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Epicutaneous vaccination with protease inhibitor-treated papain prevents papain-induced Th2-mediated airway inflammation without inducing Th17 in mice

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ABSTRACT

Environmental allergen sources such as house dust mites contain proteases, which are frequently allergens themselves. Inhalation with the exogenous proteases, such as a model of protease allergen, papain, to airways evokes release and activation of IL-33, which promotes innate and adaptive allergic airway inflammation and Th2 sensitization in mice. Here, we examine whether epicutaneous (e.c.) vaccination with antigens with and without protease activity shows prophylactic effect on the Th airway sensitization and Th2-medated airway inflammation, which are driven by exogenous or endogenous IL-33. E.c. vaccination with ovalbumin restrained ovalbumin-specific Th2 airway sensitization and/or airway inflammation on subsequent inhalation with ovalbumin plus papain or ovalbumin plus recombinant IL-33. E.c. vaccination with papain or protease inhibitor-treated papain restrained papain-specific Th2 and Th9 airway sensitization, eosinophilia, and infiltration of IL-33-responsive Th2 and group 2 innate lymphoid cells on subsequent inhalation with papain. However, e.c. vaccination with papain but not protease inhibitor-treated papain induced Th17 response in bronchial draining lymph node cells. In conclusions, we demonstrated that e.c. allergen vaccination via intact skin in mice restrained even protease allergen-activated IL-33-driven airway Th2 sensitization to attenuate allergic airway inflammation and that e.c. vaccination with protease allergen attenuated the airway inflammation similar to its derivative lacking the protease activity, although the former but not the latter promoted Th17 development. In addition, the present study suggests that modified allergens, of which Th17-inducing e.c. adjuvant activity such as the protease activity was eliminated, might be preferable for safer clinical applications of the e.c. allergen administration.

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1. Introduction

IL-33 is an epithelial or endothelial cytokine, which is important in both the innate and adaptive allergic responses [1]. A recent study reported that IL-33 breaks tolerance induced by repeated intranasal (i.n.) administration of an experimental model allergen ovalbumin (OVA) [2], suggesting IL-33-mediated breakdown of airway tolerance, which can be generally established by respiratory exposure to innocuous antigens [3].

Allergen sources such as house dust mites, insects, fungi, pollen, and so on contain proteases, which are frequently allergens themselves [4]. The proteolytic activity of allergens involves in the pathogenesis of allergies [1,4–6]. The delivery of the exogenous proteases to airways evokes the release of IL-33 [7–9]. Allergen proteases directly process and activate IL-33 by cleaving it within the protease-sensor domain [1]. Repeated i.n. administration with a model protease allergen papain induces Th2 differentiation and allergic airway inflammation in a manner dependent on IL-33 and

Abbreviations: BAL, bronchial alveolar lavage; DLN, draining lymph node; e.c., epicutaneous; ILC2, group 2 innate lymphoid cells; i.n., intranasal; OVA, ovalbumin; rIL, recombinant interleukin; ST2, a subunit for the IL-33 receptor.

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group 2 innate lymphoid cells (ILC2), which express ST2, a subunit for the IL-33 receptor [7,10,11]. The model is also highly dependent on adaptive immunity [7,12]. Epicutaneous (e.c.) [13–15] or subcutaneous papain administration [16,17] promotes Th2/Th17 or Th2 sensitization, respectively, however, in a manner independent from IL-33.

E.c. exposure to allergens through the skin is considered to be an important route of allergic sensitization, which leads to allergic diseases such as asthma and food allergy [18]. In contrast, recent studies showed that e.c. administration of antigens can induce prophylactic or therapeutic effects on allergic diseases [19,20] or their murine models [21–23]. However, whether the e.c. antigen administration has a potential to suppress the IL-33-driven responses and how different effects adjuvants contained in e.c. administered antigens, such as protease activity, show are unknown. Here, we examine whether prophylactic e.c. antigen vaccination can attenuate airway allergic sensitization and inflammation driven by exogenous IL-33 and papain-activated endogenous IL-33 and compare effects between e.c. vaccination with papain and that with protease inhibitor-treated papain.

2. Materials and methods

2.1. Mice

7- to 12-week-old C57/BL6J female mice (Sankyo Lab Service Corporation, Ibaraki, Japan) were maintained in a specific pathogen free animal facility at Juntendo University and were used in accordance with the guidelines of the Institutional Committee on animal experiments.

2.2. Antigens

Papain was purchased from Calbiochem (San Diego, CA). The covalent complex between the cysteine protease inhibitor E-64 (Peptide Institute, Osaka, Japan) and papain (E64-papain) was prepared as described previously [7]. Papain, which was incubated similarly to E64-treated papain (but without the addition of E-64) and dialyzed, was prepared and used for comparisons with E-64-treated papain in e.c. vaccination. OVA (Grade V; Sigma-Aldrich, St. Louis, MO) and that with low endotoxin content (EndoGrade OVA; Hyglos GmbH, Regensburg, Germany) were used in e.c. administration and ELISA, and in i.n. administration and stimulation of DLN cells, respectively.

2.3. E.c. vaccination

OVA (Sigma), E64-papain, dialyzed papain, or vehicle (PBS containing 0.5% Tween 20) was applied to both sides of the surface of both ears of lightly anesthetized mice using a micropipette (12.5 μl/side, concentration 2 mg/ml; 100 μg/50 μl/animal) three times a week, and this procedure was repeated for 4 weeks.

2.4. I.n. administration

After the e.c. vaccination, mixture of low-endotoxin OVA (20 μg) and recombinant mouse IL-33 (rIL-33) (BioLegend, San Diego, CA) (1.5 ng), mixture of OVA (20 μg) and papain (15 μg), or papain alone (15 μg) was i.n. administered to lightly anesthetized mice (50 μl/animal) twice with a seven day-interval. Sera, bronchial alveolar lavage (BAL) fluid and bronchial draining lymph nodes (DLNs) (mediastinal LNs) were collected 4 days after the last i.n. administration.

2.5. BAL

BAL and differential cell count for eosinophils, neutrophils, lymphocytes and monocytes of specimens prepared on glass slides were performed as described previously. Th2, $ST2^+$ Th2 and ILC2 were analyzed by flow cytometry as described previously [12,24].

2.6. Restimulation of bronchial DLN cells

Single-cell suspensions of DLN cells were prepared according to the methods described previously [12,24]. Cells were stimulated with antigens in 96-well, flat-bottomed, tissue culture plates (Corning Life Sciences, Corning. NY) (5×10^5/200 μL/well). Mixtures of all DLN cells from the mice of each group were restimulated with low-endotoxin OVA or E64-papain (25 μg/mL) in the presence or absence of rIL-33 (10 ng/mL). To assess cytokine production, culture supernatants were collected at 96 h (Figs. 1 and 2) or 72 h (Fig. 4) and subjected to ELISA using kits (R&D Systems). For IL-9, the kit was purchased from BioLegend.

2.7. ELISA for serum total IgE and antigen-specific antibodies

Serum total IgE [25] and papain-specific and OVA-specific antibodies [13] were detected on plates, which were coated with 2 μg/ml anti-murine IgE monoclonal antibodies (mAb) (clone R35-72, BD Biosciences, San Jose, CA), 10 μg/ml papain or 1 mg/ml OVA resolved in PBS (50 μl/well; 4 °C overnight), and blocked as described previously [13,25] with minor modifications as follows. Briefly, sera, detection antibodies, and streptavidin-conjugated horseradish peroxidase were diluted with PBS containing 0.05% Tween 20 and 5% ImmunoBlock (DS Pharma, Osaka, Japan)(50 μl/well).

Total IgE was measured by sandwich ELISA. After an incubation with diluted sera, plates were incubated with HRP-conjugated anti-murine IgE mAb (clone LO-ME-2; Technopharm Biotechnology, Paris, France) (dilution: 1/5000; 37 °C for 80 min). Monoclonal IgE specific to TNP (purified mouse IgE isotype control, clone IgE-3; BD Biosciences) was used as the standard. Regarding papain-specific IgE, after the incubation with diluted sera (serum dilution: 1/50; 4 °C, overnight), plates were incubated with biotin-conjugated anti-murine IgE mAb (clone R35-118, BD Biosciences) (dilution: 1/500; room temperature for 60 min). After being washed, plates were further incubated with avidin-HRP (BD Biosciences) (dilution: 1/2500; room temperature for 30 min). Regarding papain-specific IgG1, after the incubation with diluted sera (serum dilution: 1/5000; 37 °C for 80 min), plates were incubated with HRP-conjugated anti-murine IgG1 mAb (clone X56, BD Biosciences) (dilution: 1/5000; 37 °C for 80 min).

Plates were washed and were color developed and the reaction was stopped as described previously. Absorbance at 450 nm, from which that at 570 nm was subtracted, was used as the signal.

2.8. Statistical analysis

Student's *t*-test (two-tailed), the Mann-Whitney *U* test (two-tailed), or one-way ANOVA with the Tukey *post hoc* test was used as indicated in the figure legends. A value of $p < 0.05$ was regarded as statistically significant.

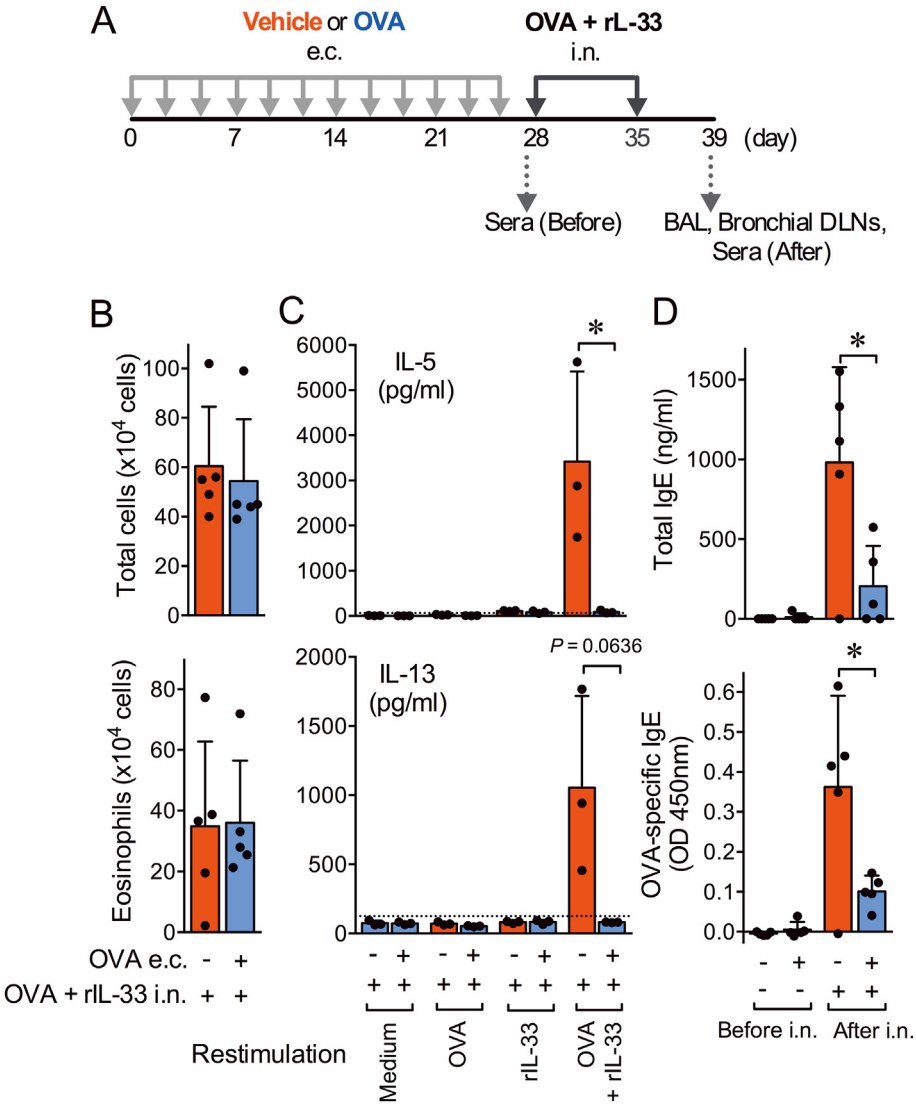

Fig. 1. Epicutaneous vaccination with OVA attenuated airway Th2 sensitization and serum OVA-specific IgE levels in mice inhaled with OVA plus rIL-33. A: Timeline. **B-D:** Responses induced by inhalation of OVA (20 μg) plus rIL-33 (1.5 ng). **B**: Airway inflammation. **C**: Cytokine production in restimulated bronchial DLN cells. **D**: Serum IgE responses. *$P < 0.05$ by *t*-test. Data are representative of two or more independent experiments with similar results. *e.c.*: epicutaneous administration of OVA (2 mg/ml) or vehicle on intact ear skin. *i.n.*: intranasal administration.

3. Results

3.1. E.c. vaccination with OVA attenuated airway Th2 sensitization and serum OVA-specific IgE levels in mice inhaled with OVA plus IL-33

In the present study, we examined whether e.c. exposure (vaccination) to antigens prophylactically restrains IL-33-driven airway sensitization and inflammation in three adaptive immunity-dependent models of i.n. sensitization (Fig. 1: the i.n. OVA-rIL-33 model; Fig. 2: the i.n. OVA-papain model; and Figs. 3 and 4: the adaptive i.n. papain model).

A recent study reported that prophylactic i.n. vaccination with OVA failed to prevent Th2 sensitization and allergic airway inflammation induced by inhalation with OVA plus rIL-33 [2]. Therefore, first we examined e.c. vaccination with OVA has a prophylactic potential in the modified i.n. OVA-rIL-33 model (Fig. 1 and Fig. E1). I.n. sensitization with OVA plus rIL-33 (Fig. 1) but not OVA alone [7] nor rIL-33 alone (data not shown) induced sensitization to OVA associated with allergic airway inflammation. The e.c. OVA vaccination to intact ear skin did not affect airway inflammation (Fig. 1B and Fig. E1A, B), however, it attenuated IL-5 and IL-13 production in bronchial DLN cells restimulated with OVA in the presence of rIL-33 (Fig. 1C). The e.c. OVA vaccination did not induce antibody responses before the i.n. sensitization and showed attenuation of serum levels of total IgE and OVA-specific IgE induced after the i.n. sensitization compared to the vehicle vaccination (Fig. 1A, D and Fig. E1C). Thus the e.c. vaccination showed prophylactic effects on OVA-specific Th2 responses in the exogenous IL-33-driven model although it did not affect airway inflammation.

3.2. E.c. vaccination with OVA attenuated airway inflammation, airway Th2 sensitization and serum OVA-specific IgE levels in mice inhaled with OVA plus papain

Although the e.c. OVA vaccination did not attenuate airway inflammation in the OVA-rIL-33 model, the administration of

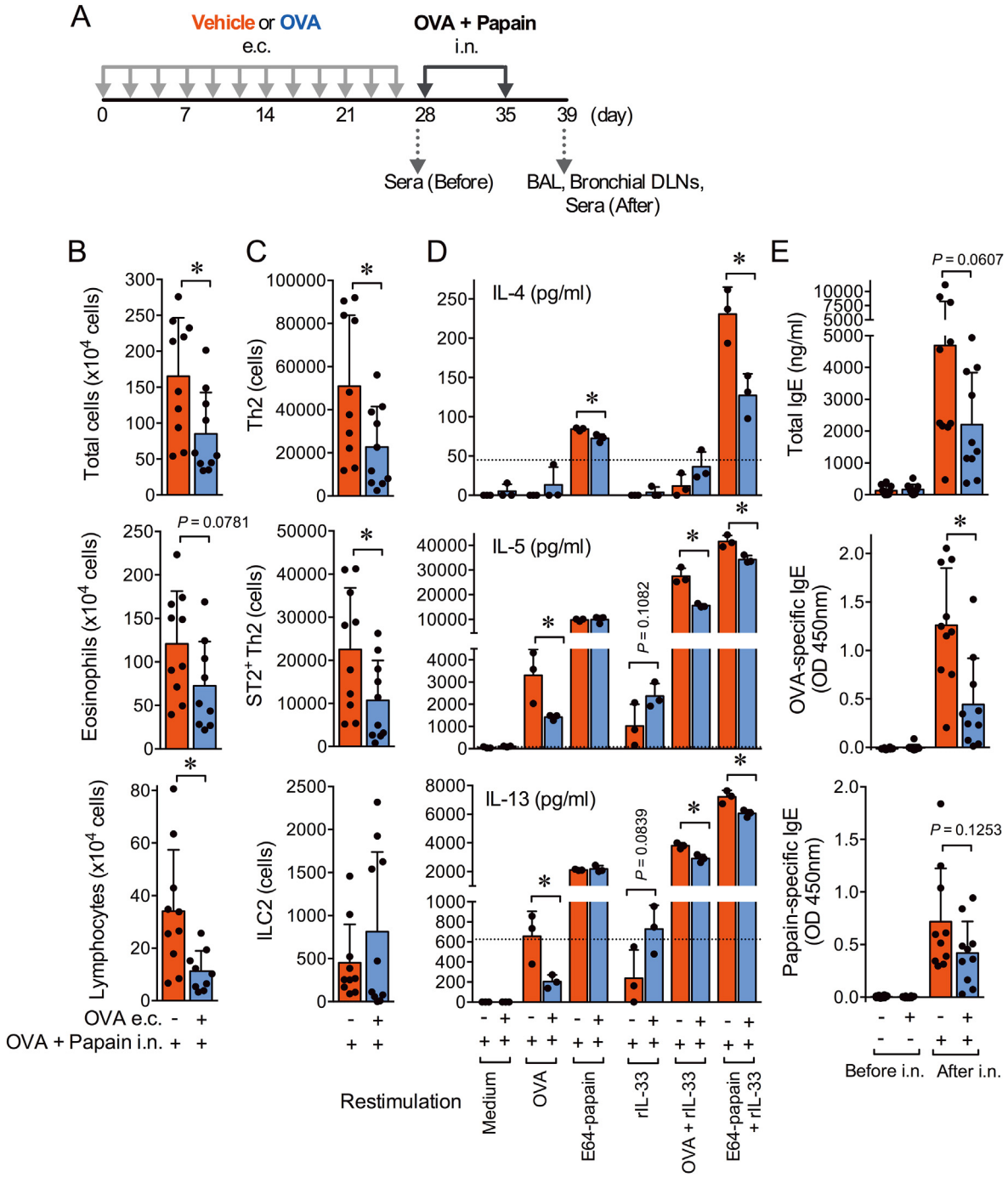

Fig. 2. Epicutaneous vaccination with OVA attenuated airway inflammation, airway Th2 sensitization and serum OVA-specific IgE levels in mice inhaled with OVA plus papain. **A**: Timeline. **B-E:** Responses induced by inhalation of OVA (20 μg) plus papain (15 μg). **B, C**: Airway inflammation. **D**: Cytokine production in restimulated bronchial DLN cells. **E:** Serum Ig**E** responses. *$P < 0.05$ by *t*-test. Data are representative of two or more independent experiments with similar results. *e.c.*: epicutaneous administration of OVA (2 mg/ml) or vehicle on intact ear skin. *i.n.*: intranasal administration.

exogenous rIL-33 in the model is not the best relevant to mimic IL-33 release and activation on allergen exposure (Fig. 1). Therefore, we next examined effects of e.c. OVA vaccination in the i.n. OVA-papain model, which is driven by papain-activated endogenous IL-33 (Fig. 2 and Fig. E2). The e.c. OVA vaccination attenuated airway inflammation (Fig. 2B) and infiltration of Th2 and ST2$^+$ Th2 (Fig. 2C) and slightly attenuated bronchial alveolar lavage (BAL) fluid levels of IL-5 and a Th2 chemokine CCL22 (Fig. E2B), and also attenuated production of IL-5 and IL-13 in bronchial DLN cells restimulated with OVA in the presence or absence of rIL-33 (Fig. 2D) and serum levels of OVA-specific IgE induced by the i.n. sensitization (Fig. 2E, *After i.n.*), indicating its prophylactic effects on not only OVA-specific Th2 responses but also airway inflammation in the model.

The e.c. OVA vaccination slightly attenuated production of IL-4, IL-5 and IL-13 on the bronchial DLN cell restimulation with E64-papain, the covalent complex between papain and the protease inhibitor E-64, in the presence of rIL-33, IL-4 production on restimulation with E64-papain (Fig. 2D) and serum papain-specific

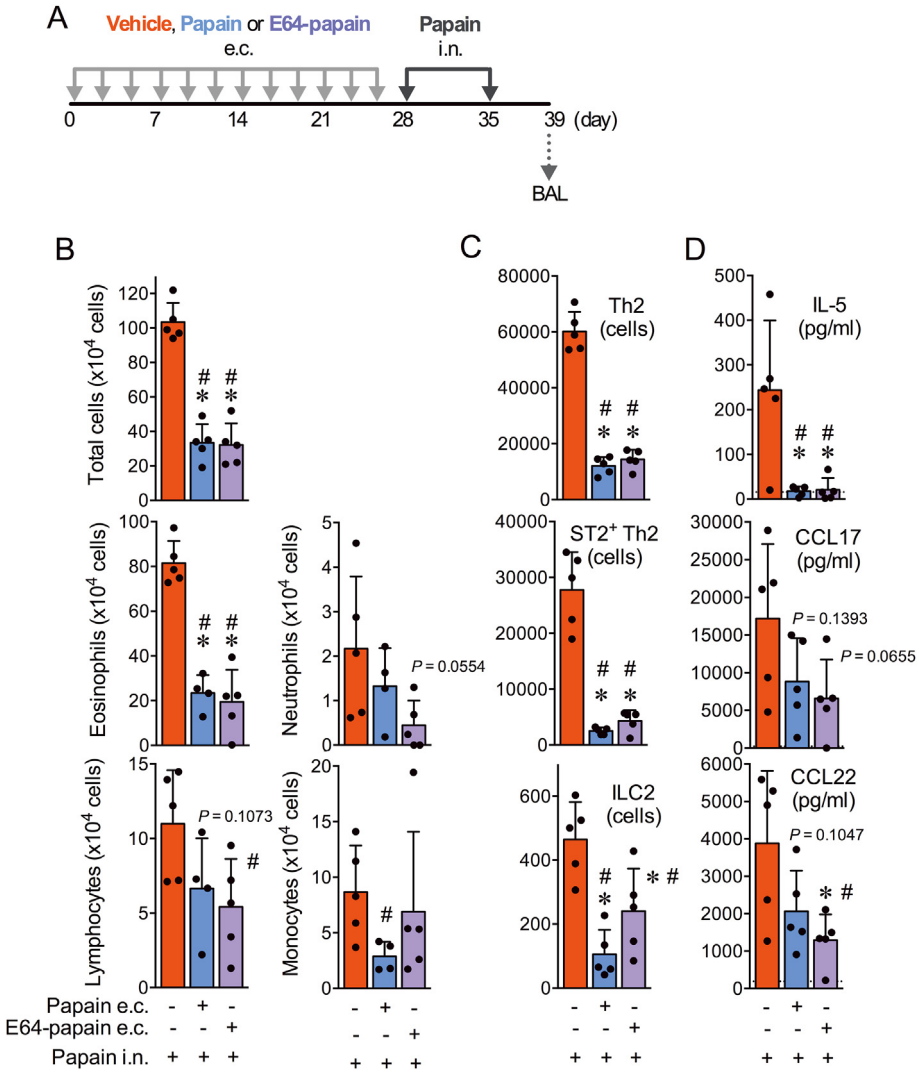

Fig. 3. Epicutaneous vaccination with papain with or without protease activity attenuated airway inflammation in mice inhaled with papain. A: Timeline. **B, C:** Airway inflammation induced by inhalation of papain (15 μg). **D**: BAL fluid IL-5 and Th2-attracting chemokines. $^{*}P < 0.05$ and indicated P values by t-test and $^{\#}P < 0.05$ by ANOVA. Data are representative of two or more independent experiments with similar results. *e.c.*: epicutaneous administration of papain, E64-papain (2 mg/ml) or vehicle on intact ear skin. *i.n.*: intranasal administration.

IgE levels (Fig. 2E), suggesting modest bystander effects on responses specific to antigens not administered in the e.c. vaccination. We used E64-papain as the antigen for restimulation to avoid potential protease activity-dependent effects. The covalent binding of the small molecule irreversible inhibitor, E-64, to papain does not affect T-cell recall cytokine responses and antibody reactivity, indicating that E64-papain retains almost intact T/B-cell epitope structures [12,26].

3.3. E.c. vaccination with papain with or without its protease activity attenuated airway inflammation

E.c. OVA vaccination was effective to attenuate responses including airway inflammation driven by papain-activated endogenous IL-33 (Fig. 2). The prophylactic effects were significant on OVA-specific responses (Fig. 2D and E), however, they were modest or weak on papain-specific responses. Therefore, next we examined effects of e.c. vaccination with papain in the adaptive i.n. papain model (Figs. 3 and 4). As our previous studies showed that e.c. papain administration in a shorter period significantly promoted sensitization in a manner dependent on its protease activity [13–15], we also examined e.c. vaccination with E64-papain, which lacks the protease activity. E.c. vaccination with papain or E64-papain attenuated airway inflammation (Fig. 3B), infiltration of Th2, ST2^{+} Th2 and ILC2 (Fig. 3C), and BAL fluid levels of IL-5 and Th2 chemokines, CCL17 and CCL22 (Fig. 3D).

3.4. E.c. vaccination with protease inhibitor-treated papain attenuated Th2/Th9 airway sensitization without inducing Th17 in mice inhaled with papain

E.c. vaccination with papain or E64-papain also attenuated production of Th2 (IL-4, IL-5, IL-13 and amphiregulin, which contributes to induction of airway fibrotic responses [27]) and Th9 (IL-9) cytokines in bronchial DLN cells restimulated with E64-papain in the presence or absence of rIL-33, and production of IL-5, IL-13 and IL-9 on restimulation with rIL-33 alone (Fig. 4B), although it did not significantly affect the antibody responses induced by the i.n. sensitization (Fig. 4C, *After i.n.*).

E.c. vaccination with papain and that with E64-papain showed

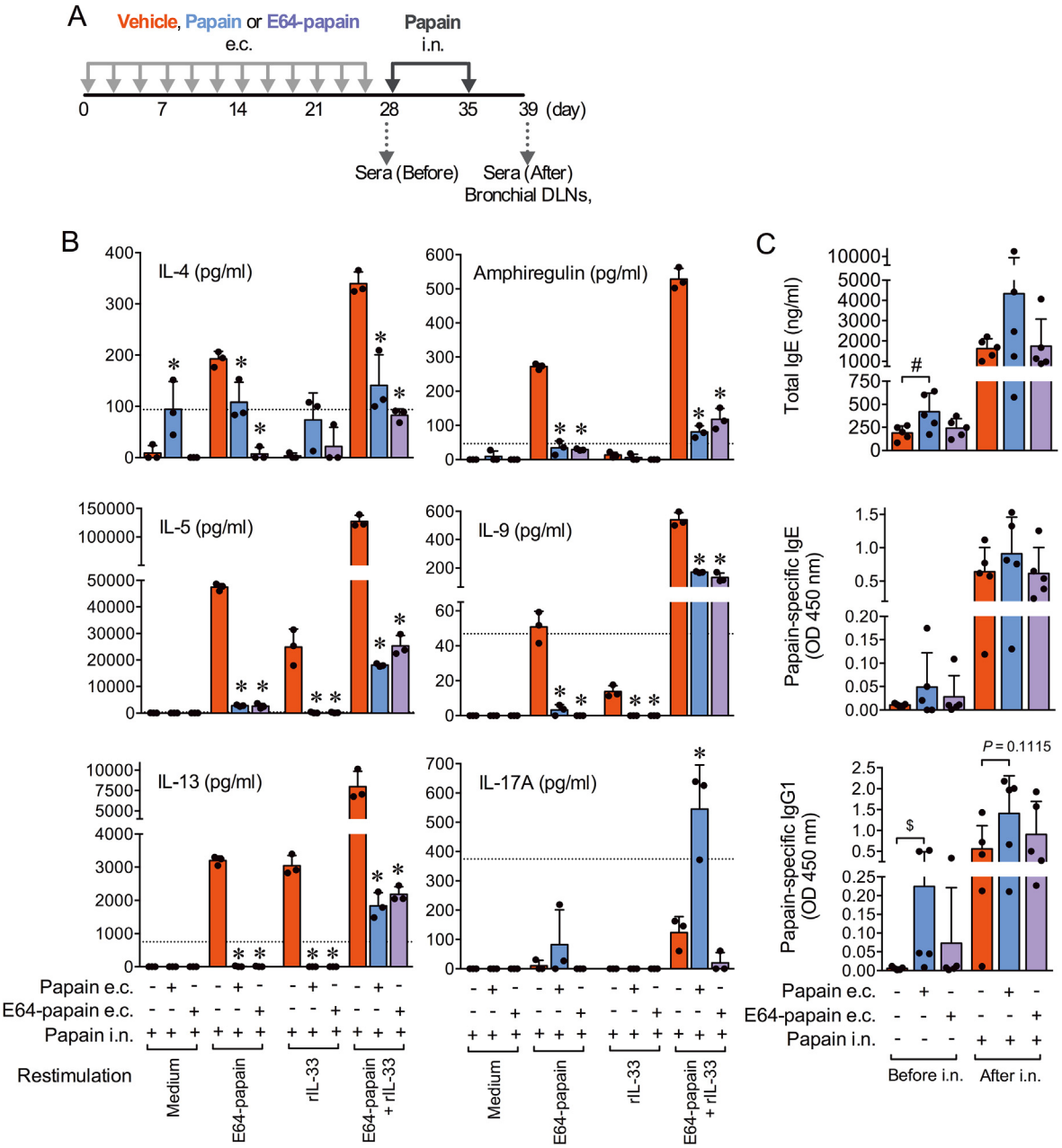

Fig. 4. Epicutaneous vaccination with protease inhibitor-treated papain attenuated Th2 and Th9 airway sensitization without inducing Th17 in mice inhaled with papain. A: Timeline. **B, C:** Responses induced by inhalation of papain (15 μg). **B**: Cytokine production in restimulated bronchial DLN cells. **C**: Serum antibody responses. $*P < 0.05$ and indicated P values by t-test, $^{\#}P < 0.05$ by ANOVA and $^{\$}P < 0.05$ by Mann-Whitney U test,. Data are representative of two or more independent experiments with similar results. *e.c.*: epicutaneous administration of papain, E64-papain (2 mg/ml) or vehicle on intact ear skin. *i.n.*: intranasal administration.

some different effects. Before inhalation with papain, e.c. vaccination with papain but not E64-papain induced serum papain-specific IgG1, and small levels of total and papain-specific IgE (Fig. 4C, *Before i.n.*). Interestingly, e.c. vaccination with papain but not E64-papain induced IL-17A production on bronchial DLN cell restimulation with E64-papain in the presence of rIL-33 (Fig. 4B).

4. Discussion

In the i.n. OVA-papain (Fig. 2) and adaptive i.n. papain models (Figs. 3 and 4), e.c. antigen vaccination prophylactically restrained IL-33-driven airway Th2 sensitization and allergic airway inflammation (Figs. 2–4). In the i.n. OVA-rIL-33 model driven by exogenous IL-33, e.c. vaccination restrained Th2 sensitization but not airway inflammation (Fig. 1), which might be due to possible more significant contribution of IL-33-responsive innate cells when stimulated with the abundant exogenous rIL-33 than limited amount of endogenous IL-33. E.c. vaccination with papain or E64-papain was effective to attenuate Th2 and Th9 responses in bronchial DLN cells (Fig. 4) and airway inflammation (Fig. 3). The airway inflammation in the i.n. OVA-papain and adaptive i.n. papain models without e.c. antigen vaccination is considered to be highly dependent on IL-33-driven Th2 immunity, being supported by that infiltration of $ST2^{+}$ Th2 into BAL fluid was approximately half of Th2 and 40- or 60-fold more than ILC2 (Figs. 2C and 3C). These results indicated that e.c. vaccination has capacity to attenuate airway Th2

sensitization and inflammation, which is driven by papain-activated endogenous IL-33.

The skin is an immunologic organ, which maintains the balance among immunologic effector responses, sensitization and tolerance [28]. Recent studies suggested involvement of Langerhans cells and regulatory T cells in prophylactic and therapeutic models with e.c. OVA administration [29,30]. Interestingly, e.c. administration of papain with protease activity promotes sensitization [12–15] or induces tolerance (Figs. 3 and 4) according to the different experimental settings. The bronchial DLN Th17 response after the airway papain exposure in mice e.c. vaccinated with papain but not E64-papain (Fig. 4) is considered to be due to the fact that e.c. papain sensitization for a shorter period promotes Th17 differentiation in skin DLNs [13]. However, the e.c. papain sensitization also promotes Th2 differentiation in skin DLNs [13]. The mechanism of the prophylaxis for Th2/Th9 but not Th17 development in bronchial DLNs by the e.c. papain vaccination for the longer period should be addressed in future studies.

Recently, e.c. immunotherapy or prophylactic e.c. vaccination has been shown to be effective in treatment of allergic diseases and their animal models [19–23]. However, as far as we know, a risk for Th17 sensitization induced by e.c. antigen administration seems to have been overlooked. Recent studies suggest Th17 contributes to neutrophilic inflammation in severe asthma [31,32]. The number of neutrophils in BALF in the adaptive i.n. papain model was relatively small, although e.c. E64-papain vaccination showed a tendency of attenuation of the neutrophil infiltration (Fig. 3B). The pathophysiological significance for the Th17 induction in e.c. papain administration should be addressed in future studies. Environmental proteases including protease allergens can cause skin barrier dysfunction [4,15,33,34], stimulate various types of cells [4–6,35–38] and promote the e.c. sensitization process [12–15]. We suggest that modified allergens, of which Th17-inducing e.c. adjuvant activity such as the protease activity was eliminated, might be preferable for safer e.c. vaccination or e.c. immunotherapy. Advantages of e.c. administration with environmental allergens such as house duct mites, pollens and so on, from which protease activity was removed, should be evaluated in future studies.

In conclusions, we demonstrated that e.c. allergen vaccination via intact skin in mice restrained even the protease allergen-activated, IL-33-driven airway Th2 sensitization to attenuate airway inflammation, and that e.c. vaccination with protease allergen showed prophylactic effects similar to its derivative lacking the protease activity although the former (papain) but not the latter (E64-papain) promoted Th17 development in bronchial DLNs, which is undesirable to safer clinical applications. Murine models, which mimic IL-33-driven airway sensitization to protease-containing environmental allergens, are relevant to natural airway allergen exposure and may assist in elucidating mechanisms behind sensitization and tolerance induced by the e.c. allergen exposure/administration. Therapeutic approaches to the IL-33-driven airway inflammation by the e.c. antigen administration would be addressed in future studies.

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Declaration of competing interest

The authors state 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.bbrc.2020.12.090.

Author contributions

S. Kunimine, TT and S. Kamijo wrote the manuscript. TT organized the study. S. Kunimine, TT, S. Kamijo, NM, TK, YM, TY, PS and SS performed the experiments, analyzed the data, and/or interpreted the data. S. Kunimine, TT, S. Kamijo, HO, KO and SI contributed to the study design. All the authors read and approved the final manuscript.

References

[1] C. Cayrol, A. Duval, P. Schmitt, et al., Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33, Nat. Immunol. 19 (2018) 375–385.
[2] C.C. Chen, T. Kobayashi, K. Iijima, et al., IL-33 dysregulates regulatory T cells and impairs established immunologic tolerance in the lungs, J. Allergy Clin. Immunol. 140 (2017), 1351-63e7.
[3] O. Akbari, R.H. DeKruyff, D.T. Umetsu, Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen, Nat. Immunol. 2 (2001) 725–731.
[4] T. Takai, S. Ikeda, Barrier dysfunction caused by environmental proteases in the pathogenesis of allergic diseases, Allergol. Int. 60 (2011) 25–35.
[5] N. Serhan, L. Basso, R. Sibilano, et al., House dust mites activate nociceptor-mast cell clusters to drive type 2 skin inflammation, Nat. Immunol. 20 (2019) 1435–1443.
[6] C. Perner, C.H. Flayer, X. Zhu, et al., Substance P release by sensory neurons triggers dendritic cell migration and initiates the type-2 immune response to allergens, Immunity 53 (2020) 1063–1077, e7.
[7] S. Kamijo, H. Takeda, T. Tokura, et al., IL-33-mediated innate response and adaptive immune cells contribute to maximum responses of protease allergen-induced allergic airway inflammation, J. Immunol. 190 (2013) 4489–4499.
[8] K. Hara, K. Iijima, M.K. Elias, et al., Airway uric acid is a sensor of inhaled protease allergens and initiates type 2 immune responses in respiratory mucosa, J. Immunol. 192 (2014) 4032–4042.
[9] H. Kouzaki, K. Iijima, T. Kobayashi, et al., The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses, J. Immunol. 186 (2011) 4375–4387.
[10] T.Y. Halim, C.A. Steer, L. Matha, et al., Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation, Immunity 40 (2014) 425–435.
[11] T.Y. Halim, Y.Y. Hwang, S.T. Scanlon, et al., Group 2 innate lymphoid cells license dendritic cells to potentiate memory TH2 cell responses, Nat. Immunol. 17 (2016) 57–64.
[12] I. Nishioka, T. Takai, N. Maruyama, et al., Airway inflammation after epicutaneous sensitization of mice requires protease activity of low-dose allergen inhalation, J. Allergy Clin. Immunol. 141 (2018) 2271–2273, e7.
[13] S. Shimura, T. Takai, H. Iida, et al., Epicutaneous allergic sensitization by cooperation between allergen protease activity and mechanical skin barrier damage in mice, J. Invest. Dermatol. 136 (2016) 1408–1417.
[14] H. Ochi, T. Takai, S. Shimura, et al., Skin treatment with detergent promotes protease allergen-dependent epicutaneous sensitization in a manner different from tape stripping in mice, J. Invest. Dermatol. 137 (2017) 1578–1582.
[15] H. Iida, T. Takai, Y. Hirasawa, et al., Epicutaneous administration of papain induces IgE and IgG responses in a cysteine protease activity-dependent manner, Allergol. Int. 63 (2014) 219–226.
[16] S. Kamijo, M. Hara, M. Suzuki, et al., Innate IL-17A enhances IL-33-independent skin eosinophilia and IgE response on subcutaneous papain sensitization, J. Invest. Dermatol. 141 (2021) 105–113, e14.
[17] S. Kamijo, M. Suzuki, M. Hara, et al., Subcutaneous allergic sensitization to protease allergen is dependent on mast cells but not IL-33: distinct mechanisms between subcutaneous and intranasal routes, J. Immunol. 196 (2016) 3559–3569.
[18] A.S. Paller, J.M. Spergel, P. Mina-Osorio, et al., The atopic march and atopic

multimorbidity: many trajectories, many pathways, J. Allergy Clin. Immunol. 143 (2019) 46–55.
[19] H.A. Sampson, W.G. Shreffler, W.H. Yang, et al., Effect of varying doses of epicutaneous immunotherapy vs placebo on reaction to peanut protein exposure among patients with peanut sensitivity: a randomized clinical trial, J. Am. Med. Assoc. 318 (2017) 1798–1809.
[20] G. Senti, S. von Moos, F. Tay, et al., Epicutaneous allergen-specific immunotherapy ameliorates grass pollen-induced rhinoconjunctivitis: a double-blind, placebo-controlled dose escalation study, J. Allergy Clin. Immunol. 129 (2012) 128–135.
[21] W. Li, Z. Zhang, A. Saxon, et al., Prevention of oral food allergy sensitization via skin application of food allergen in a mouse model, Allergy 67 (2012) 622–629.
[22] S. Siebeneicher, S. Reuter, M. Krause, et al., Epicutaneous immune modulation with Bet v 1 plus R848 suppresses allergic asthma in a murine model, Allergy 69 (2014) 328–337.
[23] L. Mondoulet, V. Dioszeghy, E. Puteaux, et al., Specific epicutaneous immunotherapy prevents sensitization to new allergens in a murine model, J. Allergy Clin. Immunol. 135 (2015) 1546–15457 e4.
[24] N. Maruyama, T. Takai, S. Kamijo, et al., Cyclooxygenase inhibition in mice heightens adaptive- and innate-type responses against inhaled protease allergen and IL-33, Allergy 74 (2019) 2237–2240.
[25] T. Takai, T. Kato, M. Ota, et al., Recombinant Der p 1 and Der f 1 with in vitro enzymatic activity to cleave human CD23, CD25 and alpha1-antitrypsin, and in vivo IgE-eliciting activity in mice, Int. Arch. Allergy Immunol. 137 (2005) 194–200.
[26] Y. Kikuchi, T. Takai, T. Kuhara, et al., Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses, J. Immunol. 177 (2006) 1609–1617.
[27] Y. Morimoto, K. Hirahara, M. Kiuchi, et al., Amphiregulin-Producing pathogenic memory T helper 2 cells instruct eosinophils to secrete osteopontin and facilitate airway fibrosis, Immunity 49 (2018) 134–150.e6.
[28] E. Guttman-Yassky, L. Zhou, J.G. Krueger, The skin as an immune organ: tolerance versus effector responses and applications to food allergy and hypersensitivity reactions, J. Allergy Clin. Immunol. 144 (2019) 362–374.
[29] Y. Luo, S. Wang, X. Liu, et al., Langerhans cells mediate the skin-induced tolerance to ovalbumin via Langerin in a murine model, Allergy 74 (2019) 1738–1747.
[30] V. Dioszeghy, L. Mondoulet, L. Laoubi, et al., Antigen uptake by Langerhans cells is required for the induction of regulatory T cells and the acquisition of tolerance during epicutaneous immunotherapy in OVA-sensitized mice, Front. Immunol. 9 (2018) 1951.
[31] A. Ray, J.K. Kolls, Neutrophilic inflammation in asthma and association with disease severity, Trends Immunol. 38 (2017) 942–954.
[32] K. Hirose, A. Iwata, T. Tamachi, et al., Allergic airway inflammation: key players beyond the Th2 cell pathway, Immunol. Rev. 278 (2017) 145–161.
[33] T. Nakamura, Y. Hirasawa, T. Takai, et al., Reduction of skin barrier function by proteolytic activity of a recombinant house dust mite major allergen Der f 1, J. Invest. Dermatol. 126 (2006) 2719–2723.
[34] Y. Hirasawa, T. Takai, T. Nakamura, et al., *Staphylococcus aureus* extracellular protease causes epidermal barrier dysfunction, J. Invest. Dermatol. 130 (2010) 614–617.
[35] T. Kato, T. Takai, K. Mitsuishi, et al., Cystatin A inhibits IL-8 production by keratinocytes stimulated with Der p 1 and Der f 1: biochemical skin barrier against mite cysteine proteases, J. Allergy Clin. Immunol. 116 (2005) 169–176.
[36] T. Ogawa, T. Takai, T. Kato, et al., Upregulation of release of granulocyte-macrophage colony-stimulating factor from keratinocytes stimulated with cysteine protease activity of recombinant major mite allergens, Der f 1 and Der p 1, Int. Arch. Allergy Immunol. 146 (2008) 27–35.
[37] T. Kato, T. Takai, T. Fujimura, et al., Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes, Allergy 64 (2009) 1366–1374.
[38] S. Kamijo, S. Nunomura, C. Ra, et al., Innate basophil IL-4 responses against allergens, endotoxin, and cytokines require the Fc receptor gamma-chain, J. Allergy Clin. Immunol. 137 (2016) 1613–1615 e2.

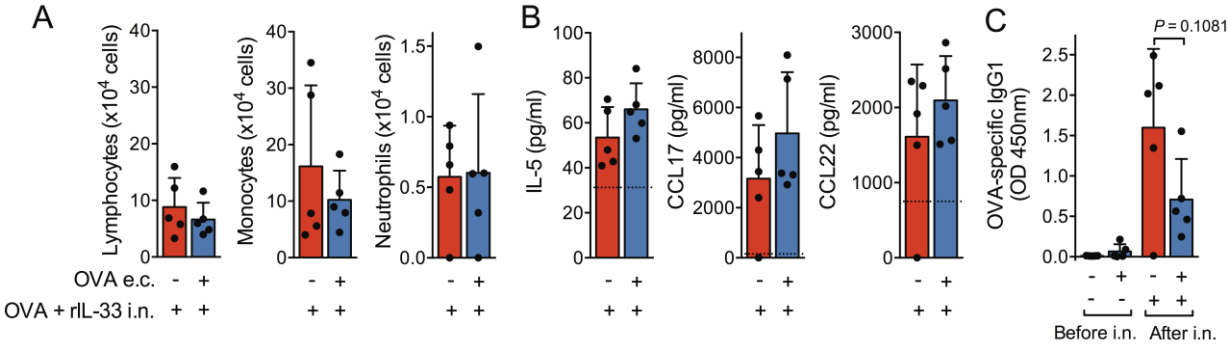
Supplementary Figure Legends

Fig. E1. Responses in mice inhaled with OVA plus rIL-33 with or without epicutaneous vaccination with OVA.

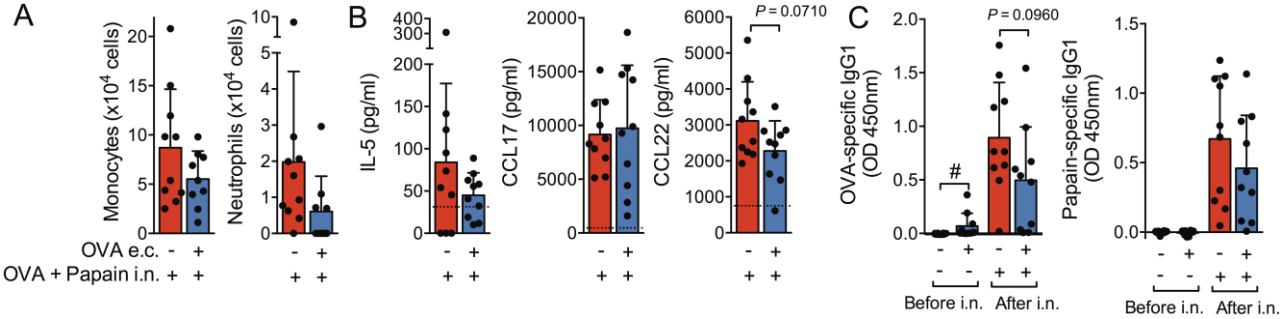
Fig. E1 is supplementary to Fig. 1. **A** Airway inflammation. **B**: BAL fluid IL-5 and Th2-attracting chemokines. **C**: Serum antigen-specific IgG1.

Fig. E2. Responses in mice inhaled with OVA plus papain with or without epicutaneous vaccination with OVA.

Fig. E2 is supplementary to Fig. 2. **A** Airway inflammation. **B**: BAL fluid IL-5 and Th2-attracting chemokines. **C**: Serum antigen-specific IgG1.



Kunimine *et al.* Figure E1



Kunimine *et al.* Figure E2