

Letter to the Editor

Airway inflammation after epicutaneous sensitization of mice requires protease activity of low-dose allergen inhalation

To the Editor:

Epicutaneous exposure to allergens through the skin is considered to be an important route of sensitization.^{1,2} Allergen sources, such as mites, insects, fungi, and pollen, contain proteases, which are frequently allergens themselves.³ Recent studies using murine models of sensitization via skin⁴ or airways⁵⁻⁷ revealed that the protease activity of a model protease allergen, papain, is essential to the induction of inflammation at the sensitization sites and serum IgE/IgG₁ responses. However, the role of allergen protease activity in effector-phase responses against low-dose antigen encounters after epicutaneous sensitization currently remains unknown. We herein examine the dependency of the onset of airway inflammation on the protease activity of antigens inhaled after epicutaneous presensitization in mice.

We compared the dose dependency of responses to the intranasal administration of papain between epicutaneously presensitized (Fig 1, A-C) and naive mice (Fig 1, D-F) (for further information, see this article's Online Repository at www.jacionline.org). The epicutaneous sensitization to papain induced elevations in total IgE and papain-specific IgE/IgG₁ levels (Fig 1, C, Before; see Fig E1, A, in this article's Online Repository at

www.jacionline.org). IgE levels spontaneously decreased without a subsequent intranasal papain challenge (Fig 1, C, After); the intranasal papain challenge dose-dependently increased papain-specific IgE levels and that even with 0.5 μ g papain resulted in higher levels of papain-specific IgE than with the vehicle inhalation (Fig 1, C, lower panel, After); that with 2.5 μ g induced airway eosinophilia and maintained IgE levels; and that with 12.5 μ g induced more severe eosinophilia and upregulated IgE (Fig 1, B and C). Epicutaneously presensitized mice (Fig 1, A-C; see Fig E2, A, in this article's Online Repository at www.jacionline.org) showed airway eosinophilia as well as the maintenance of or increases in IgE levels on the inhalation of a lower dose of papain by 1 order of magnitude that in naive mice (Fig 1, D-F, and Fig E2, B).

We then performed an intranasal challenge with a covalent complex between papain and the protease inhibitor E-64 (E64-papain). We confirmed that E64-papain was enzymatically inert in a protease assay. The inhalation of a low dose of E64-papain (3 μ g) in epicutaneously presensitized mice negligibly induced airway inflammation, the severity of which was markedly less than that with the inhalation of papain (Fig 2, A; see Fig E3, A, in this article's Online Repository at www.jacionline.org), whereas the inhalation of papain or E64-papain maintained IgE levels (Fig 2, B). Airway inflammation was still negligible following the inhalation of a higher dose of E64-papain (10 μ g and 30 μ g), and no significant increase was observed in IgE levels,

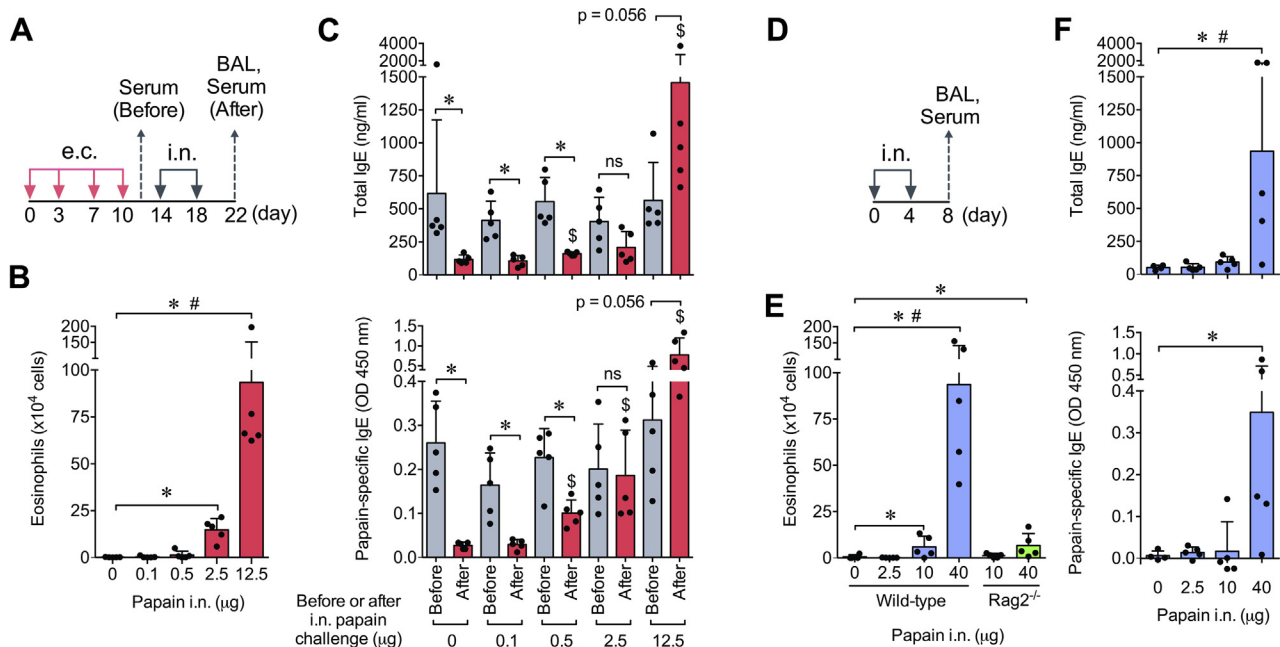
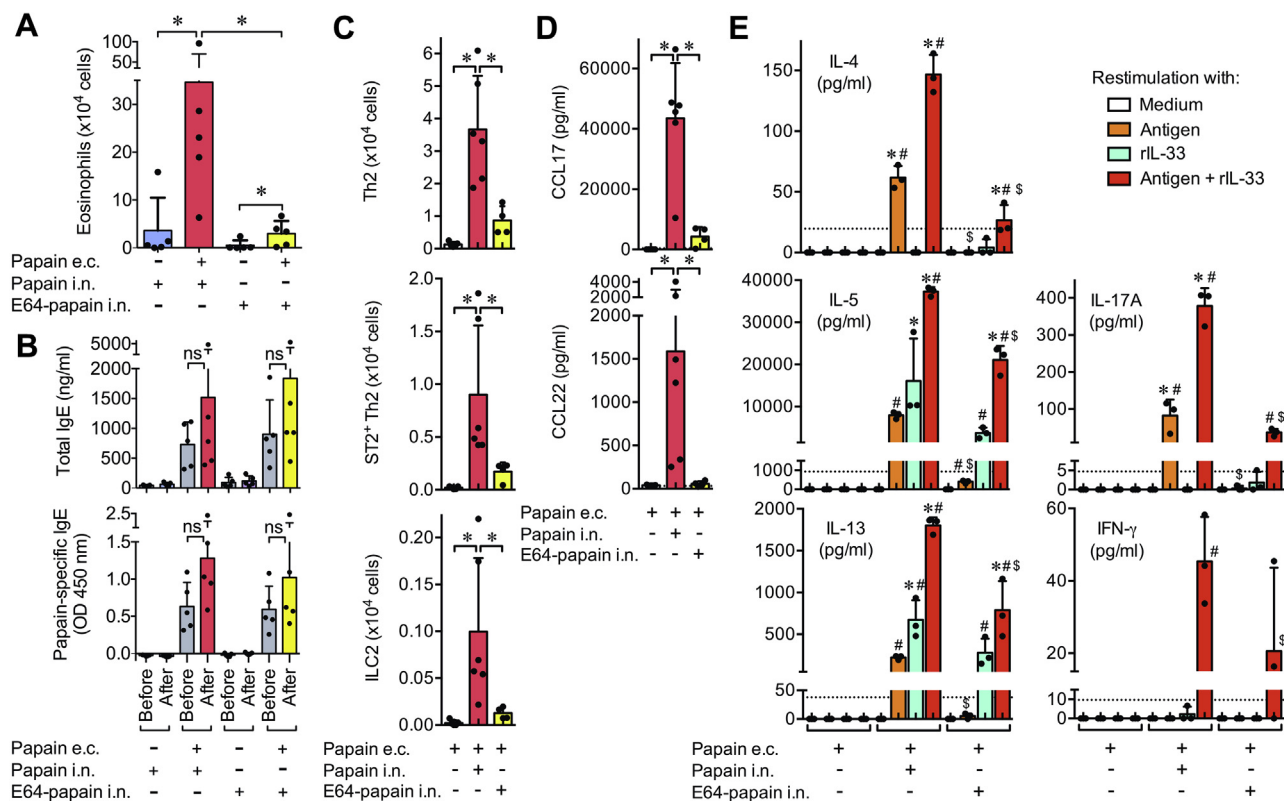


FIG 1. Allergic airway inflammation and maintenance of IgE levels induced by airway exposure to a low dose of papain in epicutaneously presensitized mice. Papain was intranasally administered to epicutaneously presensitized (A-C) or naive mice (D-F). A and D, Time lines. B and E, Airway eosinophilia. C and F, Serum total and papain-specific IgE levels (before [day 12] or after the intranasal challenge [day 22]). Data are indicated as the mean $\pm$ SD of 5 or 4 mice per group and are representative of 3 independent experiments with similar results. * $P < .05$ by Mann-Whitney U test, # $P < .05$ by ANOVA, and \$ $P < .05$ versus the intranasal vehicle challenge (after) by t test. ns, Not significantly different (C). BAL, bronchial alveolar lavage; e.c., epicutaneous sensitization by painting tape-stripped ear skin with papain; i.n., intranasal; Rag2^{-/-}, Rag2-deficient mice, which lack adaptive immune cells.



whereas the inhalation of the same dose of papain upregulated IgE (Fig E3, B). We confirmed that the covalent binding of E-64 to papain did not affect T-cell recall cytokine responses or antibody reactivity (see Fig E4 in this article's Online Repository at www.jacionline.org). These results indicate that the addition of protease activity to the antigen architecture is essential for triggering prominent allergic airway inflammation with the inhalation of a minimal amount of papain.

Airway inflammation induced by the intranasal challenge with the low dose of papain after epicutaneous presensitization was associated with the infiltration of T_H2 cells including an IL-33 receptor-expressing (ST2⁺) population (ST2⁺ T_H2) and ST2⁺ group 2 innate lymphoid cells (ILC2s) (Fig 2, C; see Fig E5 in this article's Online Repository at www.jacionline.org) and the enhanced release of the T_H2 -attracting chemokines (Fig 2, D). The bronchial draining lymph node (DLN) cells of the intranasal papain-challenged group produced IL-4, IL-5, IL-13, and IL-17A on antigen restimulation; the costimulation with IL-33 synergistically promoted their production as well as a small amount of IFN- γ , whereas the stimulation with IL-33 alone induced the production of IL-5 and IL-13 (Fig 2, E). The vehicle-challenged group showed no responses, whereas the E64-papain-challenged

group showed no or weaker responses. We consider T_H cells including ST2⁺ T_H2 cells to have mediated cytokine production in an antigen-dependent or IL-33-dependent manner, but cannot exclude the potential contribution of ILC2, which may be contained in DLN samples, to the IL-33-dependent production of IL-5 and IL-13. Recent studies demonstrated that antigen-specific ST2⁺ but not ST2[−] memory T_H2 cells were responsible for airway eosinophilia in mice⁸ and also that IL-33 stimulated CD4⁺ T cells from the nasal polyps of patients with eosinophilic chronic rhinosinusitis to upregulate the expression of IL-5 and/or IL-13.^{8,9}

The delivery of exogenous proteases evokes an enzyme-mediated "alarm," such as the release of IL-33, presumably via damage to the lung epithelium.⁵ In intranasal models with the inhalation of 10 to 30 μ g of papain, the IL-33-ILC2 axis has been reported to contribute to T_H2 differentiation, IgE production, the recruitment of T_H2 cells, and airway eosinophilia.^{5–7} We analyzed effector-phase responses in a tissue (lung) different and distant from the sensitization site (ear skin). Recently we reported that in the epicutaneous sensitization-intranasal challenge model with the inhalation of a low dose (2.5–3 μ g) of papain, airway eosinophilia and the maintenance of IgE levels induced

by the subsequent inhalation of a minute amount of papain were dependent on IL-33, whereas IL-33 was dispensable for skin inflammation and antibody responses in the epicutaneous sensitization phase.⁴ In the present study, we demonstrated that (1) the onset of allergic airway inflammation after epicutaneous sensitization was critically dependent on allergen protease activity contained in the low-dose airway encounter (Fig 2, A, and Fig E3, A) and (2) 10-fold more ST2⁺ T_H2 cells than ILC2s infiltrated bronchial alveolar lavage fluid with abundant amounts of T_H2-attracting chemokines (Fig 2, C and D) and the combination of the antigen and IL-33 synergistically stimulated DLN cells to upregulate cytokine production (Fig 2, E), suggesting the contribution of the “antigen plus IL-33-T_H2 axis.” In addition, the observation that the protease activity was dispensable for the maintenance of IgE levels upon the low-dose inhalation (Fig 2, B, and Fig E3, B) but necessary for the further upregulation of antigen-specific IgE upon higher-dose inhalation (Fig E3, B, papain-specific IgE) may be relevant to upregulation of allergen-specific IgE in allergic patients upon natural seasonal/transient exposure to airborne allergens such as pollen.

In conclusion, we herein demonstrated that the effector-phase responses of the onset of allergic airway inflammation after the establishment of epicutaneous sensitization in mice not only need intact T/B-cell epitope structures but also the crucial commitment of the protease activity of the low-dose inhalation and the possible contribution of IL-33-responsive, antigen-specific T_H2 cells. The present results suggest that the protease activity contained in airborne allergens contributes more prominently to the onset of allergic airway inflammation in epicutaneously sensitized individuals, including the “atopic march,”^{1,2} than previously considered, and that targeting ST2⁺ T_H2 cells along with ILC2s may be a promising strategy to regulate protease-dependent, IL-33-dependent effector-phase responses in airways.

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Izumi Nishioka, MD, PhD^{a,b}

Toshiro Takai, PhD^a

Natsuko Maruyama, MSc^{a,b}

Seiji Kamijo, PhD^a

Punyada Suchiva, MD, MSc^{a,b}

Mayu Suzuki, MSc^a

Shinya Kunimine, MD^{a,b}

Hirono Ochi, MD, PhD^{a,b}

Sakiko Shimura, MD, PhD^{a,b}

Katsuko Sudo, PhD^c

Hideoki Ogawa, MD, PhD^b

Ko Okumura, MD, PhD^a

Shigaku Ikeda, MD, PhD^{a,b}

From ^aAtopy (Allergy) Research Center and ^bthe Department of Dermatology and Allergology, Juntendo University Graduate School of Medicine, and ^cAnimal Research Center, Tokyo Medical University, Tokyo, Japan. E-mail: t-takai@juntendo.ac.jp.

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METHODS

Mice

Six- to 12-week-old C57/BL6J mice (Sankyo Lab Service Corporation, Ibaraki, Japan) and Rag2^{-/-} mice^{E1} 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. Female mice were used in experiments, except for intraperitoneal immunization, for which male mice were used (Fig E4, D).

Papain

Papain was purchased from Calbiochem (San Diego, Calif). E-64 (Peptide Institute, Osaka, Japan) is a small protease inhibitor that irreversibly binds to the catalytic site of papain family proteases without altering their tertiary structure. To prepare papain treated with E-64, similar to our previous studies,^{E2-E6} papain was reacted with an excess of E-64 after the addition of L-cysteine and, after the reaction, L-cysteine and unbound free E-64 were removed by dialysis. In some experiments (Fig 2 and Figs E3 and E4), 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 E64-treated papain. In the covalent complex between E-64 and papain (E64-papain) and dialyzed papain, purity, no degradation, and no aggregation were confirmed by electrophoresis, protease activity was analyzed using synthetic fluorogenic substrates in the presence or absence of a reducing reagent,^{E7,E8} and protein concentrations were assessed by a kit (Micro BCA protein assay kit; Thermo Scientific, Waltham, Mass).

Epicutaneous papain sensitization

The method used was as described previously.^{E4} Briefly, papain (concentration, 1 mg/mL) was applied to both sides of the surface of both ears of each mouse after a pretreatment with tape-stripping 3 times using cellophane tape (Cellotape, Nichiban, Tokyo, Japan), and this procedure was repeated twice per week for a total of 4 times.

Intranasal papain challenge

Epicutaneously sensitized or unsensitized mice were intranasally challenged with papain (50 μ L/mouse). Sera and bronchial alveolar lavage fluid (BALF) were collected 4 days after the last intranasal administration. Cellular infiltration was analyzed by differential cell counting as described previously.^{E2,E4} In some experiments (Fig 2, C-E), BALF and bronchial draining, mediastinal lymph nodes (DLNs) were recovered 3 days after the last intranasal administration. CCL17 and CCL22 concentrations in BALF were measured by ELISA using kits (R&D Systems, Minneapolis, Minn).

Flow cytometric analysis

In analyses of ILC2s and CD4⁺ T cells in BALF, cells were stained using the following antibodies: phycoerythrin (PE)-Cy7-conjugated anti-mouse lineage markers (B220 [clone RA3-6B2], CD3 [clone 145-2C11], CD11b [clone M1/70], CD16/CD32 [clone 2.4G2], TER-119 [clone TER-119] [BD Biosciences, San Jose, Calif], CD14 [clone Sa2-8], CD49b [clone DX5] [eBioscience, San Diego, Calif], CD11c [clone N418], Fc ϵ RI α [clone MAR-1] [BioLegend, San Diego, Calif], allophycocyanin (APC)-conjugated anti-mouse CD25 (clone PC61), fluorescein isocyanate (FITC)- or PerCP/Cy5.5-conjugated anti-mouse/human CD44 (clone IM7), streptavidin-APC/Cy7 (BioLegend), biotinylated anti-T1/ST2 (clone DJ8) (MD Bioproducts; Zürich, Switzerland), Alexa Fluor e660-conjugated anti-mouse GATA-3 (clone TWAJ), FITC-conjugated anti-mouse CD62L (clone MEL-14) (eBioscience), PE-conjugated anti-mouse CD4 (clone GK1.5) (TONBO Bioscience, San Diego, Calif), and streptavidin-PE (BD Biosciences). Before cell surface staining, Fc receptors were blocked using purified anti-mouse CD16/CD32 (clone 2.4G2) (TONBO Bioscience). The True Nuclear Transcription Factor Buffer Set (BioLegend) was used for the intracellular staining of GATA3. Acquisition and analyses were performed using the FACS Verse cell sorter (BD

Biosciences) and FlowJo software (FlowJo, Ashland, Ore). The number of CD4⁺CD62L⁻CD44^{Lo}GATA3⁺ cells (T_H2), CD4⁺CD62L⁻CD44^{Lo}GATA3⁺ST2⁺ cells (ST2⁺ T_H2), and lineage⁻CD25⁺CD44⁺ST2⁺ cells (ILC2s) was shown as data.

ELISA for serum total IgE and antigen-specific IgE/IgG₁

Serum total IgE^{E9} and papain-specific antibodies^{E4} were detected on plates, which were coated with 2 μ g/mL anti-murine IgE mAb (clone R35-72, BD Biosciences, San Jose, Calif) or 10 μ g/mL papain resolved in PBS (50 μ L/well; 4°C overnight), and blocked with 20% ImmunoBlock (DS Pharma Biomedical, Osaka, Japan) diluted with pure water (200 μ L/well; 37°C for 90 minutes), as described previously^{E4,E9} with minor modifications as follows. Briefly, sera, detection antibodies, and streptavidin-conjugated horseradish peroxidase (avidin-HRP) were diluted with PBS containing 0.05% Tween20 and 5% ImmunoBlock (50 μ L/well).

The total IgE level was measured by sandwich ELISA. After an incubation with diluted sera (serum dilution: 1/50 and 1/250; 37°C, 80 minutes), 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 minutes). 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 minutes). After being washed, plates were further incubated with avidin-HRP (BD Biosciences) (dilution: 1/2500; room temperature for 30 minutes).

Regarding papain-specific IgG₁, after the incubation with diluted sera (serum dilution: 1/5000; 37°C 80 minutes), plates were incubated with HRP-conjugated anti-murine IgG₁ mAb (clone X56, BD Biosciences) (dilution: 1/5000; 37°C for 80 minutes).

Plates were washed with PBS containing 0.05% Tween20 after each of the steps. The plates were color developed with tetramethyl benzidine (BD-optEIA kit, BD Biosciences) (100 μ L/well; room temperature for 20 minutes) and the reaction was stopped by adding 2N sulfuric acid (50 μ L/well). Absorbance at 450 nm, from which that at 570 nm was subtracted, was used as the signal.

Comparison of antibody reactivities between papain and E64-papain

To compare the reactivities of papain and E64-papain to papain-specific antibodies, the binding of IgE and IgG₁ contained in serially diluted serum to ELISA plates coated with dialyzed papain or E64-papain (10 μ g/mL) was analyzed according to the method described above.

Restimulation of DLN cells and splenocytes

Single-cell suspensions of DLN cells and splenocytes were prepared according to the methods described previously^{E5,E7,E10} with minor modifications. Briefly, DLNs and spleens were aseptically recovered. Single-cell suspensions were prepared by gently teasing apart the tissue in RPMI1640 medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and antibiotics, that is, the homogenization of spleens by passage through EASYstrainer (70- μ m pore size, Greiner Bio-One, Tokyo, Japan) or homogenization of DLNs by sandwiching and crushing them between 2 glass slides and passage through EASYstrainer. After centrifugation at 500g (splenocytes) or 300g (DLN cells) at 4°C for 5 minutes, cells were resuspended in ACK buffer at room temperature (splenocytes: 1 mL, 5 minutes; DLN cells: 2 mL, shorter period) to deplete red blood cells. After washing with the medium, cells were stimulated with antigens in 96-well, flat-bottomed, tissue culture plates (Corning Life Sciences, Corning, NY) (5 $\times$ 10⁵/200 μ L/well). Mixtures of all DLN cells from the mice of each group or mixtures of

equivalent number of splenocytes from each mice of each group were restimulated with E64-papain or dialyzed papain (25 $\mu\text{g/mL}$ or the concentrations indicated) in the presence or absence of recombinant mouse IL-33 (R&D Systems) (10 ng/mL) (Fig 2, E, and Fig E4, B-D). To assess cytokine production, culture supernatants were collected at 96 hours (DLN cells) or 72 hours (splenocytes) and subjected to ELISA using kits (R&D Systems).

Statistical analysis

A 1-way ANOVA with the Tukey *post hoc* test, the Mann-Whitney *U* test (2-tailed), or Student *t* test (2-tailed) was used. A *P* value of less than .05 was regarded as significant.

DISCUSSION

Spontaneous decrease in IgE levels

Although the epicutaneous sensitization to papain induced elevations in total IgE and papain-specific IgE/IgG₁ levels (Fig 1, C, Before, and Fig E1, A), IgE levels (total IgE and papain-specific IgE) spontaneously decreased without a subsequent intranasal papain challenge (Fig 1, C, After). A possible explanation for the decrease is that serum IgE in mice is produced mainly by short-lived IgE plasma cells with minor contribution of long-lived IgE plasma cells.^{E11} Recently, Haniuda et al^{E12} proposed that formation of IgE memory B cells or long-lived IgE plasma cells according to abnormal B-cell signaling may cause allergic diseases.

Cytokine production in DLN cells restimulated *in vitro*

We consider T_H cells including ST2⁺ T_H2 cells to have mediated cytokine production in an antigen-dependent or IL-33-dependent manner (Fig 2, E), but cannot exclude the potential contribution of ILC2s, which may be contained in bronchial DLN samples, to the IL-33-dependent production of IL-5 and IL-13. Recent studies demonstrated that inhalation of ovalbumin in mice, to which ovalbumin-specific ST2⁺ but not ST2⁻ memory T_H2 cells were transferred, induced airway eosinophilia^{E13} and that T_H cells can respond to IL-33-dependent, TCR-independent stimuli^{E14} and also IL-33-stimulated CD4⁺ T cells from the nasal polyps of patients with eosinophilic chronic rhinosinusitis^{E13,E15} to upregulate the expression of IL-5 and/or IL-13.

Murine naive T cells cocultured with dendritic cells in the presence of IL-33 have been reported to produce IL-5 and IL-13^{E16,E17} but DLN cells from mice intranasally challenged with vehicle after epicutaneous sensitization showed no cytokine response in the presence of IL-33 (Fig 2, E). We cannot exclude the potential indirect contribution of IL-33 via activation of dendritic cells^{E16,E17} to the T-cell cytokine production in mice intranasally challenged with papain after epicutaneous sensitization.

Different levels of protease dependency among models

Different levels of protease dependency in the effector phase among models with different sensitization routes^{E5,E18} may be

due to various differences between epicutaneous and other sensitization routes such as the magnitude of sensitization, pattern of T_H differentiation, and mediators released from sensitization sites.

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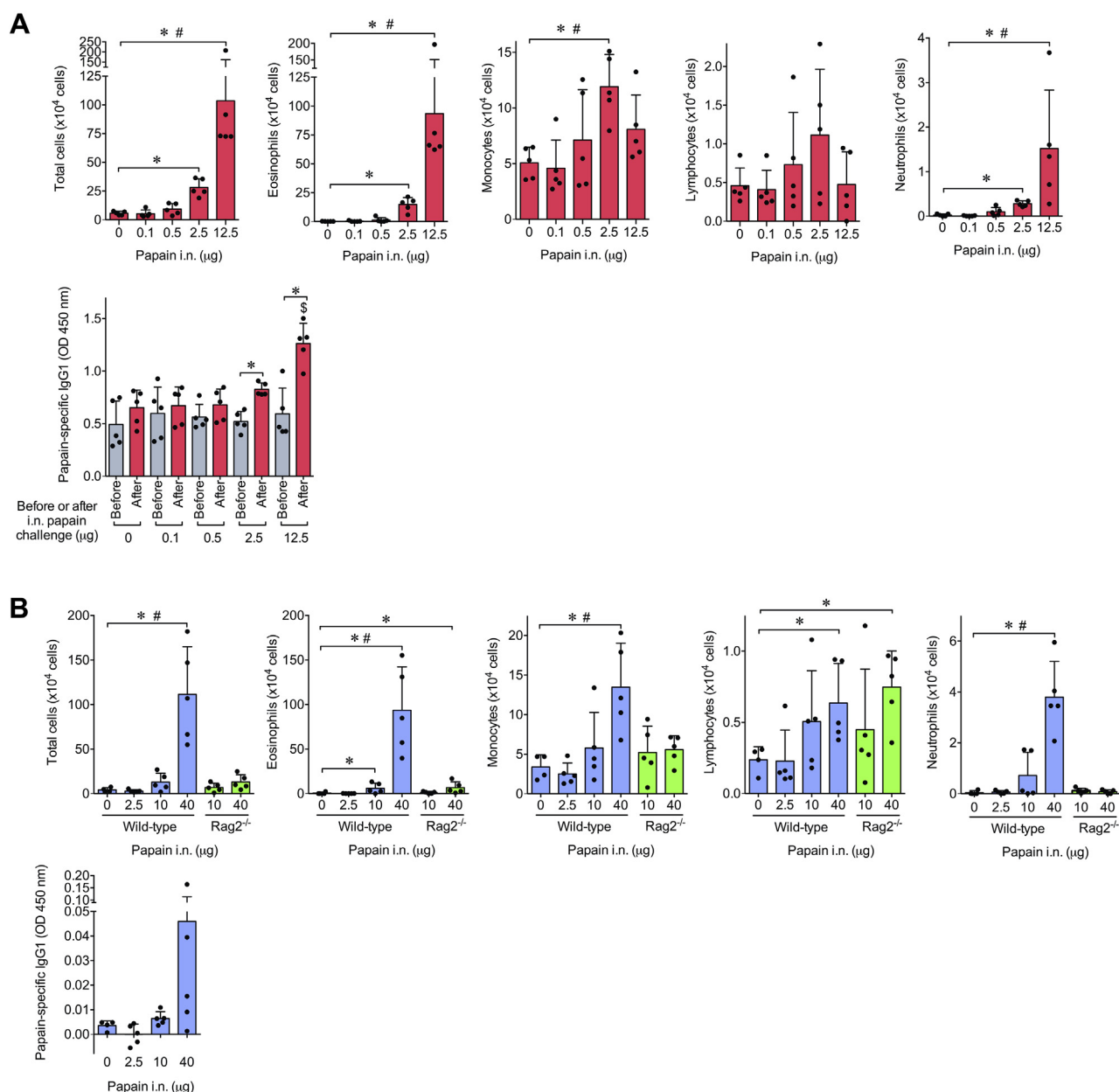


FIG E1. Airway inflammation and papain-specific IgG₁ responses induced by the inhalation of papain with a 4-day interval. **A**, Epicutaneously presensitized mice. **B**, Naive mice. Fig E1, A and B, are supplementary to Fig 1, A-C and D-F, respectively. Data shown in Fig 1, B and E, are included here for comparison. *i.n.*, Intranasal. Data are indicated as the mean $\pm$ SD of 5 or 4 mice per group and are representative of 3 independent experiments with similar results. * $P < .05$ by the Mann-Whitney *U* test, # $P < .05$ by ANOVA, and \$ $P < .05$ versus the intraperitoneal vehicle challenge (*After*) by the *t* test. Even without epicutaneous presensitization, adaptive immune cells play an important role because Rag2^{-/-} mice showed markedly less severe lung eosinophilia, similar to a 7-day interval model.^{E2}

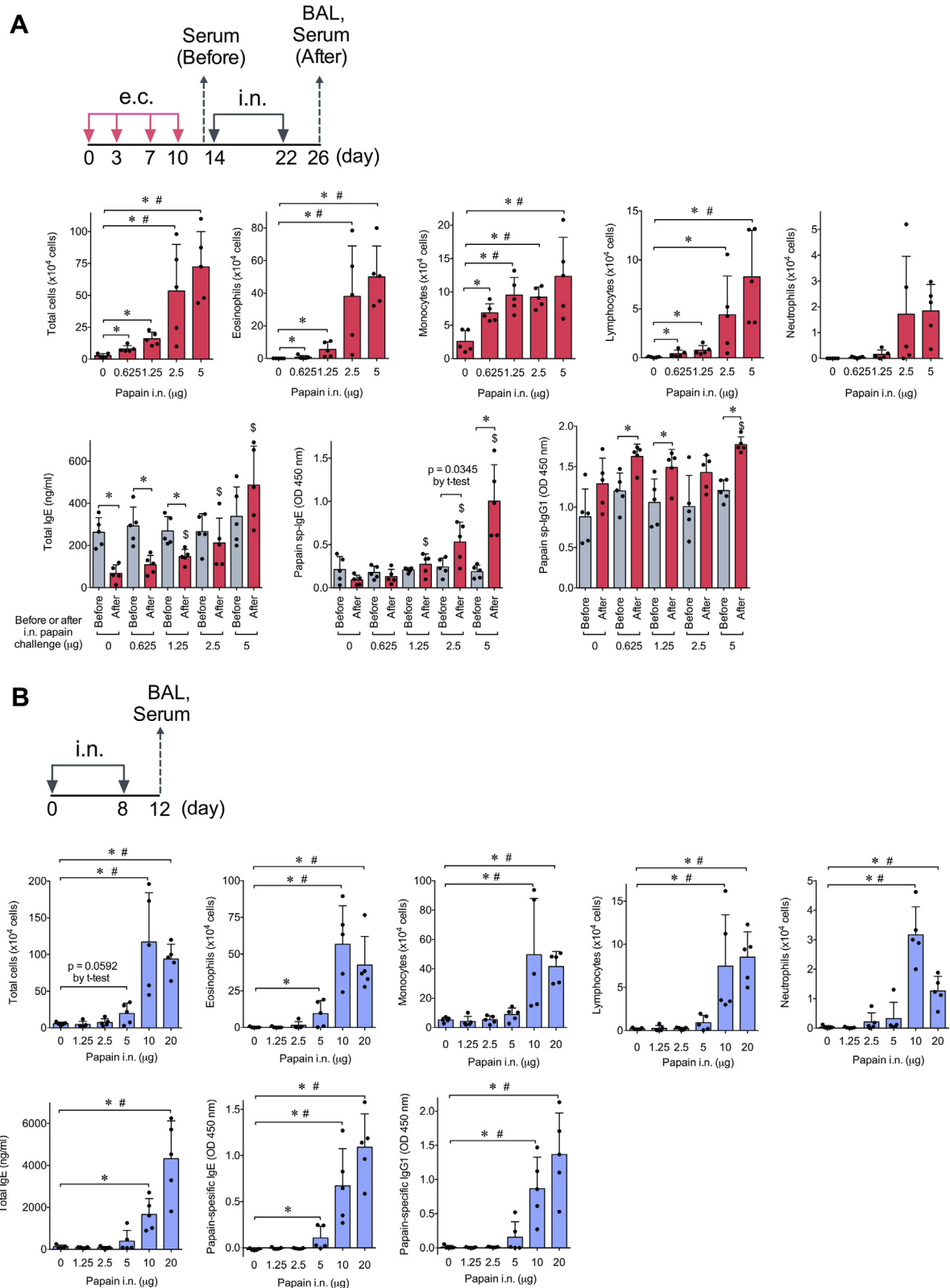


FIG E2. Airway inflammation and antibody responses induced by the inhalation of papain with an 8-day interval. **A**, Epicutaneously presensitized mice. **B**, Naive mice. Data are indicated as the mean $\pm$ SD of 5 mice per group. Data are representative of 3 independent experiments with similar results. * $P < .05$ by the Mann-Whitney U test, # $P < .05$ by ANOVA, and \$ $P < .05$ versus the intranasal vehicle challenge (After) by the t test. e.c., Epicutaneous; i.n., intranasal.

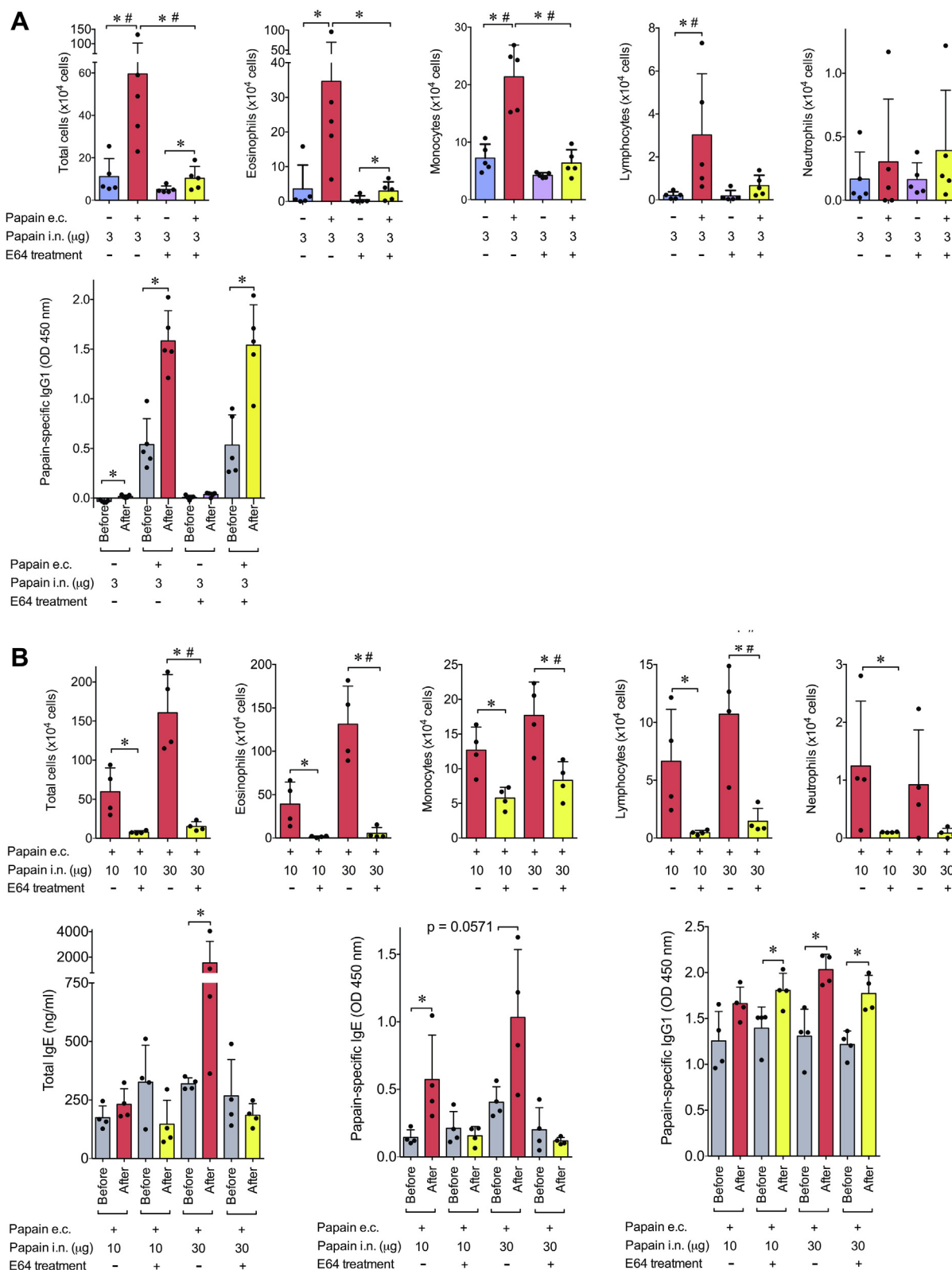


FIG E3. Enzymatic activity of inhaled papain is necessary for eosinophilic airway inflammation induced by the inhalation of papain with a 4-day interval in epicutaneously sensitized mice. **A**, Supplementary data for Fig 2, A and B. **B**, Mice epicutaneously sensitized to papain were intranasally challenged with papain or E-64-papain (10 or 30 μ g). BAL, Bronchial alveolar lavage; e.c., epicutaneous; i.n., intranasal. Data are indicated as the mean $\pm$ SD of 5 or 4 mice per group. Data are representative of 2 independent experiments with similar results. * $P < .05$ by the Mann-Whitney U test, # $P < .05$ by ANOVA, and \$ $P < .05$ versus the intranasal vehicle challenge (After) by the t test. Before: day 13. After: day 22.

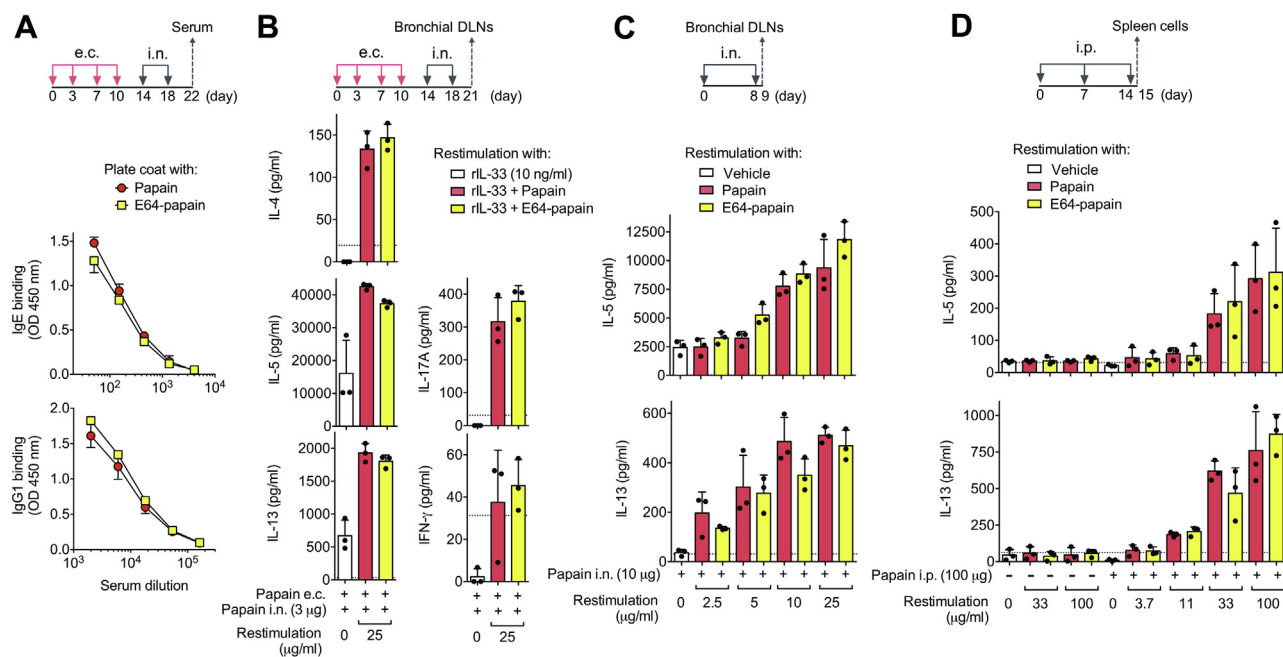


FIG E4. Covalent binding of E-64 to papain does not affect T-cell recall cytokine responses or antibody reactivity against T/B-cell epitope structures. Serum antibody binding (**A**) and cytokine production (**B**) in restimulated bronchial DLN cells from mice epicutaneously presensitized-intranasally challenged with papain. **C**, Cytokine production in restimulated bronchial DLN cells from mice intranasally administered papain. **D**, Responses of restimulated splenocytes from mice intraperitoneally immunized with papain. *e.c.*, Epicutaneous; *i.n.*, intranasal; *i.p.*, intraperitoneal. Data are indicated as the mean $\pm$ SD of 3 wells per group. Data are representative of 2 independent experiments with similar results.

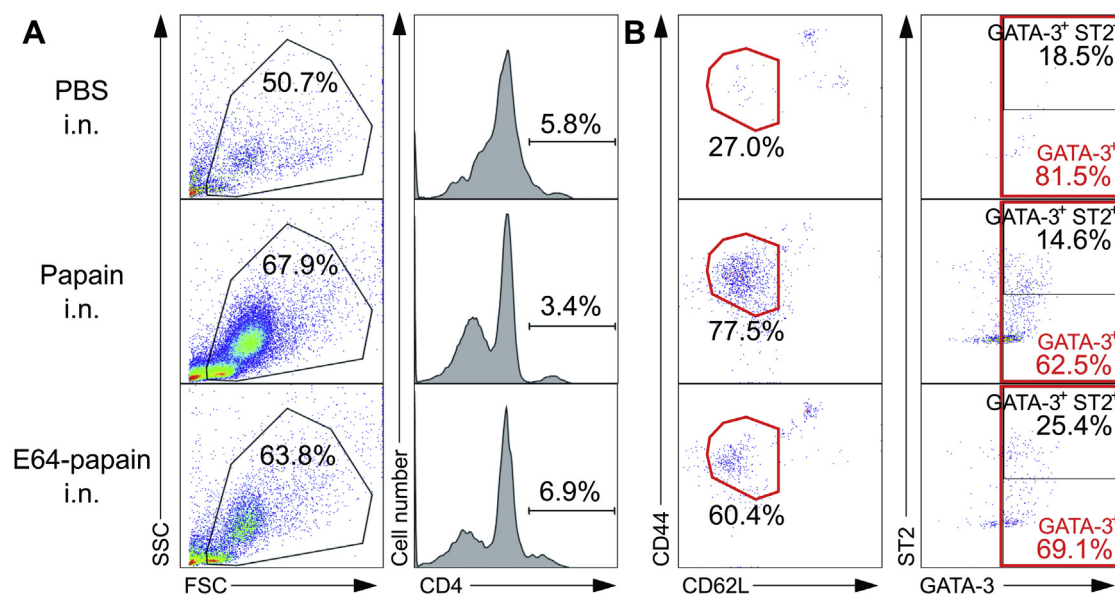


FIG E5. Gating strategy in the flow cytometric analysis of T_H2 cells. Cells contained in BALF were recovered 3 days after the last intranasal challenge (day 21) and analyzed with flow cytometry. **A**, Total live cells were gated on the basis of FSC and SSC and CD4-positive cells were then gated. **B**, $CD4^+CD62L^-CD44^{Lo}$ cells were gated and the expression of GATA3 and ST2 in the population was then analyzed. The $CD4^+CD62L^-CD44^{Lo}GATA3^+$ population is indicated as T_H2 and its $ST2^+$ population is indicated as $ST2^+ T_H2$ in Fig 2. **C**, Representative data of 1 mice of each group are shown. *i.n.*, Intranasal; FSC, forward scatter; SSC, side scatter.