, Black R, Loh WK, Stevens JL, Storozynsky E, et al. In vivo behavior of peptide-specific T cells during mucosal tolerance induction: antigen introduced through the mucosa of the conjunctiva elicits prolonged antigen-specific T cell priming followed by anergy. *J Immunol* 2000;164:4543–50.
- [55] Barbosa FL, Xiao Y, Bian F, Coursey TG, Ko BY, Clevers H, et al. Goblet cells contribute to ocular surface immune tolerance-implications for dry eye disease. *Int J Mol Sci* 2017;18.
- [56] Ko BY, Xiao Y, Barbosa FL, de Paiva CS, Pflugfelder SC. Goblet cell loss abrogates ocular surface immune tolerance. *JCI Insight* 2018;3.
- [57] Gunawan H, Takai T, Ikeda S, Okumura K, Ogawa H. Protease activity of allergenic pollen of cedar, cypress, juniper, birch and ragweed. *Allergol Int* 2008;57:83–91.
- [58] Bacsí A, Dharajiyi N, Choudhury BK, Sur S, Boldogh I. Effect of pollen-mediated oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in allergic conjunctivitis. *J Allergy Clin Immunol* 2005;116:836–43.
- [59] Gao B, Wang Y, Tsan MF. The heat sensitivity of cytokine-inducing effect of lipopolysaccharide. *J Leukoc Biol* 2006;80:359–66.
- [60] Leonardi A, Daull P, Garrigue JS, Cavarzeran F, Docquier M, Di Stefano A, et al. Conjunctival transcriptome analysis reveals the overexpression of multiple pattern recognition receptors in vernal keratoconjunctivitis. *Ocul Surf* 2021;19:241–8.
- [61] Fukushima A, Ozaki A, Jian Z, Ishida W, Fukata K, Ueno H, et al. Dissection of antigen-specific humoral and cellular immune responses for the development of experimental immune-mediated blepharoconjunctivitis in C57BL/6 mice. *Curr Eye Res* 2005;30:241–8.
- [62] Leonardi A, Fregona IA, Gismondi M, Daniotti E, Carniel G, Secchi AG. Correlation between conjunctival provocation test (CPT) and systemic allergometric tests in allergic conjunctivitis. *Eye* 1990;4(Pt 5):760–4.
- [63] Abu El-Asrar AM, Al-Mansouri S, Tabbara KF, Missotten L, Geboes K. Immunopathogenesis of conjunctival remodelling in vernal keratoconjunctivitis. *Eye* 2006;20:71–9.
- [64] Kunert KS, Keane-Myers AM, Spurr-Michaud S, Tisdale AS, Gipson IK. Alteration in goblet cell numbers and mucin gene expression in a mouse model of allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2001;42:2483–9.
- [65] Bielory L, Meltzer EO, Nichols KK, Melton R, Thomas RK, Bartlett JD. An algorithm for the management of allergic conjunctivitis. *Allergy Asthma Proc* 2013;34:408–20.
- [66] Pitt AD, Smith AF, Lindsell L, Voon LW, Rose PW, Bron AJ. Economic and quality-of-life impact of seasonal allergic conjunctivitis in Oxfordshire. *Ophthalmic Epidemiol* 2004;11:17–33.

Online Supporting Information

Pollen shells and soluble factors play non-redundant roles in the development of allergic conjunctivitis in mice

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Supplementary Figure E1-E5

Supplementary Methods

Preparation of ragweed pollen extract and shells

To prepare RW pollen extract, pollen grains were suspended in deionized water and homogenized using a bead crusher TissueLyser II (Qiagen, Germany). Ten times concentrated PBS was added to adjust the final concentration to 200 mg/mL in PBS. Soluble factors were extracted by rotating at 4°C overnight, and insoluble debris was removed by repeating a one-hour centrifugation at 14,000 x g three times. The supernatant was filtered through a 0.22 µm membrane (Merck, NJ, USA) to obtain the sterile RW extract.

The shells of RW pollen were produced following a previously published protocol ¹. Briefly, 1 g of RW pollens was defatted with 9 mL of acetone under reflux at 60°C overnight. The defatted grains were filtered and air-dried overnight. This preparation was used as “defatted” pollens. The defatted pollens were suspended in 20 mL of phosphoric acid and incubated at 60 °C for 7 d. The pollen grains were separated by vacuum filtration, and washed with hot water, followed by acetone, 2 M HCl, 2 M NaOH, water, acetone, and ethanol. After drying overnight at room temperature, washed pollens were incubated in 17.5 mL of 6% w/v KOH solution at 80 °C for 6 h. The pollens were separated by centrifugation and resuspended in 17.5 mL of fresh KOH solution to be incubated for another 6 h at 80°C. Pollen shells were then filtered, washed with hot water, followed by acetone. After air drying, pollen shells were weighed and suspended in PBS or RW extract. The solution was introduced into the shells by freezing at -30°C. The shell particle concentration was adjusted to the same particle number per mL as the raw RW pollen preparations.

To prepare the crushed pollens, raw pollen grains were bead-homogenized using TissueLyser II.

Crushed pollens were resuspended in PBS and broken morphology was confirmed under the microscope.

Heated pollen was prepared by incubating the raw pollen suspension in PBS at 72°C for 10 min.

After cooling, the suspension was administered to the mice.

Antigen stimulation of lymph node cells

Cervical lymph nodes were collected in a sterile manner and single cell suspension prepared from them was cultured in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), non-essential amino acids (ThermoFisher Scientific, USA), sodium pyruvate (FujiFilm, Japan), β -mercaptoethanol, penicillin, and streptomycin. Cells were seeded at 5×10^6 cells/mL and stimulated by 20 μ g/mL of ragweed (RW) extract for 120 h. The supernatant was harvested and frozen at -30°C until analysis.

Flow cytometry

The mouse conjunctiva was dissected using surgical scissors. The tissues were minced and digested in RPMI1640 supplemented with 10% FCS, 1 mg/mL collagenase (FujiFilm) and 0.5 mg/mL DNase I with continuous stirring for 1 h at 37°C. The dispersed cell suspension was filtered through a nylon mesh, and cells were counted. The washed cells were blocked with anti-CD16/CD32 antibody (clone 2.4G2) for 10 min at 4°C. The cells were further incubated with surface-staining antibodies at 4°C for 20 min.

Fluorescence intensity was measured by FACSVerse (BD Biosciences, USA). Data were analyzed using the FlowJo software (BD Biosciences).

Histology

After euthanasia, a mouse conjunctival biopsy was obtained. Giemsa staining was performed for the paraformaldehyde-fixed paraffin-embedded sections. For IL-33 staining, the mouse conjunctiva was fixed with paraformaldehyde in situ for 5 min and collected thereafter. After a total of 25 minutes fixation, the tissue was thoroughly rinsed with PBS and embedded in OCT compound (Sakura Finetech, Japan). Cryosections were blocked with 2% skim milk and incubated with anti-IL-33 antibody (AF3626, R&D Systems), AlexaFluor 647-conjugated anti-Ki67 antibody (clone 11F6, Biolegend, CA, USA), and/or AlexaFluor 488-conjugated anti-EpCAM antibody (clone G8.8, Biolegend), followed by Dylight649 anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA). After staining with DAPI, the coverslips were mounted with SlowFade Diamond (ThermoFisher Scientific). Fluorescence was acquired using LSM-700 confocal microscope system (Carl Zeiss, Germany) and IL-33 positivity was quantified using inForm software version 2.4 (PerkinElmer, MA, USA).

RNA extraction and quantitative reverse-transcription PCR

Total RNA was extracted from mouse conjunctival tissues by RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Complementary DNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Japan). Real-time PCR was performed using QuantiNova SYBR Green PCR Kit (Qiagen) on StepOnePlus System (Thermo Fisher Scientific).

Enzyme-linked Immunosorbent Assay (ELISA)

Chemiluminescence enzyme immunoassay were performed to analyze mouse sera and lymph node cell culture supernatants. 384-well black microplates (Greiner Bio-one, Germany) were coated with 5 µg/mL of RW extract or the capturing antibodies. After blocking with 30% ImmunoBlock reagent (KAC, Japan), diluted mouse serum or the culture supernatant was applied to the wells and incubated for 2 h at room temperature. After washing, the plates were incubated with biotin-conjugated detection antibodies together with avidin-HRP (BioLegend, CA, USA) for 1 h. After extensive washing, Western Lightning ECL Pro (PerkinElmer) was added as a substrate, and luminescence was measured by GloMax luminescence plate reader (Promega, USA). For the analysis of tissue IL-33 and IgE content, colorimetric ELISA was performed for the tissue homogenate. The conjunctival tissue was collected, weighed, and homogenized in PBS supplemented with 0.1% NP40 and Halt Protease Inhibitor Cocktail (Thermofisher) using BioMasher II (Nippi, Tokyo, Japan). After centrifuge, the supernatant was collected. The protein concentration was measured by BCA protein assay (BioRad, CA, USA). The protein concentration was adjusted to 250 µg/mL before analysis.

Statistics

Statistical analysis was performed using Prism 8 software (GraphPad, CA, USA). Data are presented as mean $\pm$ S.E.M. in all figure parts in which error bars are shown. Groups were analyzed by two-tailed t-test with Welch's correction or ANOVA with Dunnett's multiple comparison unless otherwise indicated. *P* values of <0.05 were considered statistically significant.

Supplementary References

1. Gonzalez-Cruz P, Uddin MJ, Atwe SU, Abidi N, Gill HS. A chemical treatment method for obtaining clean and intact pollen shells of different species. *ACS Biomater Sci Eng.* 2018;4(7):2319-2329.

Supplementary Figure E1

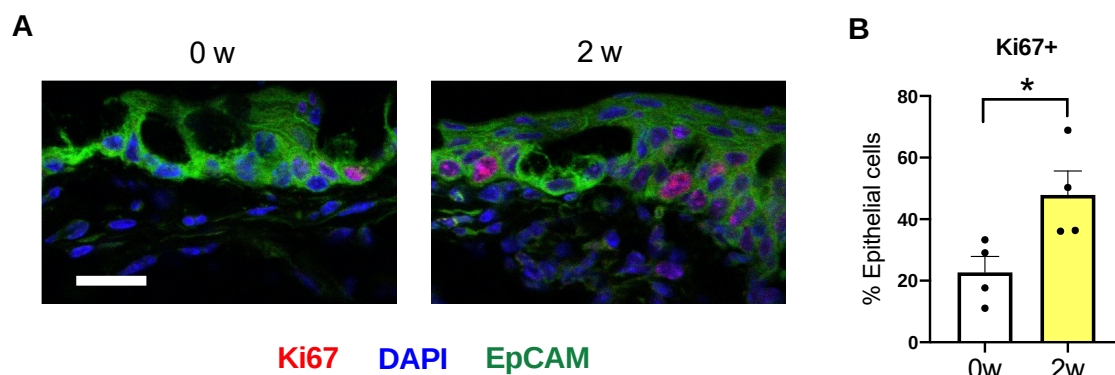


Figure E1. Topical challenges induce epithelial proliferative response.

(A) Immunofluorescence staining of Ki67 in the conjunctiva. Bar, 20 μm . (B) Quantification of Ki67 positive cells. * $p < 0.05$ by Welch's t-test.

Supplementary Figure E2.

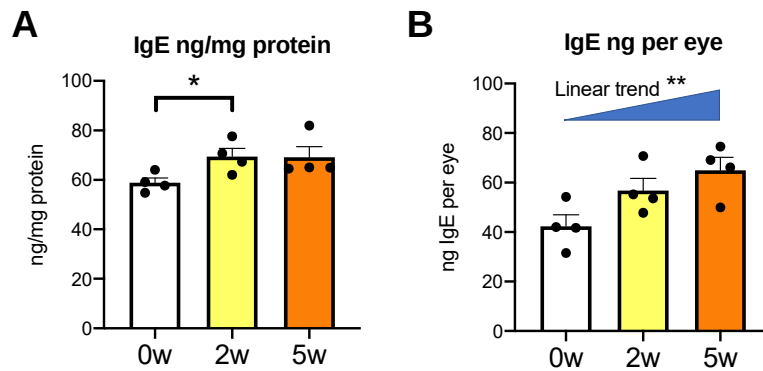


Figure E2. Local tissue IgE content start to increase in two weeks.

The IgE tissue content in the conjunctiva was measured by ELISA. IgE content normalized by protein concentration (A) and that in the whole conjunctiva (B) are shown. * $p < 0.05$ by Welch's t-test. ** $p < 0.01$ by ANOVA for linear trend.

Supplementary Figure E3.

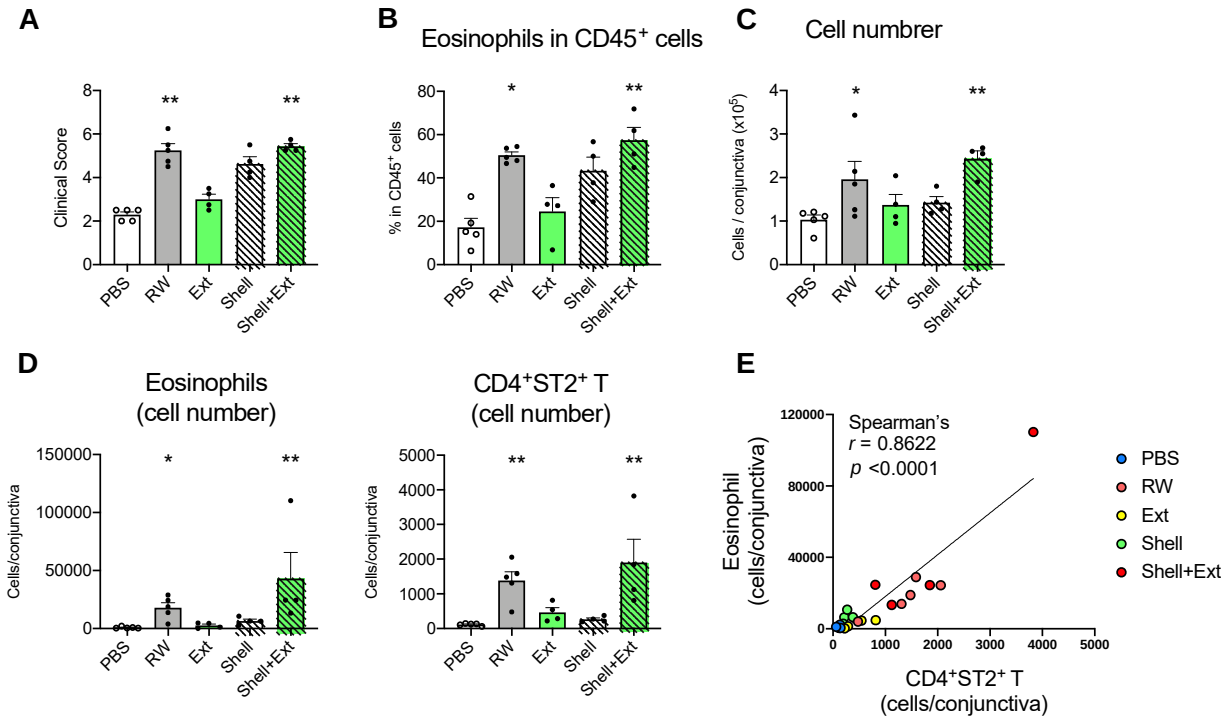


Figure E3. Pollen shells are required for the elicitation phase of conventional allergic conjunctivitis model.

(A) Clinical score. (B) Conjunctival eosinophil populations among CD45⁺ cells. (C,D) Cell numbers of total (C) or indicated populations (D) in the conjunctiva. (E) Correlation of CD4⁺ST2⁺ T cell and eosinophil numbers in the conjunctiva. * $p < 0.05$ and ** $p < 0.01$ by Kruskal-Wallis test with Dunn's multiple comparison against PBS group.

Supplementary Figure E4.

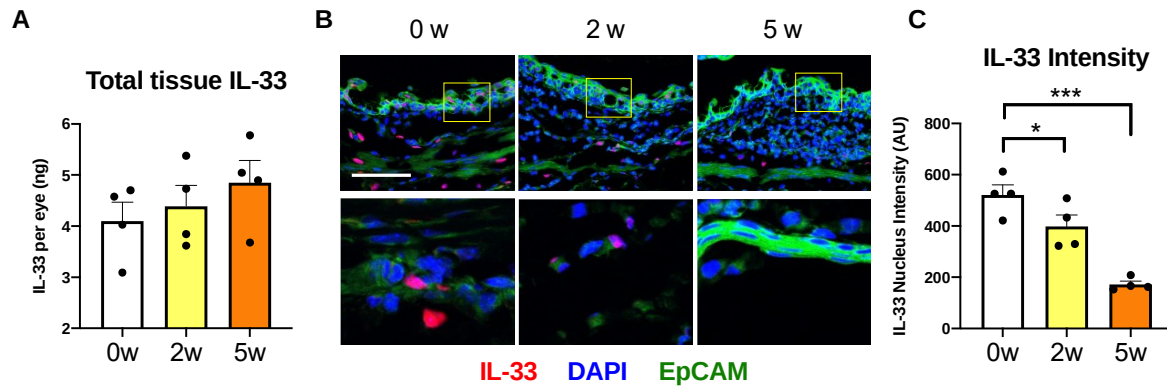


Figure E4. IL-33 is mobilized by the induction of allergic conjunctivitis.

(A) The IL-33 tissue content in the conjunctiva was measured by ELISA. (B) Immunofluorescence staining of IL-33 in the conjunctiva after indicated periods of pollen administration. Bar, 100 μ m. (C) The IL-33 staining intensity was quantified. *p<0.05, ***p<0.001 by ANOVA with Holm-Sidak's multiple comparisons.

Supplementary Fig E5.

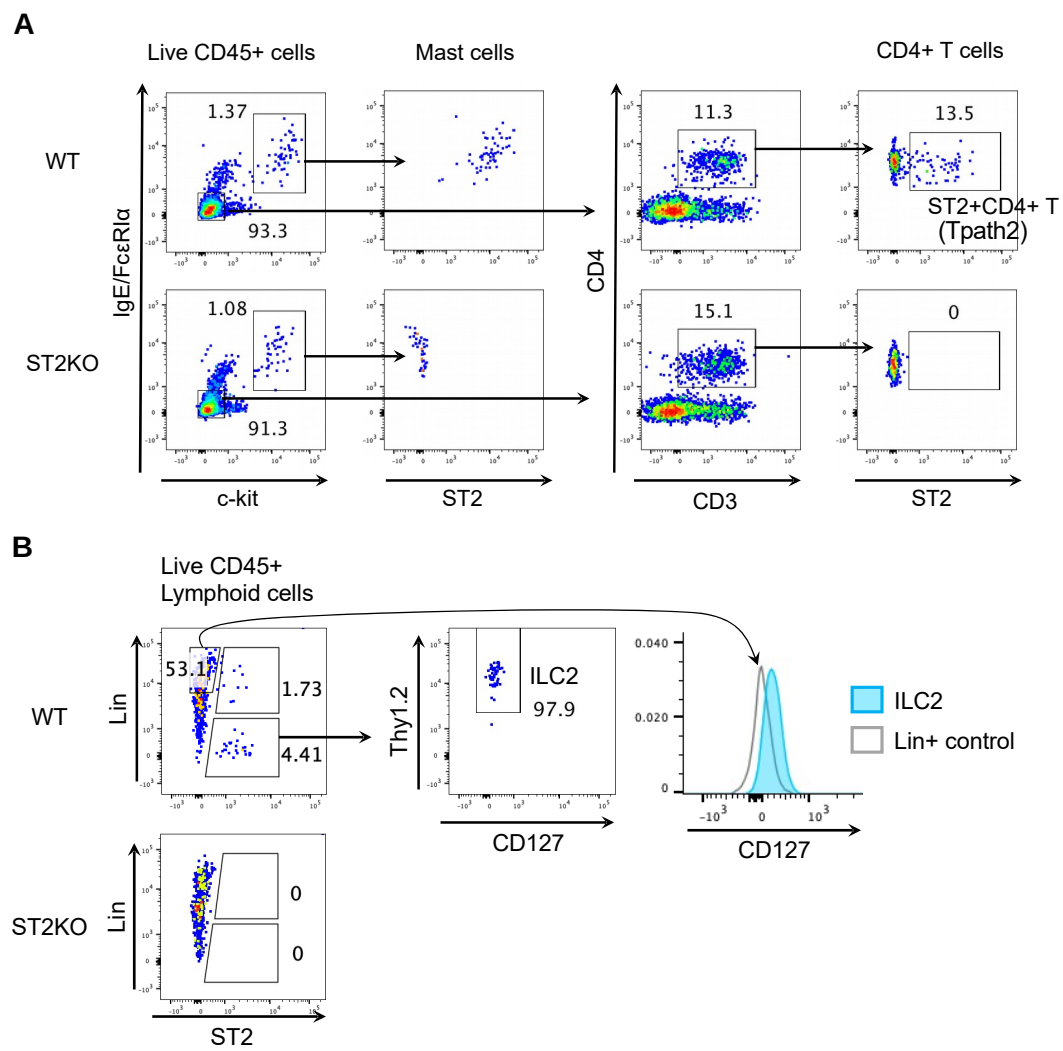


Figure E5. Mast cells, CD4⁺ T cells, and ILC2s express ST2 in the conjunctiva.

Gating strategies for measuring ST2 expressions in mast cells and CD4⁺ T cells (A) or in ILC2s (B). Lin, lineage.