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IL-33–Mediated Innate Response and Adaptive Immune Cells Contribute to Maximum Responses of Protease Allergen–Induced Allergic Airway Inflammation

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How the innate and adaptive immune systems cooperate in the natural history of allergic diseases has been largely unknown. Plant-derived allergen, papain, and mite allergens, Der f 1 and Der p 1, belong to the same family of cysteine proteases. We examined the role of protease allergens in the induction of Ab production and airway inflammation after repeated intranasal administration without adjuvants and that in basophil/mast cell stimulation in vitro. Papain induced papain-specific IgE/IgG1 and lung eosinophilia. Der f 1 induced Der f 1–specific IgG1 and eosinophilia. Although papain-, Der f 1–, and Der p 1–stimulated basophils expressed allergy-inducing cytokines, including IL-4 in vitro, basophil-depleting Ab and mast cell deficiency did not suppress the papain-induced in vivo responses. Protease inhibitor–treated allergens and a catalytic site mutant did not induce the responses. These results indicate that protease activity is essential to Ab production and eosinophilia in vivo and basophil activation in vitro. IL-33–deficient mice lacked eosinophilia and had reduced papain-specific IgE/IgG1. Coadministration of OVA with papain induced OVA-specific IgE/IgG1, which was reduced in IL-33–deficient mice. We demonstrated IL-33 release, subsequent IL-33–dependent IL-5/IL-13 release, and activation of T1/ST2-expressing lineage[–]CD25⁺CD44⁺ innate lymphoid cells in the lung after papain inhalation, suggesting the contribution of the IL-33–type 2 innate lymphoid cell–IL-5/IL-13 axis to the papain-induced airway eosinophilia. Rag2-deficient mice, which lack adaptive immune cells, showed significant, but less severe, eosinophilia. Collectively, these results suggest cooperation of adaptive immune cells and IL-33–responsive innate cells in protease-dependent allergic airway inflammation. *The Journal of Immunology*, 2013, 190: 000–000.

Protease activity of allergens has been suspected to be an important property that endows an allergen with its allergenicity (1–4). Protease activity of house dust mite (HDM) major group 1 allergens (Der f 1 and Der p 1), two of the most important allergens common worldwide, has been shown to cause

barrier dysfunction, to induce the production of proinflammatory cytokines in epithelial cells and keratinocytes, to cleave various molecules, to modulate functions of various cell types, and to induce Th2 responses (1–11). Papain, a papaya fruit–derived occupational protease allergen, stimulates epithelial cells (12, 13) and mouse basophils in vitro (14, 15) and induces Th2 responses (14–17) and IL-33–dependent innate responses of lung eosinophilia in mice (18). HDM-derived Der f 1 and Der p 1 and plant-derived papain belong to the same family of cysteine proteases, family C1 of clan CA (19, 20).

Recently, the importance of basophils in type 2 immune responses has received much attention (14, 15, 21, 22). In vitro stimulation of human basophils with Der p 1 induces IL-4 expression (23), and Sokol et al. (14) reported that the Th2 differentiation induced by papain in mice is mediated by IL-4 derived from papain-stimulated basophils. However, Tang et al. (17) proposed a reactive oxygen species–mediated model of papain-induced Th2 immune responses, in which basophils act as an amplifier rather than an initiator, and Hammad et al. (24) reported that the true initiator of the HDM-induced Th2 response is not basophils, but inflammatory dendritic cells. Thus, the contribution of basophils to Th2 induction is controversial among different mouse models.

IL-33, an IL-1 family cytokine, contributes to type 2 immune responses (25, 26). In mice, intranasal (i.n.) administration of IL-33 induces asthma-like symptoms in a T/B cell–independent manner (27). Attenuated eosinophil influx in IL-33–deficient (IL-33^{–/–}) mice was shown in the inhalation models of HDM extract and papain (18). Airway eosinophilia induced by short-term i.n. administration (once per day for 3 consecutive days) of high-dose

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchial alveolar lavage; BM, bone marrow–derived; HDM, house dust mite; ILC, innate lymphoid cell; i.n., intranasal/intranasally.

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papain (100 µg/20 µl per animal) occurred in Rag2^{-/-} mice, but not in IL-33^{-/-} mice and IL-4^{-/-}/IL-13^{-/-} mice, suggesting that the papain-induced “innate” allergic airway inflammation is IL-33 dependent and that innate cell derived, but not T cell derived, IL-4 or IL-13 or both are important for the event. However, the contribution of IL-33 and adaptive immune cells to protease allergen-dependent allergic airway inflammation associated with the Ab response, in which the adaptive immune system should be activated, is unknown.

In the current study, we examined the contribution of enzyme activity of protease allergens, basophils, IL-33, and adaptive immune cells to Ab production and lung eosinophilia in an allergic airway inflammation model induced by repeated inhalation of plant- and HDM-derived cysteine protease allergens.

Materials and Methods

Mice

Female 7- to 10-wk-old C57BL/6 mice (Charles River Japan, Yokohama, Japan; or Japan SLC, Shizuoka Japan), IL-33^{-/-} mice (18), and Rag2^{-/-} mice (28) (C57BL/6 background), as well as mast cell-deficient WBB6F1-W/W^v mice and control wild-type WBB6F1-^{+/+} mice (Japan SLC) were maintained in a specific pathogen-free animal facility at Juntendo University (Tokyo, Japan) and used in accordance with the guidelines of the institutional committee on animal experiments.

Ag

Papain was purchased from Calbiochem (San Diego, CA). Recombinant Der p 1 and Der f 1 were prepared as previously described (29, 30), with some modifications. Briefly, proforms of recombinant proteins were secreted into the culture supernatant of yeast *Pichia pastoris* and converted into their prosequence-removed mature forms, Der p 1-N52Q (rDer p 1), Der f 1-N53Q (rDer f 1), Der f 1-WT, and rDer f 1-C35S (11, 29, 30). The mature forms were then purified by anion exchange column and size-exclusion column chromatography. Papain, rDer f 1, and rDer p 1 were treated with an irreversible inhibitor specific to cysteine proteases, E64 (Peptide Institute, Osaka, Japan), as described previously (10), with minor modifications. After the treatment, free E64 was removed by dialysis. Protease allergens, which were incubated similarly to E64-treated allergens (but without the addition of E64) and dialyzed, were prepared and used for in vivo and in vitro experiments for comparison with E64-treated ones. Purity, no degradation, and no aggregation were confirmed by electrophoresis, and protease activity was analyzed using synthetic fluorogenic substrates (10, 31). Chicken OVA (Grade V; Sigma-Aldrich, St. Louis, MO) and that with low endotoxin content (EndoGrade OVA; Hyglos GmbH, Regensburg, Germany) were used in ELISA and i.n. administration, respectively.

i.n. administration of protease allergens to mice

Mice were lightly anesthetized with an i.p. injection of pentobarbital (Somnopenyl; Kyoritsu Pharmaceutical, Tokyo, Japan) and allowed to inhale 40 µl allergens following alternate application to both nares with a pipette. Papain and OVA were each i.n. administered twice per week, and rDer f 1 was i.n. administered three times every 2 wk. At 3 d after the last i.n. administration, sera and bronchial alveolar lavage cells were collected.

Bronchial alveolar lavage

The tracheas of terminally anesthetized mice were cannulated, and the internal airspaces were lavaged with 1000 µl PBS containing 5% FCS. Fluids were centrifuged at 500 g for 10 min, and the pellets were recovered for cellular analysis. Bronchial alveolar lavage (BAL) specimens were prepared on glass slides by Cytospin 4 (Thermo Shandon, Cheshire, UK) followed by Diff-Quick (Sysmex, Kobe, Japan) staining. Differential cell counts were performed with a minimum of 200 cells.

For detecting cytokines in BAL fluid, the internal airspaces were lavaged three times with 500 µl, 250 µl, and 250 µl HBSS. Fluids were centrifuged at 500 g for 10 min, and the cell-free supernatants were subjected to cytokine-specific ELISA (DuoSet; R&D Systems, Minneapolis, MN).

ELISA for serum total IgE and allergen-specific Abs

Serum total IgE and allergen-specific Abs were measured by a sandwich ELISA, as described previously (10, 32–35) with minor modifications. Plates were coated with papain (30 µg/ml for the experiment of Fig. 1 and 5 µg/ml for the other experiments), Der f 1-WT (5 µg/ml), or OVA (1 mg/ml); and blocked with BlockAce (Megmilk Snow Bland, Sapporo, Japan),

in the case of papain, or ImmunoBlock (DS Pharma Biomedical, Osaka, Japan), in the case of Der f 1-WT or OVA. Allergen-specific Abs were detected with HRP-conjugated Abs specific to the mouse IgE and IgG subclasses, as described previously, with some modifications. Sera and detection Abs were diluted with Solutions 1 and 2 of CanGetSignal (TOYOBO, Osaka, Japan), respectively. For detecting allergen-specific IgE, serum dilutions were 1/200 in Figs. 1A, 1C, 2C, and 5D; and 1/50 in Fig. 2A. For detecting allergen-specific IgG1, serum dilutions were 1/5000 in Figs. 1A, 2A, 4D, and 5B; 1/10000 in Figs. 1C and 2C; and 1/2500 in Figs. 5D, 8B, and Supplemental Fig. 1D. For detecting allergen-specific IgE, incubation with serum or a detection Ab was for 15 h at 4°C or for 2 h at room temperature, respectively. For detecting allergen-specific IgG1, incubation with serum or a detection Ab was for 1.5 h at 37°C. In some experiments (Figs. 4D, 5B, 8B, and Supplemental Fig. 1D), papain-specific IgE was measured as follows. For the measurement of papain-specific IgE, plates were coated with 10 µg/ml papain and blocked with ImmunoBlock; then serum samples diluted (serum dilution was 1/40) with PBS containing 0.05% Tween 20 and 1/20 volume ImmunoBlock were applied to each well of the plates. Biotinylated anti-mouse IgE (clone: R35-118; BD Biosciences, San Diego, CA), followed by incubation with HRP-conjugated streptavidin (BD Biosciences), was used for detecting papain-specific IgE.

Generation and stimulation of bone marrow-derived basophils

Mouse bone marrow-derived (BM) basophils were generated as previously described (14). In brief, bone marrow cells prepared from the tibias and femurs of mice were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 30 ng/ml recombinant mouse IL-3 (Peprotech, Rocky Hill, NJ), 2 mM L-glutamine, 10% (v/v) heat-inactivated FCS, 0.05 mM 2-ME, and antibiotics at a density of 5×10^6 cells per milliliter (day 0). Every 3–4 d, cells were replated at a density of 1×10^6 cells per milliliter. On day 14, cells were collected and subjected to magnetic separation for the CD49b⁺ population using anti-CD49b microbeads and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the separated cells was assessed by flow cytometry. A total of 92% of the cells were CD49b⁺FcεRIα⁺ after the magnetic separation. CD49b⁺ cells suspended in fresh medium containing 5% FCS were plated onto 96-well culture plates (3×10^5 cells per 80 µl per well). Finally, 20 µl solution containing protease allergens was added to each of the wells (total, 100 µl per well). To crosslink the IgE receptors, CD49b⁺ cells were sensitized with 10 µg/ml anti-trinitrophenol IgE mAb (clone IgE-3; BD Biosciences) for 1 h, washed, and then suspended in fresh medium containing 5% FCS. Cells were stimulated with 10 µg/ml anti-IgE mAb (clone R35-72; BD Biosciences).

Generation and stimulation of BM mast cells

Bone marrow cells prepared from tibias and femurs of C57BL/6 mice were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.1 mM 2-ME, 0.01 mM MEM-nonessential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 ng/ml mouse IL-3. After 5–8 wk of culture, cells suspended in fresh medium containing 5% FCS and 10 ng/ml mouse IL-3 were plated onto 96-well culture plates (5×10^5 cells per 80 µl per well). Finally, a 20 µl solution containing protease allergens was added to each of the wells.

Quantitative PCR

Total RNA was extracted from cells using an RNeasy Plus Micro Kit (Qiagen, Clifton Hill, Australia). First-strand cDNA was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed with the TaqMan method and an ABI 7500 apparatus (Applied Biosystems). The mRNA levels of the target gene were normalized to the gene expression of GAPDH and are shown as relative expression levels to the control group.

Cytokine ELISA of the culture supernatants of BM basophils and BM mast cells

After stimulation for 24 h, culture supernatants were recovered by centrifugation at 310 g for 5 min. Cytokine concentrations were measured with ELISA kits (Quantikine or DuoSet; R&D Systems).

Flow cytometric analysis of in vivo basophil depletion and lung innate lymphoid cells

Mice were i.p. injected with anti-FcεRIα mAb, and the depletion of basophils was confirmed by flow cytometry (22). The following Abs were used: anti-mouse FcεRIα mAb (clone MAR-1), Armenian hamster IgG (eBioscience, San Diego, CA), PE-conjugated anti-mouse CD49b (clone HMα2), PE-conjugated anti-mouse CD200R3 (clone Ba160) (BioLegend,

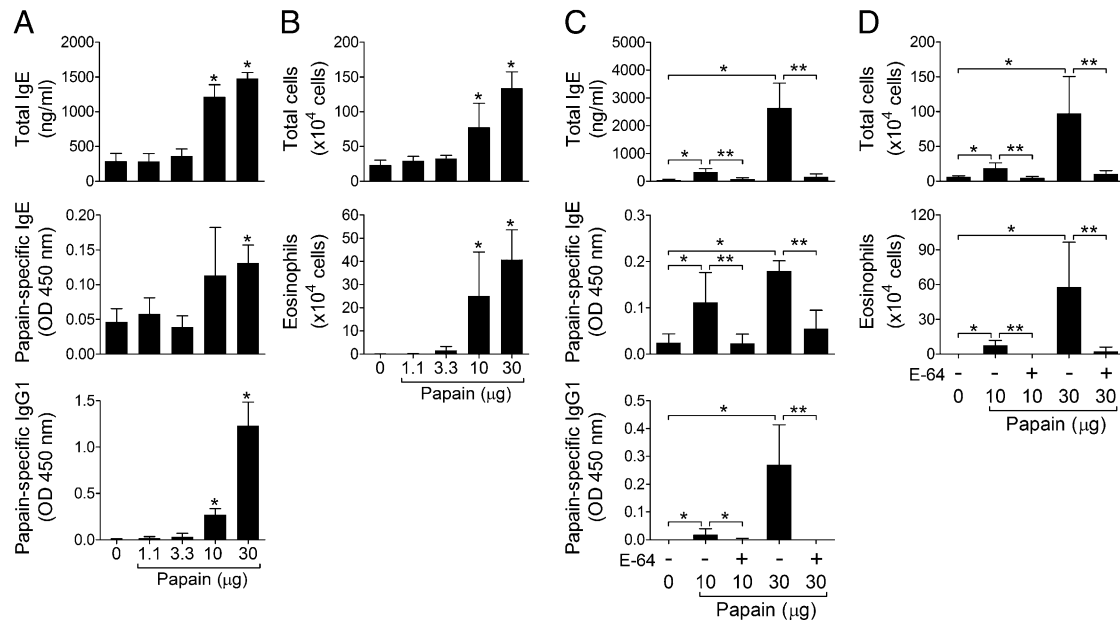


FIGURE 1. Protease-dependent induction of Ab production and eosinophilic airway inflammation by inhalation of papain. C57BL/6 mice were i.n. administered papain or E64-treated papain (1.1–30 μ g/animal, days 0 and 7). BAL fluid and serum were collected 3 d after the last administration. Dose-dependent (**A**, **B**) and protease-dependent (**C**, **D**) induction of serum Ab production (**A**, **C**) and airway inflammation (**B**, **D**). Data are indicated as mean $\pm$ SD of four or five mice per group. Data are representative of three independent experiments with similar results. * p < 0.05, ** p < 0.01 versus vehicle (Papain 0) by Mann-Whitney U test.

San Diego, CA), PE-Cy7-conjugated anti-mouse CD3 (clone 145-2C11), PE-Cy7-conjugated anti-mouse B220 (clone RA3-6B2), and anti-mouse CD16/CD32 (clone 2.4G2) mAbs (BD Pharmingen, San Diego, CA), mouse IgE (clone SPE-7) (Sigma-Aldrich), FITC-conjugated anti-mouse IgE (clone: 23G3) mAb (Southern Biotech, Birmingham, AL), and allophycocyanin-conjugated anti-mouse CD49b (clone DX5) mAb (BioLegend). C57BL/6 mice were treated i.p. with 5 μ g MAR-1 or control Armenian hamster IgG twice per day for 3 consecutive days (22). At 1 or 5 d after the last i.p. injection, a single-cell suspension of splenocytes was prepared and stained with PE- or allophycocyanin-conjugated anti-CD49b, PE-Cy7-conjugated anti-CD3, PE-Cy7-conjugated anti-B220, IgE and

FITC-conjugated anti-IgE, or PE-conjugated anti-CD200R3 after blocking the Fc receptors with anti-CD16/CD32. Depletion of basophils was assessed by detection of the CD3⁺B220⁺CD49b⁺IgE⁺ or the CD3⁺B220⁺CD49b⁺CD200R3⁺ cell population.

Lineage⁺CD25⁺CD44⁺ innate lymphoid cells (ILCs) in the lung were analyzed as follows. At 3 or 5 h after the last i.n. administration of PBS or papain, single-cell suspensions were prepared from the lungs digested with collagenase type 4 (1.6 mg/ml; Worthington Biochemical, Lakewood, NJ) at 37°C for 1 h with gentle shaking. The lung cells were stained and analyzed using the following Abs: PE-Cy7-conjugated anti-mouse lineage markers [B220 (clone RA3-6B2), CD3 (clone 145-2C11), CD11b (clone

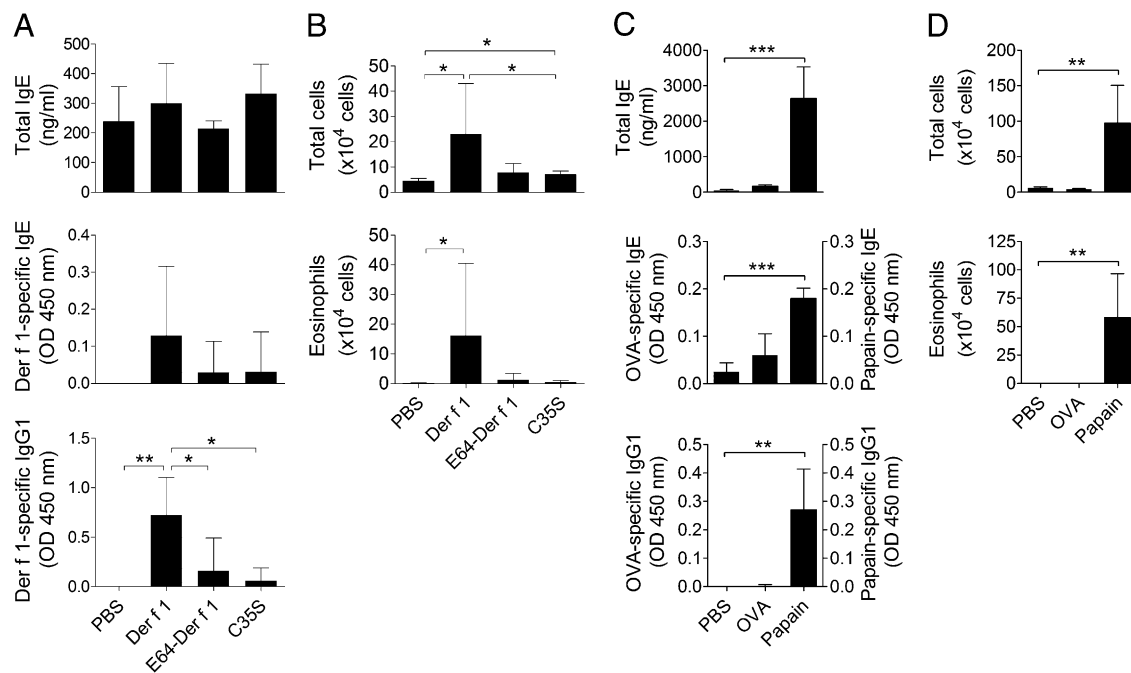


FIGURE 2. Protease-dependent induction of Ab production and eosinophilic airway inflammation by inhalation of mite major protease allergen Der f 1 and no responses to OVA. C57BL/6 mice were i.n. administered rDer f 1, E64-treated rDer f 1, or rDer f 1-C35S (days 0, 7, and 14) in (**A**) and (**B**) and with OVA or papain (days 0 and 7) in (**C**) and (**D**) (30 μ g per animal). BAL fluid and serum were collected 3 d after the last administration. (**A** and **C**) Serum Ab levels. (**B** and **D**) Airway inflammation. Data are indicated as mean $\pm$ SD of four or five mice per group. Data are representative of three independent experiments with similar results. * p < 0.05, ** p < 0.01, *** p < 0.001 versus PBS by Mann-Whitney U test (**A**, **B**) and by one-way ANOVA with the Tukey post hoc test (**C**, **D**).

M1/70), CD16/CD32 (clone 2.4G2) (BD Pharmingen), and CD14 (clone Sa2-8) (eBioscience), allophycocyanin-conjugated anti-mouse CD25 (clone PC61), FITC-conjugated anti-mouse/human CD44 (clone IM7) (BioLegend), PE-conjugated anti-mouse c-Kit (clone 2B8), PE-conjugated anti-mouse CD127 (clone A7R34) (eBioscience), PE-conjugated anti-mouse CD45 (clone 30-F11) (BioLegend), PE-conjugated anti-mouse Sca-1 (clone E13-161.7), PE-conjugated anti-mouse Thy1.2 (clone 30-H12) (BD Pharmingen), biotinylated anti-mouse T1/ST2 (clone DJ8) (MD Bioproducts, Zürich, Switzerland), and streptavidin-PE (BD Pharmingen). Acquisition and analyses were performed using a FACSCalibur cell sorter and Cell Quest software (BD Biosciences).

Statistical analysis

One-way ANOVA with the Tukey post hoc test was used to analyze the data from *in vitro* experiments. One-way ANOVA with the Tukey post hoc test, unpaired *t* test, and Mann-Whitney *U* test (two-tailed) were used to analyze data from *in vivo* experiments. A *p* < 0.05 was regarded as statistically significant.

Results

Intranasally administered papain and an HDM major protease allergen induced Ab production and lung eosinophilia in a cysteine protease-dependent manner

Differentiation of Th2 cells in mice immunized s.c. with a plant-derived protease allergen, papain, with no adjuvants has been

reported (14); however, the mechanism by which protease allergen inhalation leads to allergic diseases is not well investigated. We analyzed mouse allergic airway inflammation induced by repeated i.n. administration of protease allergens without using adjuvants such as Alum. In preliminary experiments (S. Kamijo, T. Tokura, and T. Takai, unpublished observations), even a single i.n. administration of papain at a higher dose (100 µg/40 µl per animal) resulted in hemorrhage in BAL fluid collected 1 d after papain administration. These results indicate that the protease activity of high-dose papain may have caused tissue injury; therefore, we selected lower doses (≤30 µg/40 µl per animal) for our experiments.

When i.n. administered twice, with a 7 d-interval, to wild-type C57BL/6 mice (days 0 and 7), papain at the doses of 30 µg and 10 µg/40 µl per animal increased the serum level of total IgE and induced papain-specific IgE and IgG1 (Fig. 1A), along with infiltration of inflammatory cells mainly consisting of eosinophils into the lung (Fig. 1B) at day 10. Inhalation of papain treated with an irreversible cysteine protease inhibitor E64 did not lead to production of IgE and IgG1 or eosinophil infiltration (Fig. 1C, 1D). When i.n. administered three times at 7-d intervals (days 0, 7, and 14), a recombinant form of HDM major protease allergen, Der f 1, induced Der f 1-specific IgG1 (Fig. 2A) and lung eosinophilia (Fig. 2B) at day 17. E64-treated rDer f 1 and a protease activity–

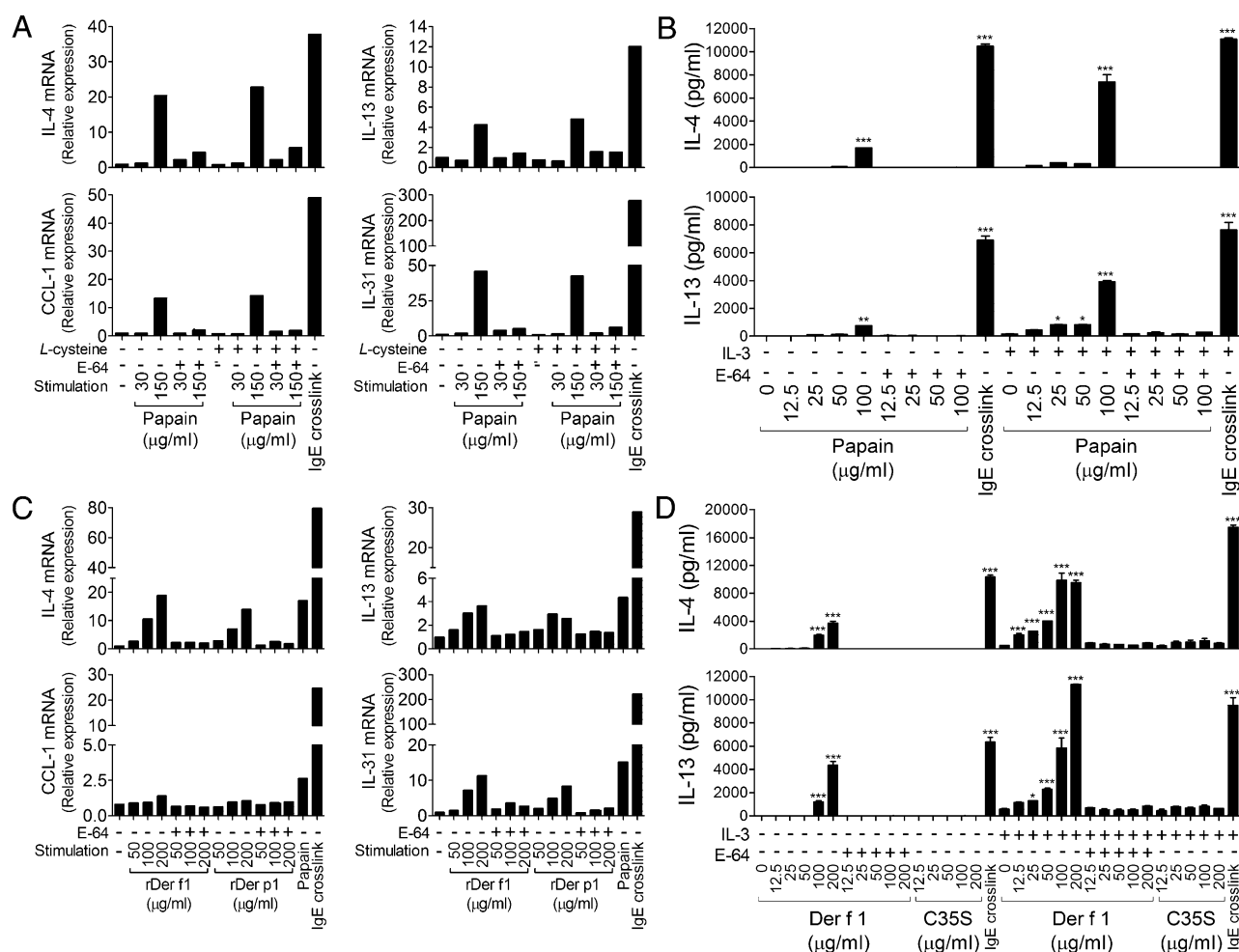


FIGURE 3. Protease allergen-induced cytokine expression in BM basophils. C57BL/6BM basophils were stimulated with papain (**A, B**), rDer p 1, rDer f 1, and rDer f 1-C35S (**C, D**), with or without treatment with E64 in the presence [(A, C) and (B, D), IL-3⁺] or absence of IL-3 (10 ng/ml) [(B, D), IL-3⁻]. Stimulation with crosslinking of the FcεRI with IgE and anti-IgE Ab was performed as a positive control (IgE crosslink). After 2 h, mRNA expression was measured by quantitative real-time PCR (A, C). After 24 h, culture supernatant was collected and subjected to ELISA (B, D). The final concentration of L-cysteine was 1 mM. The concentration of papain in (C) was 150 µg/ml. Data are indicated as mean ± SD. Data are representative of two [(A, C), IL-13 and CCL-1] or three independent experiments with similar results. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus vehicle by one-way ANOVA with the Tukey post hoc test.

deficient mutant (rDer f 1-C35S) (Fig. 2A, 2B), and OVA (Fig. 2C, 2D), which have no enzyme activity, failed to induce the responses. These results indicate that repeated i.n. administration of plant-derived and HDM-derived protease allergens in mice can induce allergic airway inflammation associated with eosinophilia and induction of IgE and/or IgG1, the class-switch to which Th2 cytokines contribute to, and that cysteine protease activity of the allergens is essential for this process.

Cysteine protease activity of papain and HDM major protease allergens triggered expression of allergy-inducing cytokines in BM basophils in vitro in a cysteine protease-dependent manner

Some reports described that basophils play a pivotal role in Th2 immunity via IL-4 production (14, 22). As papain and rDer f 1 showed their capacity to induce IgE and/or IgG1 production and allergic airway inflammation (Figs. 1, 2A, 2B), we examined the expression of IL-4 and other allergy-inducing cytokines in mouse BM basophils in response to papain, rDer f 1, and rDer p 1 (Fig. 3).

Stimulation of BM basophils with papain induced mRNA expression of Th2 cytokines (IL-4 and IL-13); CCL-1, which is a chemoattractant for eosinophils (36, 37); and IL-31, a cytokine related to the itch-and-scratch behavior (38) (Fig. 3A), with or without L-cysteine, which can assist in the regeneration of cysteine protease activity by reducing the oxidized SH group at the catalytic site. Stimulation with rDer f 1 and rDer p 1 induced mRNA expression of IL-4, IL-13, and IL-31 (Fig. 3C). Stimulation with papain, rDer f 1, and IgE crosslink induced the secretion

of extremely high levels of IL-4 and IL-13 (Fig. 3B, 3D). The E64-treated protease allergens (Fig. 3) and rDer f 1-C35S (Fig. 3D) exhibited no response. In contrast, papain, rDer f 1, and rDer p 1 did not induce mRNA expression of IL-4 and IL-31 in BM mast cells (Supplemental Fig. 1A, 1B). Thus, the cysteine protease activity of rDer f 1 and rDer p 1 can induce the production of allergy-related cytokines, including IL-4, by BM basophils, but not BM mast cells, in a manner similar to papain in vitro.

Basophil-depleting mAb did not reduce Ab production and lung eosinophilia in the protease allergen-induced allergic airway inflammation model

We examined the contribution of basophils to the pathogenesis of allergic airway inflammation induced by repeated airway exposure to papain. Basophil-depleted mice were prepared by injection of a mAb specific for the α subunit of the Fc ϵ RI, Fc ϵ RI α , (clone MAR-1) before each of the two i.n. injections of papain. The deletion of basophils was confirmed by flow cytometry of spleen cells. The CD49b⁺IgE⁺ basophil population was absent in the spleens of the MAR-1-treated mice 1 d prior to the first papain administration (day -1) (Fig. 4A, MAR-1) and 3 d after the second papain administration, which is the day when BAL was performed (day 10) (Fig. 4B, MAR-1). However, when detected as CD49b⁺CD200R3⁺ cells, a small population of basophils (0.11% on day 0, 0.07% on day 10) still remained even after MAR-1 administration (Fig. 4C).

The production of total IgE and papain-specific IgE/IgG1 (Fig. 4D) and the numbers of total infiltrating cells and eosinophils (Fig.

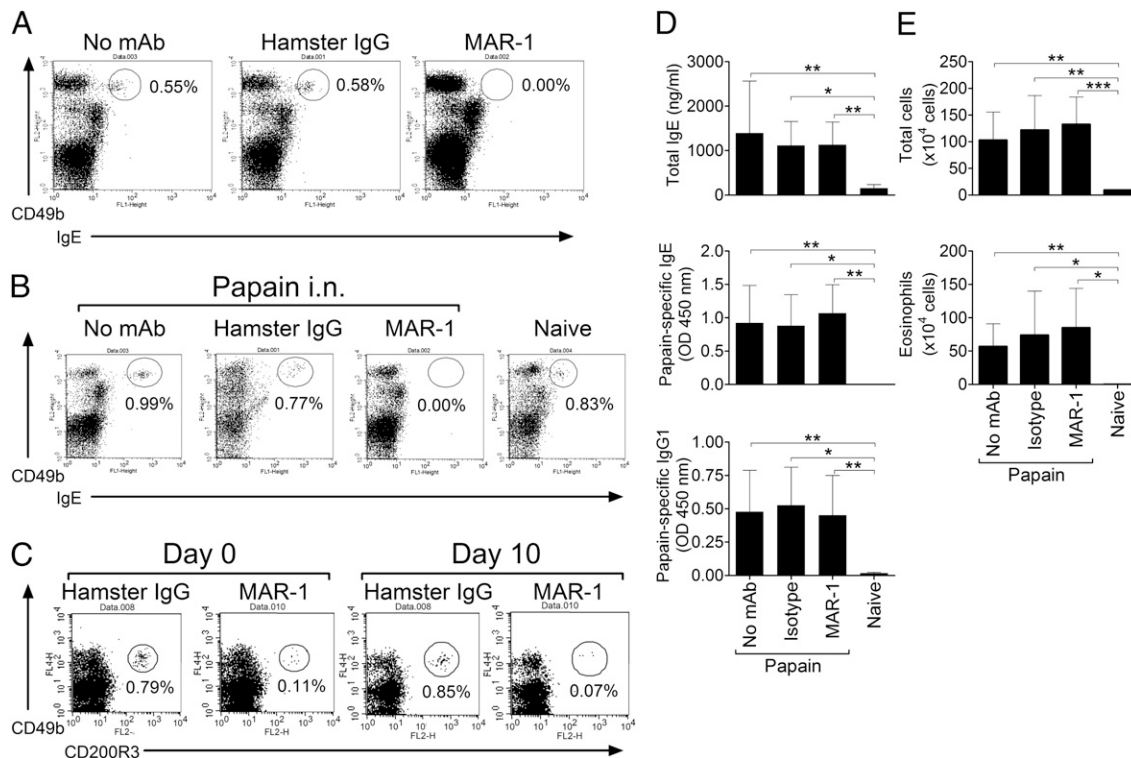


FIGURE 4. Papain-induced eosinophilic airway inflammation and Ab production in mice administered basophil-depleting mAb. C57BL/6 mice were i.p. administered MAR-1 or Armenian hamster IgG twice per day for 3 consecutive days (first week: days -4, -3, and -2; and second week: days 3, 4, and 5). Two days after the last injection in each of the weeks, the mice were treated i.n. with papain (30 μ g per animal, days 0 and 7). Single-cell suspensions of splenocytes prepared on day -1 (A), day 0 (C), and day 10 (B, C) were stained with PE-labeled anti-CD49b and IgE plus FITC-labeled anti-IgE (A, B) or with allophycocyanin-labeled anti-CD49b and PE-labeled anti-CD200R3 (C). Basophils were detected as the CD49b⁺IgE⁺CD3⁻B220⁻ cell population [(A, B), circled] or the CD49b⁺CD200R3⁺CD3⁻B220⁻ cell population [(C), circled]. BAL fluid and serum were collected 3 d after the second papain administration. (D) Serum Ab levels. * p < 0.05, ** p < 0.01 by Mann-Whitney U test. (E) Airway inflammation. Data are presented as mean $\pm$ SD of six mice per group. Data are representative of two (A–C) or three (D, E) independent experiments with similar results. * p < 0.05, ** p < 0.01, *** p < 0.001 by unpaired t test.

4E) in MAR-1-treated mice were equal to those of control hamster IgG-treated mice. The papain-induced Ab production and lung eosinophilic inflammation were equivalent between mast cell-deficient mice (WBB6F1-W/W^Y) and wild-type mice (WBB6F1-^{+/+}) (Supplemental Fig. 1C, 1D). Thus, ~90% reduction of basophils by MAR-1 administration does not affect the Ab production and lung eosinophilia induced by repeated i.n. administration of papain, and mast cells are dispensable for the model.

IL-33 was essential for lung eosinophilia and contributed to Ab production in the protease allergen-induced allergic airway inflammation model

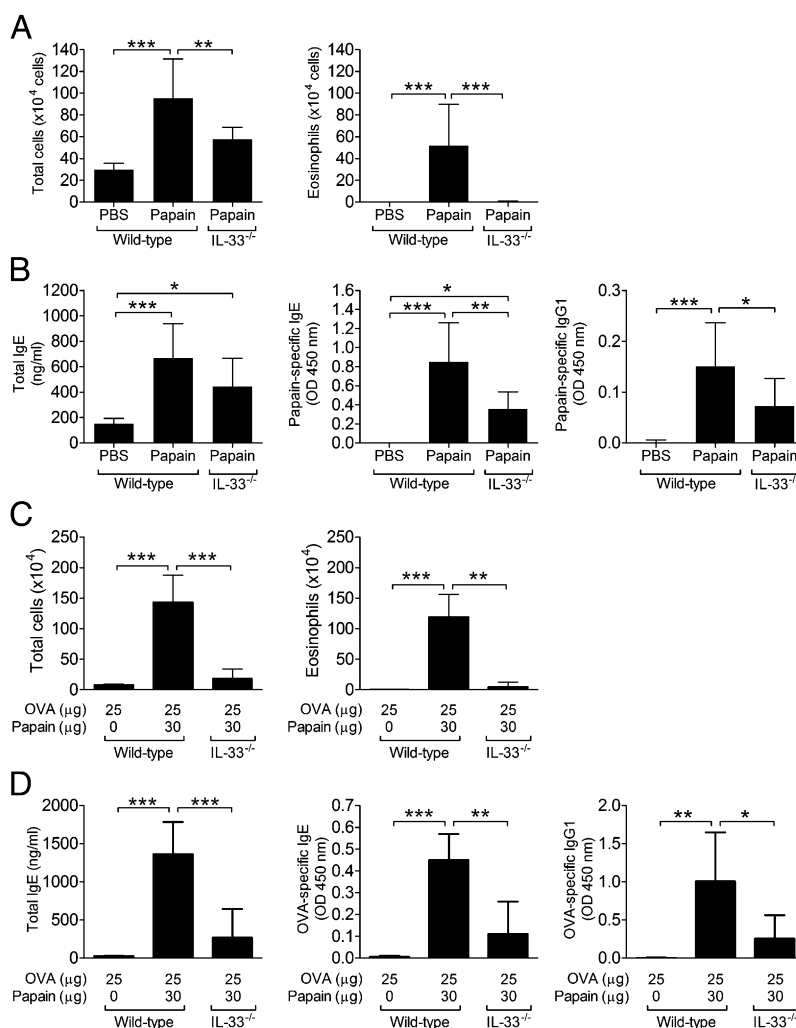
To examine the contribution of IL-33 to the pathogenesis of allergic airway inflammation associated with the Ab response induced by repeated exposure to papain, we compared the responses between wild-type mice and IL-33^{-/-} mice (Fig. 5). In papain-treated IL-33^{-/-} mice, eosinophil infiltration was almost absent (Fig. 5A), and the serum level of papain-specific IgE and IgG1 was less than that in papain-treated wild-type mice (Fig. 5B). Thus, IL-33 is essential for eosinophil infiltration and contributes to Ab production in this model of allergic airway inflammation. Co-administration of OVA with papain also induced eosinophilic lung inflammation in a manner dependent on IL-33 (Fig. 5C). Papain showed adjuvant effect in inducing OVA-specific IgE and IgG1 in wild-type mice, and the level of upregulation of OVA-specific IgE/IgG1 and total IgE was less in IL-33^{-/-} mice (Fig. 5D).

Lung IL-33 induced by cysteine protease activity of papain was essential for release of IL-5 and IL-13 in the lung

We examined cytokine release into the BAL fluid after the first (Fig. 6A) or second (Fig. 6B) i.n. papain administration (30 µg per animal). IL-33 release was observed 1 h after the first administration and peaked at 3 h, then subsided within 24 h (Fig. 6A, IL-33). Induction of lung IL-5 and IL-13 followed the release of IL-33 (Fig. 6A, IL-5 and IL-13). Whereas IL-5 persisted for 24 h, IL-13 rapidly subsided after peaking at 3 h (Fig. 6A). The time course and concentration of IL-33 after the second i.n. papain administration were similar to those observed after the first papain administration (Fig. 6B, IL-33). However, much greater amounts of IL-5 and IL-13 were released at a later time point (24 h) after the second administration (Fig. 6B, IL-5 and IL-13). The induction of IL-5 and IL-13 after the second papain administration showed two peaks; the earlier peak was at 3–5 h, and the later peak was at 24 h. Next, we examined the dose-response relationship of papain-induced release of IL-33 in the lung. A higher dose (100 µg per animal) of papain induced levels of IL-33 release similar to those induced by 33 µg per animal of papain (Fig. 6C). Papain at a dose of 11 µg per animal was not enough to induce IL-33 release that would be detectable in the BAL fluid. Pretreatment of papain with E64 abrogated the papain-induced cytokine release in the lung, and papain did not induce IL-5 and IL-13 in IL-33^{-/-} mice (Fig. 6D).

To assess the possible proteolysis of IL-5, IL-13, and IL-33 in the lung caused by inhaled papain, we treated recombinant IL-5, IL-13,

FIGURE 5. Papain-induced eosinophilic airway inflammation and Ab production in IL-33^{-/-} mice. Papain (30 µg per animal) and/or OVA (25 µg per animal) were i.n. administered to IL-33^{-/-} or wild-type C57BL/6 mice (days 0 and 7). BAL fluid and serum were collected 3 d (A, B) or 4 d (C, D) after the last administration. (A and C) Airway inflammation. (B and D) Serum Ab levels. Data are presented as mean ± SD of 10 [(A, B), wild-type], 7 [(A, B), IL-33^{-/-}], or 5 (C, D) mice per group. Data are representative of three (A, B) or two (C, D) independent experiments with similar results. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by one-way ANOVA with the Tukey post hoc test.



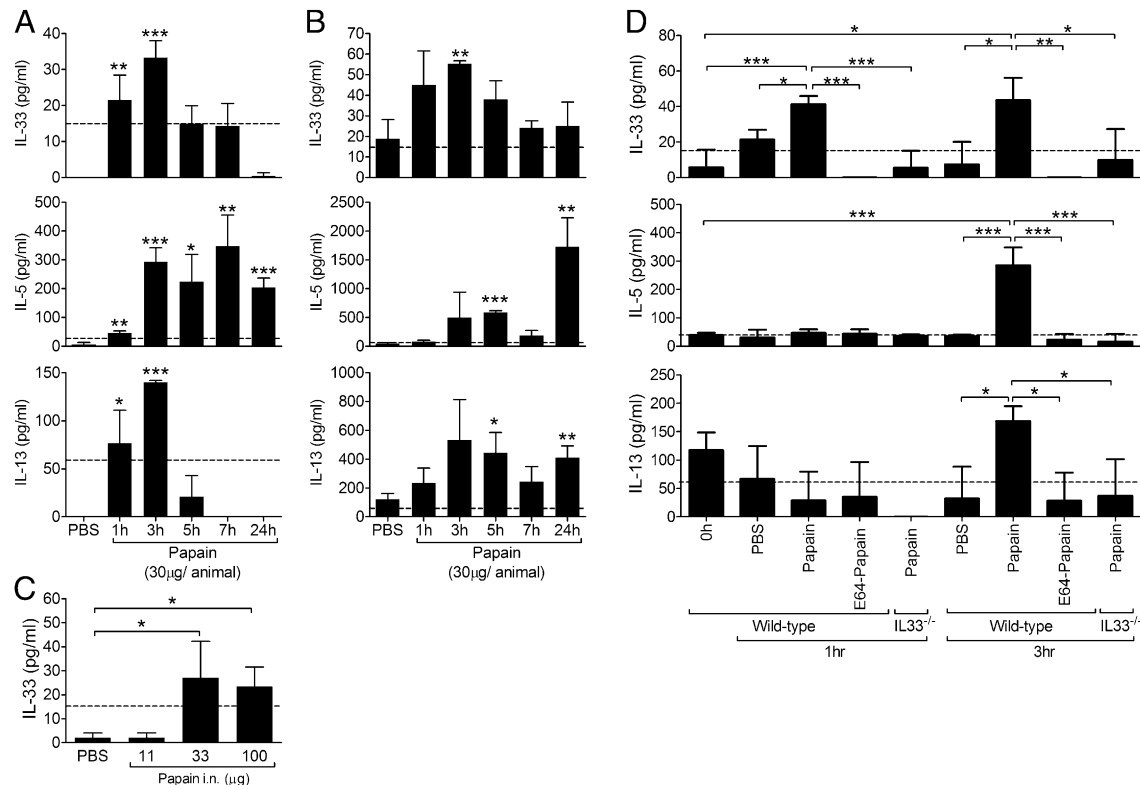


FIGURE 6. Release of IL-33, IL-5, and IL-13 in the lung of papain-treated mice. Papain or E64-treated papain [(A), (B), (D): 30 µg per animal; (C): 11 µg, 33 µg, or 100 µg per animal] was i.n. administered to wild-type C57BL/6 (A–D) or IL-33^{-/-} (D) mice once (A, C, D) or twice at a 7-d interval (B). BAL fluid was collected 1–24 h (A, B), 3 h (C), or 1 or 3 h (D) after the last papain administration, and concentrations of IL-33, IL-5, and IL-13 in the BAL fluid were measured by ELISA. (A) Time course after the single administration. (B) Time course after the second administration. (C) Dose–response after the single administration. (D) Cysteine protease–dependent and IL-33–dependent release of cytokines in the lungs of mice received the single administration. Detection limits are indicated by dotted lines (IL-33: 15.6 pg/ml; IL-5: 31.3 pg/ml; IL-13: 62.5 pg/ml). Data are presented as mean ± SD of three mice per group. Data are representative of three (A, D) or two (B, C) independent experiments with similar results. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by unpaired *t* test (A–C) or by one-way ANOVA with the Tukey post hoc test (D).

and IL-33 with papain at a high concentration (0.75 mg/ml = 30 µg/40 µl) in vitro (Supplemental Fig. 2). IL-13 and IL-33 were undetectable by ELISA within 1 h after incubation with papain and IL-5 was less susceptible to papain than IL-13 and IL-33, suggesting that the concentrations of these cytokines in the lung are underestimated owing to the proteolysis by papain even if they were initially released in higher concentrations.

These results indicate that inhalation of papain results in release of IL-33 in the lung in a manner dependent on protease activity of papain and suggest that release of IL-5 and IL-13 in response to the papain-induced IL-33 release causes the lung eosinophilia.

Papain induced activation of T1/ST2-expressing lineage⁻CD25⁺CD44⁺ ILCs in the lung

To identify the IL-33–responsive cells in the lung, we analyzed lineage⁻CD25⁺CD44⁺ ILCs, which have been reported to be present in the lung of naive as well as fungal extract–exposed mice (39). The lineage⁻CD25⁺CD44⁺ lymphoid cell population was detected in the lung of PBS- or papain-treated mice (Fig. 7A). Further analyses of cell surface marker expression revealed that the lineage⁻CD25⁺CD44⁺ lymphoid cells are positive for T1/ST2 (a subunit of IL-33 receptor), Sca-1, and Thy1.2 (Fig. 7B); all of these molecules have been reported to be expressed on type 2 ILCs (39). The lineage⁻CD25⁺CD44⁺ ILCs are also positive for CD45 and CD127 (a subunit of IL-7 receptor), which is consistent with the surface marker profile of natural helper cells reported by Moro et al. (40). The c-Kit expression on this cell population was low but positive (Fig. 7B). The T1/ST2 expression suggests that

this cell population can be activated by the papain-induced lung IL-33 (Fig. 6, IL-33). We examined the expression levels of an activation marker, CD25 (39, 40), on CD25⁺CD44⁺ ILCs in the lungs of PBS- or papain-exposed mice. The papain exposure enhanced the mean fluorescence intensity of CD25 at 3–5 h (Fig. 7C).

Adaptive immune system contributed to amplification of the magnitude of lung eosinophilia in the protease allergen–induced allergic airway inflammation model

Significant cellular infiltration mainly consisting of eosinophils occurred in papain-treated Rag2^{-/-} mice; however, the numbers of total cells and eosinophils were significantly lower in Rag2^{-/-} mice than in wild-type mice (Fig. 8A). Whereas the eosinophil infiltration was partially dependent on adaptive immune cells, the induction of serum IgE/IgG1 was absent in Rag2^{-/-} mice owing to the lack of Rag2 recombinase (Fig. 8B). The results indicate that repeated inhalation of papain can induce eosinophil infiltration even in the absence of adaptive immune cells, but that adaptive immune cells and/or allergen-specific Abs amplify the magnitude of the papain-induced lung eosinophilia.

Discussion

Exposure to allergen sources such as HDMs and their feces, insects, pollen grains, fungal spores, animal dander, and enzymes used in the food industry (such as papain) triggers a Th2-skewed immune response toward allergic diseases, which are associated with Ab production and eosinophilic inflammation. Allergen source–derived proteases, some of which are allergens (protease allergens),

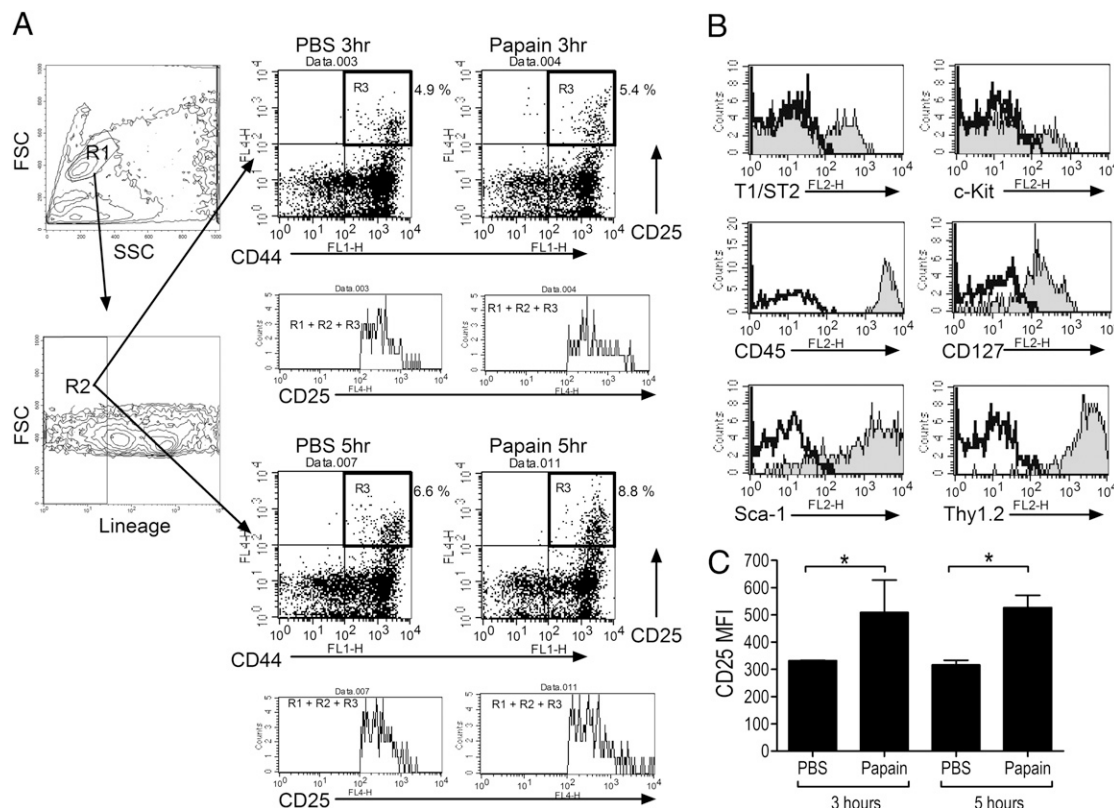


FIGURE 7. Papain-induced activation of lineage⁺CD25⁺CD44⁺ ILCs in the lung. PBS or papain (30 μ g per animal) was i.n. administered to C57BL/6 mice. Single-cell suspensions were prepared from the lungs at the time indicated. Cells were stained with PE-Cy7-labeled antilinerage markers (B220, CD3, CD11b, CD14, CD16, and CD32), allophycocyanin-labeled anti-CD25, FITC-labeled anti-CD44, PE-labeled anti-c-Kit, anti-CD45, anti-CD127, anti-Sca-1, and anti-Thy1.2, and biotinylated anti-T1/ST2 plus streptavidin-PE. **(A)** Detection of lineage⁺CD25⁺CD44⁺ ILCs in the lungs of mice administered PBS or papain. R1: Lymphocytes. R2: Lineage⁺ cells. R3: CD25⁺CD44⁺ cells. **(B)** Surface expression of T1/ST2, c-Kit, CD45, CD127, Sca-1, and Thy1.2 (gray histograms) on the lineage⁺CD25⁺CD44⁺ lymphoid cell population. Open histograms, isotype control mAbs. **(C)** Mean fluorescence intensity of CD25 on the lineage⁺CD25⁺CD44⁺ lymphoid cells in the lungs of mice administered PBS or papain. Data are presented as mean $\pm$ SD of three mice per group. Data are representative of three (A, C) or two (B) independent experiments with similar results. * p < 0.05 by one-way ANOVA with the Tukey post hoc test (C).

are able to cause breakdown of the epithelial barrier and induce innate immune responses in various cell types (1–4). By the use of E64 to inactivate the protease activity, Ab production has been shown to depend on the protease activity of the HDM group 1 major allergen Der p 1 partially (9) or critically (10) when the allergen was i.p. administered with Alum to mice. Partial reduction (14, 16) or elimination (14) of Ab production has been reported in E64-treated or heat-denatured papain, respectively, when s.c. administered with no adjuvants. In the current study, we established a mouse allergic airway inflammation model associated with IgE/IgG1 Ab production and lung eosinophilia, which were induced by repeated airway exposure of naive mice to protease allergens without using the Th2-skewing adjuvants such as Alum (Figs. 1, 2), and analyzed mechanisms of the responses. The protease activity was essential to Ab production and lung eosinophilia in vivo (Figs. 1, 2) and BM basophil activation in vitro (Fig. 3), but administration of basophil-depleting Ab or mast cell deficiency did not suppress the in vivo responses in the model (Fig. 4, Supplemental Fig. 1C, 1D). Results from IL-33^{-/-} mice (Fig. 5) and Rag2^{-/-} mice (Fig. 8) suggest that adaptive immune cells and IL-33-responsive innate cells cooperate in protease allergen-dependent allergic airway inflammation. Papain showed IL-33-dependent adjuvant effect in inducing IgE/IgG1 specific to coadministered OVA (Fig. 5). Cysteine protease activity of papain induced release of IL-33 into BAL fluid of and subsequent IL-33-dependent release of IL-5 and IL-13 (Fig. 6). Lineage⁺CD25⁺CD44⁺ ILCs (also positive for T1/ST2, c-Kit, CD45, CD127, Sca-1,

and Thy1.2) were detected in the lung of naive and papain-treated mice, and inhalation of papain resulted in enhanced expression of an activation marker, CD25 (39, 40), in the ILCs (Fig. 7).

We demonstrated that the protease activity of the inhaled allergens crucially contributes to serum Ab production and airway inflammation (Figs. 1C, 1D, 2A, 2B). We used a cysteine protease inhibitor, E64, which irreversibly binds to the catalytic site of papain, rDer f 1, or rDer p 1 without altering their tertiary structure (10, 41). The global structure of the enzymatically inactive mutant, rDer f 1-C35S, is identical to that of rDer f 1 (T. Takai and T. Kato, unpublished observations). Inactivation of protease activity by heat denaturation disrupts the tertiary structure or causes oligomerization, or both, which are likely to affect the immunochemical properties of the allergens such as Ab binding (42, 43) and T cell response (10). Our results using E64-treated protease allergens and rDer f 1-C35S (Figs. 1C, 1D, 2A, 2B) indicate that the lung eosinophilia and elevated serum Ab production are dependent on the cysteine protease activity of these allergens, but not to the conformational change or aggregation, which may cause different immune responses. OVA with no protease activity, which has been frequently used as a model allergen in combination with Alum for Th2-skewed immunization of mice, did not induce the in vivo responses in airway exposure without the use of adjuvants (Figs. 2C, 2D, 5C, 5D).

How the innate and adaptive types of immunity cooperate in the natural history of allergic diseases has been largely unknown. Our protocol of using repeated exposure to papain (30 μ g/40 μ l per

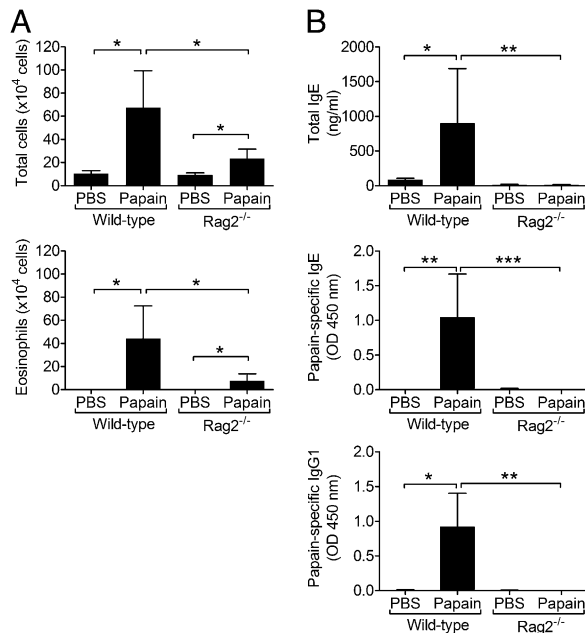


FIGURE 8. Papain-induced eosinophilic airway inflammation in adaptive immunity-deficient Rag2^{-/-} mice. Papain was i.n. administered to Rag2^{-/-} or wild-type C57BL/6 mice (30 μ g/40 μ l per animal, days 0 and 7). BAL fluid and serum were collected 3 d after the last administration. **(A)** Airway inflammation. * p < 0.05 by unpaired t test. **(B)** Serum Ab levels. Data are indicated as mean $\pm$ SD of four or five mice per group. Data are representative of four independent experiments with similar results. * p < 0.05, ** p < 0.01, *** p < 0.001 by Mann–Whitney U test.

animal at days 0 and 7; and BAL at day 10) or highly purified recombinant HDM major protease allergen (30 μ g/40 μ l per animal at days 0, 7, and 14; and BAL at day 17) induced lung eosinophilia and Ab production (Figs. 1, 2). Lung eosinophilia was absent in IL-33^{-/-} mice (Fig. 5AC) and was less severe in Rag2^{-/-} mice (Fig. 8A), and a single i.n. administration (30 μ g/40 μ l per animal at day 0 and BAL at day 3) was not sufficient to induce significant eosinophilia in wild-type mice (S. Kamijo, T. Tokura, and T. Takai, unpublished observations). Oboki et al. (18) reported that short-term i.n. administration of a higher dose of papain (100 μ g/20 μ l per animal at days 0, 1, and 2; and BAL at day 3) induced airway infiltration of eosinophils in Rag2^{-/-} mice with numbers almost equivalent to (or even higher than) those in wild-type mice, but not in IL-33^{-/-} mice, demonstrating an IL-33-dependent papain-induced “innate” allergic airway inflammation. Taken together, our results indicate that, whereas the IL-33-mediated innate immune response contributes to the initial response to the first airway exposure to protease allergens, the adaptive immune system plays an important role as an amplifier of allergic airway inflammation in the repeated exposure to protease allergens.

In our mouse model of protease allergen-dependent allergic airway inflammation, IL-33 is crucial for lung eosinophilia and contributes to Ab production (Fig. 5). Various types of cells have been reported to be target cells of IL-33 (25, 26, 44). Recently, newly identified Th2 cytokine-producing ILC populations have emerged as important players in the protection against helminth infection and the pathogenesis of allergic diseases (39, 40, 44–46). Common features of these type 2 ILCs are their capacity to produce extremely high amounts of Th2 cytokines IL-5 and IL-13 and their ability to expand in response to IL-33 and/or IL-25. Very recently, Halim et al. (47) reported that ILCs are a critical source of IL-5 and IL-13 in innate allergic airway inflammation induced by short-term (days 0, 1, and 2) administration of papain. IL-5 and

IL-13 regulate eosinophils (48). We demonstrated IL-33 release in the lung 1–3 h after the single exposure to papain, but not to E64-treated papain and subsequent, IL-33-dependent IL-5/IL-13 release (Fig. 6A, 6D) and papain-dependent activation of lung ILCs (39) (Fig. 7C), which showed the expression profile of cell surface markers similar to natural helper cells originally identified in the peritoneal fat-associated lymphoid cluster (40) (Fig. 7A, 7B).

Of interest, the lung IL-5/IL-13 release after the second papain exposure showed two peaks at 5 h and 24 h after exposure (Fig. 6B), and concentrations of IL-5 and IL-13 at 24 h (Fig. 6B) were higher than those after the single papain exposure (Fig. 6A). Possible sources of IL-5 and IL-13 at the early (within 5 h) and late (after 24 h) time points are IL-33-activated ILCs and Th2 cells, respectively. Another possibility is that adaptive immune cell-derived factors might enhance the release of IL-5/IL-13 from ILCs upon the second papain exposure. These hypotheses can provide explanations for the significant requirement of the adaptive immune system for the papain-induced lung eosinophilia in our mouse model (Fig. 8A). In a study of papain-induced lung inflammation by Wilhelm et al. (49), IL-9 production was largely restricted to ILCs; i.n. administration of IL-33 increased the number of ILCs in the lung; IL-9 production depended on IL-2 and the adaptive immune system; and IL-9 promoted the expression of IL-5, IL-6, and IL-13 in ILCs themselves. Their results indicated the indirect dependence of the Th2 cytokine production by ILCs on IL-2 and adaptive immune cells. Upon the second exposure to papain, the IL-33-activated ILCs and allergen-specific Th2 cells may synergistically contribute to the maximum responses of the protease allergen-induced allergic airway inflammation (Fig. 8) via increased production of IL-5 and IL-13.

The protease activity of inhaled protease allergens crucially contributes to the Ag-specific Ab production (Figs. 1C, 2A). Inhaled papain induced IgE/IgG1 production specific to coadministered OVA (Fig. 5D) and architecture of papain itself (Fig. 5B) in an IL-33-dependent manner. The results suggest that protease activity present in allergen sources, such as HDMs, cockroaches, pollen, and fungi (1), contributes to induction of IgE specific to nonprotease allergens coexisting in the allergen sources. IL-33-responsive cells responsible for the adjuvant effect of protease allergens are yet to be identified.

Several recent reports have highlighted the importance of basophils in the induction and promotion of the Th2 response (14, 15, 17, 21, 22). Robust protein secretion of IL-4 and IL-13 along with mRNA expression of allergy-related cytokines such as IL-4, IL-13, IL-31, and CCL-1 in BM basophils upon in vitro stimulation with papain, rDer f 1, rDer p 1, and IgE crosslink (Fig. 3) supports a previous report of mRNA expression or intracellular cytokine staining for these cytokines in papain-stimulated BM basophils (14). However, treatment of wild-type mice with the mAb MAR-1, which is known to deplete basophils in vivo, did not affect the lung eosinophilia or Ab production (Fig. 4). When detected as CD49b⁺CD200R3⁺ cells, we found a small population of basophils still remaining even after MAR-1 treatment, whereas CD49b⁺IgE⁺ cells were completely absent (Fig. 4A–C). BM mast cells did not show upregulation of cytokine mRNA expression upon stimulation with the protease allergens, and the mast cell deficiency did not affect the in vivo responses (Supplemental Fig. 1). Mast cells did not contribute to the in vivo responses of the lung eosinophilia and Ab production in our protease allergen-induced allergic airway inflammation model, and depletion of the majority of the basophil population also did not affect the in vivo responses. However, we cannot exclude the possibility that basophils contribute to inflammations and Ab production in other experimental settings with differences in the schedule, Ag dose,

and route of the sensitization via not only direct stimulation with protease activity and IL-33-mediated indirect stimulation, but also allergen-specific IgE.

Dependence on IL-33 of the lung eosinophilia in our mouse model and that by Oboki et al. (18) strongly suggested that protease allergens cause release of IL-33 in the airway epithelium. Although Halim et al. (47) showed papain-induced IL-33 release from lung explants ex vivo, cysteine protease-dependent release of lung IL-33 in vivo has not been demonstrated. We demonstrated that active papain, but not E64-treated papain, induces IL-33 release in BAL fluid in vivo (Fig. 6). Recent studies indicated important roles of epithelium-derived cytokines, such as IL-33, thymic stromal lymphopoietin, and IL-25, in Th2 responses (50–52). Papain stimulates the release of thymic stromal lymphopoietin in a human bronchial epithelial cell line (12), and papain and Der p 1 can induce upregulation of IL-25 mRNA expression in a mouse lung epithelial cell line (13). Molecular mechanisms of sensing protease allergens (mediators, sensors, and signaling molecules) to induce the release of these cytokines toward the in vivo responses have yet to be investigated. Of interest, our unpublished observations suggested involvement of uric acid in the papain-induced allergic airway inflammation (S. Kamijo and T. Takai, unpublished observations) similar to that induced by HDM extract (53).

We showed that repeated exposure of airway mucosa to cysteine protease allergens (papain and recombinant HDM group 1 allergens) in the absence of adjuvant leads to lung eosinophilia and serum IgE/IgG1 production in a manner dependent on the protease activity of the allergen, primarily, with the cooperation of adaptive immune cells and IL-33-responsive innate cells to maximize the responses. Although the model by Oboki et al. (18) with high-dose papain is considered more purely dependent on IL-33-mediated innate immunity, IL-33-responsive innate cells such as Th2 cytokine-producing ILCs and adaptive immune cells such as allergen-specific Th2 cells cooperate to maximize the response to lower doses of protease allergens in our model. Basophils showed their capacity to release extremely high amounts of IL-4 and IL-13 in response to papain and recombinant HDM group 1 allergens in vitro; however, depletion of the majority of the basophil population by treatment with anti-FcεRIα mAb did not affect the induction of airway inflammation and Ab production in our model. The molecular mechanism of sensing the protease activity and unknown roles of protease-dependent basophil activation should be addressed in future studies.

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Disclosures

The authors have no financial conflicts of interest.

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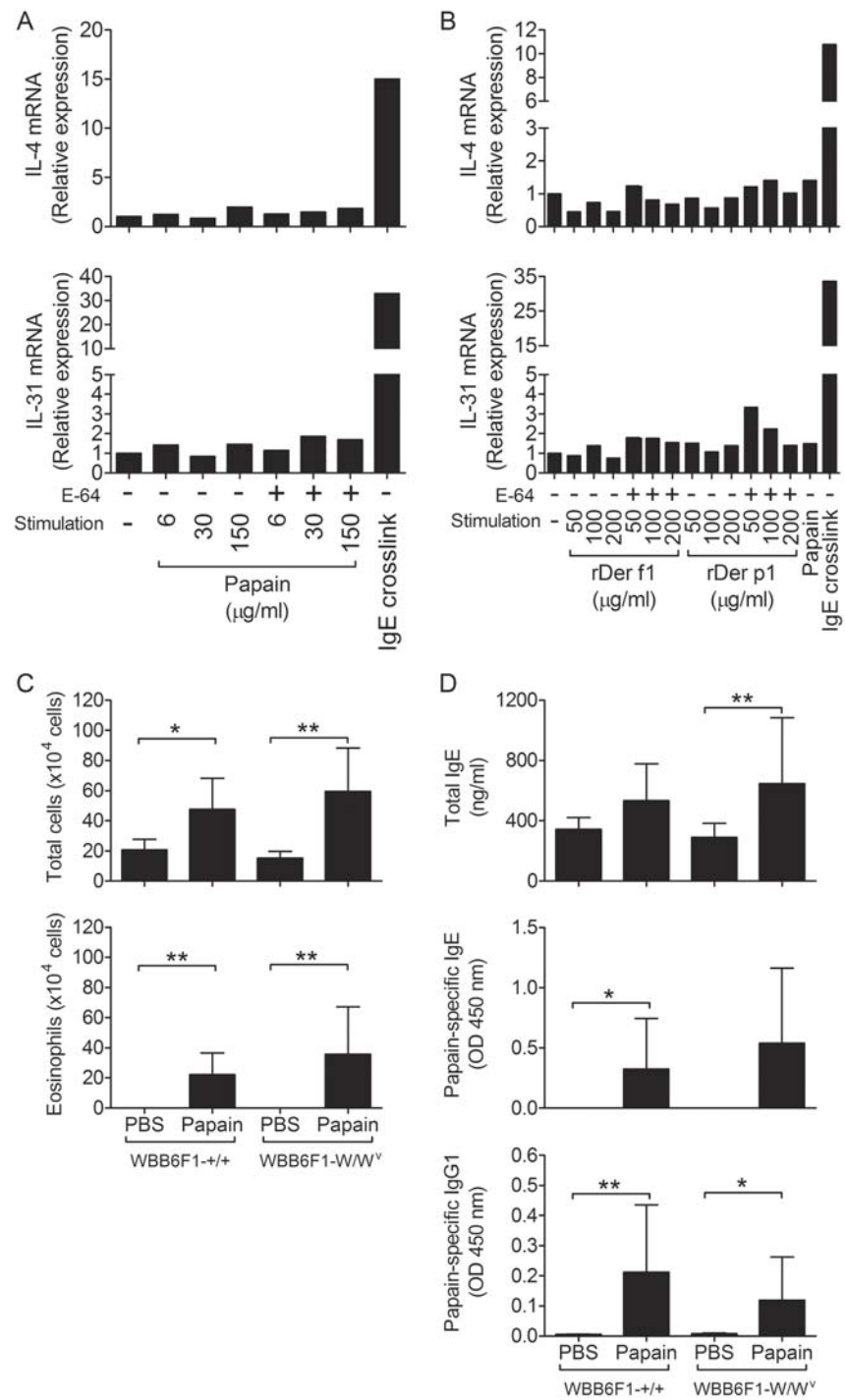
Supplemental Figure legends

Supplemental Figure 1. Cytokine mRNA expression in cysteine protease allergen-stimulated mast cells and the papain-induced airway eosinophilic inflammation/Ab production in mast cell-deficient mice.

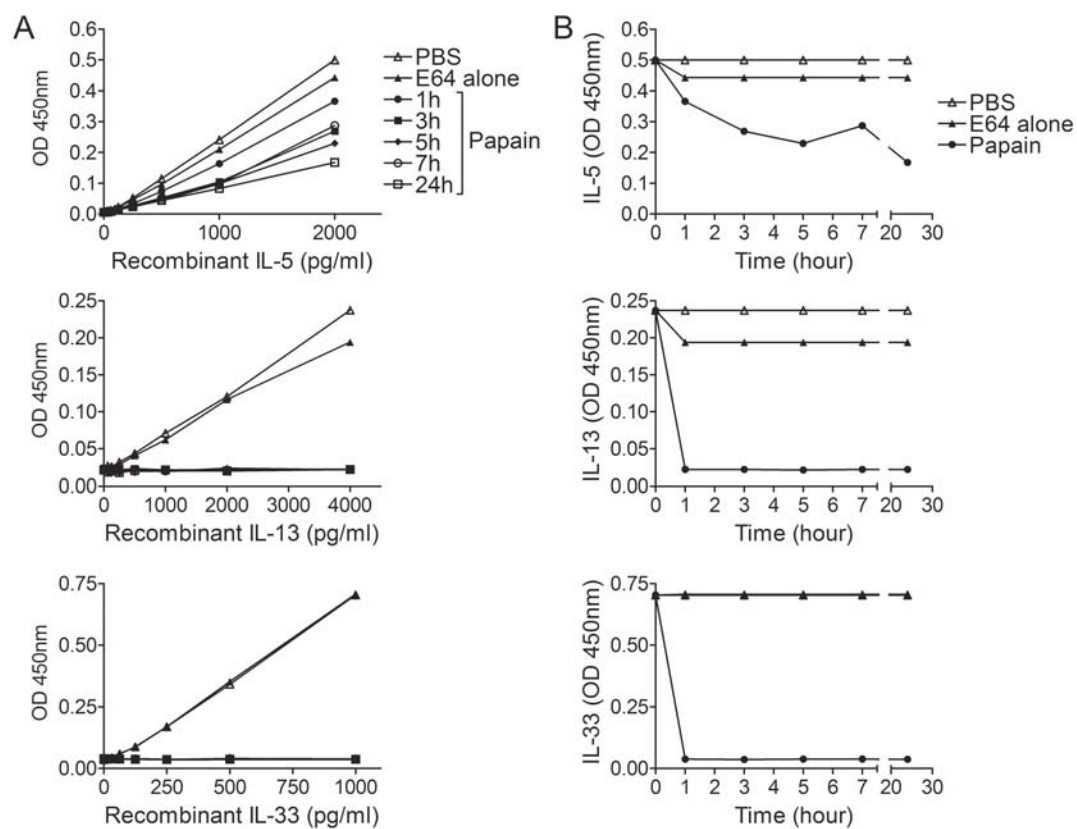
C57BL/6 bone marrow-derived mast cells were stimulated with papain (**A**), rDer f 1, and rDer p 1 (**B**) with or without treatment with E64 in the presence of IL-3. After 2 hours, mRNA expression was measured by real-time PCR (**A, B**). WBB6F1- $+/+$ or WBB6F1-W/W ^{v} mice were i.n. administered weekly papain (30 μ g/40 μ l/animal, Day 0 and 7). BAL fluid and serum were collected at 3 days after the last administration. (**C**) Airway inflammation. (**D**) Serum Ab levels were measured by ELISA. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney U test. Data are indicated as mean $\pm$ SD of 5 mice per group.

Supplemental Figure 2. Proteolysis of recombinant IL-5, IL-13, and IL-33 by papain.

Serially diluted recombinant IL-5, IL-13, and IL-33 were treated with PBS or papain (0.75 mg/ml which is equivalent to the concentration of i.n. administered papain) at 37°C for the time indicated, and then the proteolytic reaction was stopped by adding E64 (2 mM). Concentrations of recombinant IL-5, IL-13, and IL-33 after the papain treatment were measured by cytokine-specific ELISA. The level of cytokine concentrations is indicated as absorbance at wavelength 450 nm. (**A**) Proteolysis of serially diluted cytokines. (**B**) Time course of proteolysis of cytokines at the highest cytokine concentrations (IL-5: 2000 pg/ml, IL-13: 4000 pg/ml, IL-33: 1000 pg/ml).



Kamijo, S. et al. Supplemental Fig. 1



Kamijo, S. et al. Supplemental Fig. 2