

LETTER TO THE EDITOR

A mouse model of asthma-chronic obstructive pulmonary disease overlap induced by intratracheal papain

To the Editor:

Asthma-chronic obstructive pulmonary disease (COPD) overlap (ACO) is recognized as a distinct clinical disorder that can be differentiated from asthma or COPD alone, owing to the rapid disease progression, frequent exacerbations and increased comorbidities.¹ A major impediment to the elucidation of mechanisms underlying ACO pathogenesis has been the lack of development and detailed evaluation of appropriate animal models. Administration of papain, a cysteine protease from the fruit of the plant species *Carica papaya*, was shown to induce acute/subacute allergic airway inflammation (AAI) via type 2 responses.² Results from previous mouse model experiments also indicated that short-term exposure to papain induces emphysema.³ We hypothesized that weekly intratracheal aerosol administration of papain could induce a clinically feasible murine ACO model that features concurrent emphysema, persistent AAI and airway hyper-responsiveness. Further, we examined its characteristics with multifaceted analyses including comprehensive cytokine profiling, neutrophil gelatinase-associated lipocalin (NGAL) measurement and long-term observations.

We first evaluated the model with respect to pulmonary emphysema. Porcine pancreatic elastase (PPE) has been used to induce murine models of COPD.⁴ We intratracheally administered PPE or papain using a microsprayer (Figure 1A). The changes in body weight in the PPE- or papain-treated groups were not significantly different from the controls (Figure S1). Both PPE- and papain-treated groups showed increased inspiratory capacity/weight and dynamic compliance (Figure 1B); representative lung histology revealed PPE- and papain-induced emphysema (Figure 1C). The mean linear intercept was significantly increased in the PPE- and papain-treated groups (Figure 1D). As such, we concluded that both PPE and papain induced emphysema. Furthermore, goblet cell hyperplasia was detected exclusively among mice in the papain-treated group (Figure 1E).

Consistent with this finding, whole lung RNA preparations from the papain (but not PPE-) treated mice exhibited increased transcript levels of Muc5ac (Figure S2).

We then evaluated asthmatic features associated with papain administration. Representatives of bronchoalveolar lavage fluid (BALF) cytospin preparations are shown in Figure 1F. The total number of cells, macrophages and eosinophils were significantly increased in the papain-treated group (Figure 1G). The papain-treated group exhibited higher airway responsiveness to increasing doses of methacholine compared to other groups (Figure 1H); no differences in baseline airway resistance were observed (Figure 1B). Whole lung homogenates of the papain-treated mice revealed increased expression levels of Eotaxin1 and Eotaxin2 (Figure S2). Thus, our papain-treated model had both COPD-associated and asthmatic features and was considered to be an appropriate model for ACO.

For more detailed analyses, multiple cytokines/chemokines in the BALF were quantitatively detected (Figure 2A; cytokine array data are shown in Table S1). Regarding macrophage/neutrophil-related cytokines/chemokines, macrophage colony-stimulating factor (M-CSF), keratinocyte-derived chemokine (KC) and IL-6 were detected in BALF from the papain-treated mice only. With regard to type 2 inflammation-associated cytokines, IL-4 levels were not significantly different among the groups, while IL-5 and IL-13 levels were detectable only in the papain-treated mice. Collectively, our ACO model reproduced the characteristics of both asthmatic and COPD-associated airway inflammation. IL-33 concentrations were not significantly different among the groups (Figure S3). The levels of total IgE, a serum marker used to define asthma endotypes,⁵ were significantly higher in the papain-treated mice (Figure 2B). Significantly higher levels of NGAL were detected in the BALF of papain-treated mice; NGAL has been identified as a promising biomarker for clinical evaluation of ACO (Figure 2C).⁶

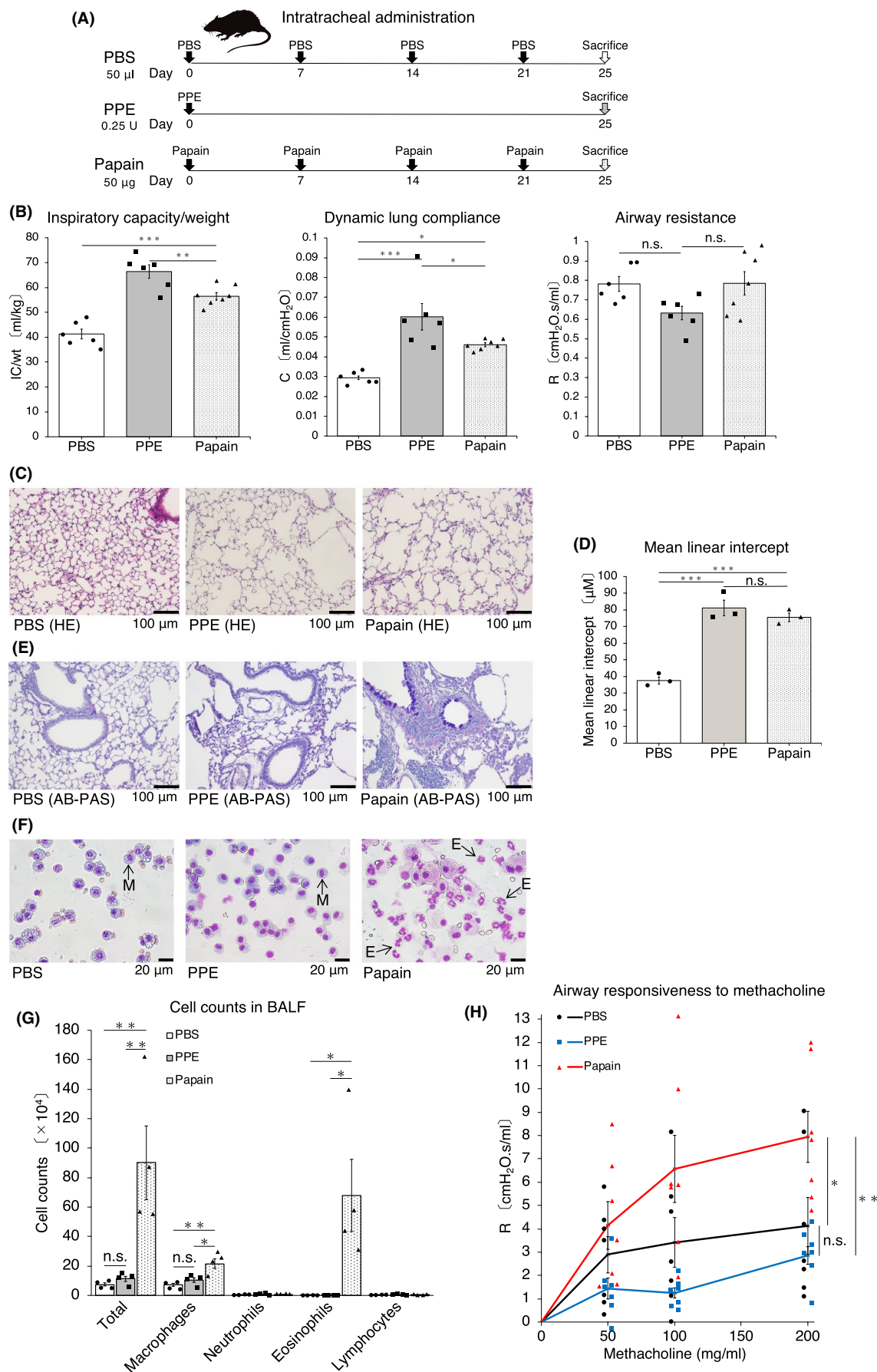


FIGURE 1 Both porcine pancreatic elastase (PPE) and papain induced pulmonary emphysema and papain reproduced the asthmatic features. (A) Protocol (B) Respiratory mechanics analysed with a computer-controlled small-animal ventilator (flexiVent, Scireq) (C) Lung tissue stained with haematoxylin and eosin (HE; 20x) or (E) Alcian blue and periodic acid-Schiff (AB-PAS; 20x) (D) Calculation of the mean linear intercept (F) Representative bronchoalveolar lavage fluid (BALF) cytospin preparations (50x); M, macrophage; E, eosinophil. (G) Differential cell counts of cytospin preparations (H) Airway responsiveness to increasing doses of methacholine measured by subtracting the baseline from each airway resistance; (B) $n = 6-7$, (D) $n = 3$, (G) $n = 4$, (H) $n = 7$ per group, per experiment; * $P < .05$, ** $P < .01$, *** $P < .001$; n.s. not significant with one-way ANOVA with the Holm-Bonferroni post hoc test. Data are duplicates-triplicates from a single experiment

We also provide a more long-term (8 weeks; day 56) assessment of the papain-treated mice (Figure 2D). BALF eosinophils remained significantly elevated, a finding that indicates prolonged eosinophilic inflammation in response to papain (Figure 2E). Inspiratory capacity/weight and lung compliance also remained elevated at the 8-week time point (Figure 2F), as did emphysema-associated lung pathology (Figure 2G). ELISA was notable for elevated serum levels of total and papain-specific IgE at both 4 and 8 weeks (Figure 2H). Taken together, our papain-induced model of ACO included prolonged eosinophilic type 2 inflammation along with emphysema. The papain-treated group at both 4 and 8 weeks similarly exhibited higher levels of NGAL in the BALF (Figure 2I).

Polyinosinic:polycytidylic acid [poly(I:C)] is a form of dsRNA that acts as a TLR3 agonist and is used to mimic viral infection-induced exacerbations of airway inflammation.⁷ In our poly(I:C)-induced exacerbation models (Figure S4A), neutrophil/macrophage-dominated inflammation was triggered, while AAI was sustained in the ACO model (Figure S4B-E). We also found administration of poly(I:C) resulted in increased levels of NGAL in both serum and BALF (Figure S4F); of note, serum NGAL levels were increased only slightly above baseline by papain administration alone (Figure 2I). Since NGAL is expressed not only in the neutrophils but also in the respiratory epithelial cells,⁸ we hypothesized that poly(I:C)-induced elevations in NGAL were largely related to the influx of neutrophils;

by contrast, NGAL resulting from papain administration alone may be result from inflammation-activated and/or damaged epithelial cells.

Paediatric asthma is a major predictor for the development of clinical ACO after middle age.⁹ As our model features administration of papain to mice at 5-6 weeks of age, our model would correspond to patients diagnosed with ACO associated with early-onset asthma. Specifically, our ACO model reflects early-onset, eosinophilic, high IgE and Th2-dominant asthma phenotypes/endotypes. We recognize that emphysema in this model develops rapidly and at an earlier time point than what might be anticipated clinically and is not associated with smoking; these are both potential limitations of this model. However, this model is simpler and easier to use than most smoking models as it does not require several months of daily procedures and special equipment.⁴

To the best of our knowledge, this is the first established murine model of ACO that includes both prolonged emphysema and asthmatic features. Additionally, this is the first model of ACO that evaluated elevated levels of NGAL and as such parallels human clinical findings. The close investigation of this model includes results of comprehensive cytokine and NGAL measurements that reflect findings from clinical reports focused on ACO.⁶ Our murine model will be useful for elucidating the pathological processes associated with ACO and for identifying new diagnostic markers and therapeutic targets.

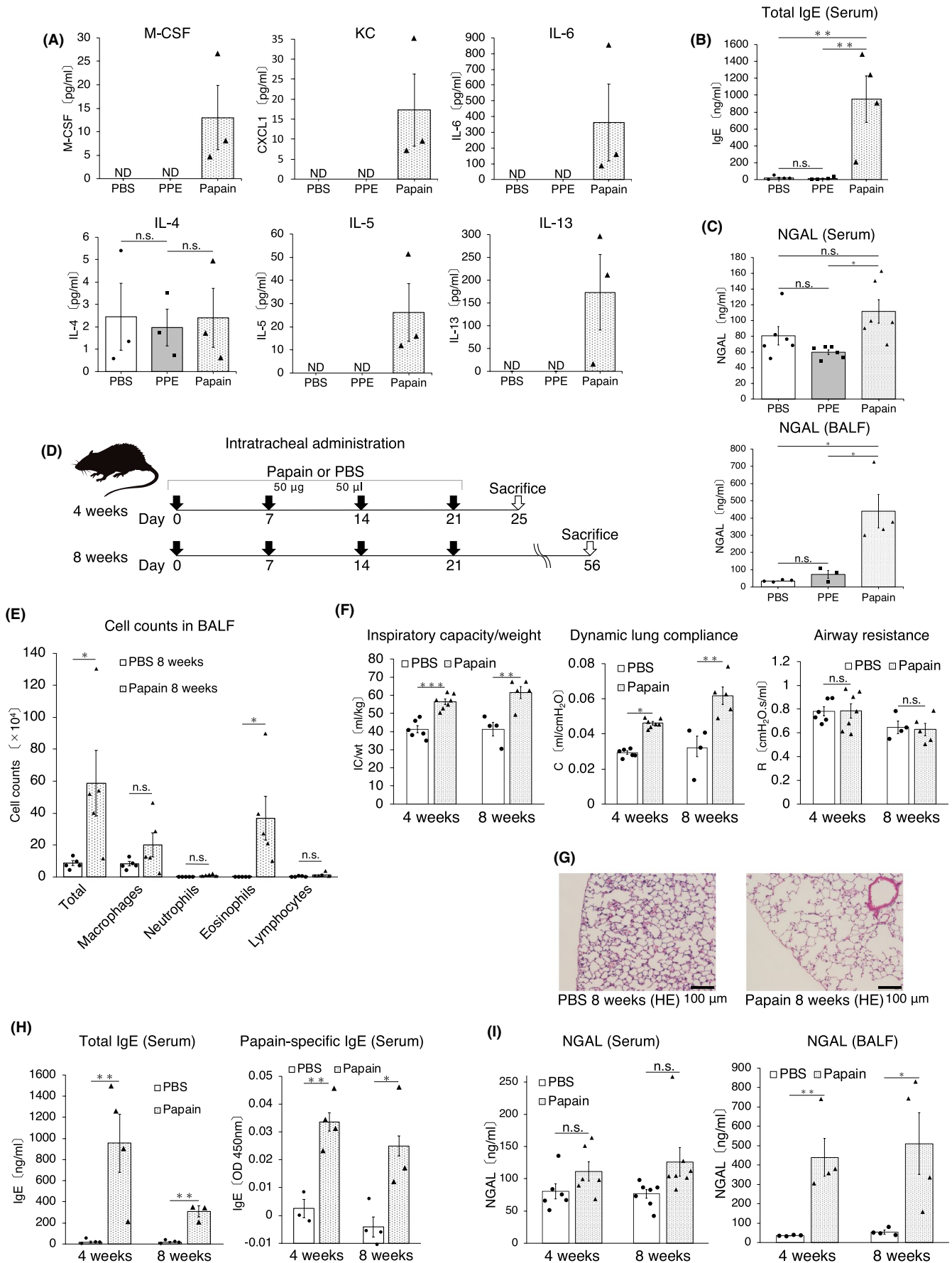


FIGURE 2 Papain induced an increase in the macrophage/neutrophil- and type 2 inflammation-associated cytokines; allergic airway inflammation and emphysema persisted for 5 wk after the final papain administration. (A) Cytokines in the BALF (B) Serum total IgE (C) Neutrophil gelatinase-associated lipocalin (NGAL) in the serum and BALF (D) Protocol (E) Differential cell counts from BALF cytopspins (F) Respiratory mechanics (G) Lung tissue stained with HE; 20x. (H, I) Total and papain-specific IgE and NGAL; (A) and (B, C, H, I) were measured by cytokine quantitative antibody array and ELISA, respectively; (A) $n = 3$, (B) $n = 4$, (C) $n = 6$ (serum) and $n = 3-4$ (BALF), (E) $n = 5$, (F) $n = 4-7$, (H) $n = 3-4$, (I) $n = 4-7$ per group, per experiment; * $P < .05$, ** $P < .01$, *** $P < .001$; ND, not detected; n.s. not significant with two-tailed Student's t test between two groups or one-way ANOVA with the Holm-Bonferroni post hoc test for more than two groups. Data are duplicates-quadruplicates from a single experiment

KEYWORDS

animal models, asthma, COPD

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CONFLICTS OF INTEREST

Dr Fukuda has nothing to disclose. Dr Matsuzaki has nothing to disclose. Dr Mikami has nothing to disclose. Dr Makita has nothing to disclose. Dr Miyakawa has nothing to disclose. Dr Miyashita has nothing to disclose. Dr Hosoki has nothing to disclose. Dr Ishii has nothing to disclose. Dr Noguchi has nothing to disclose. Dr Urushiyama has nothing to disclose. Dr Horie has nothing to disclose. Dr Mitani has nothing to disclose. Dr Yamauchi has nothing to disclose. Dr Shimura has nothing to disclose. Dr Nakae has nothing to disclose. Dr Saito has nothing to disclose. Dr Nagase has nothing to disclose. Dr Hiraishi reports grants from KAKENHI Grant of Japan Society for the Promotion of Science and grants from GSK Japan Research Grant 2019 during the conduct of the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1

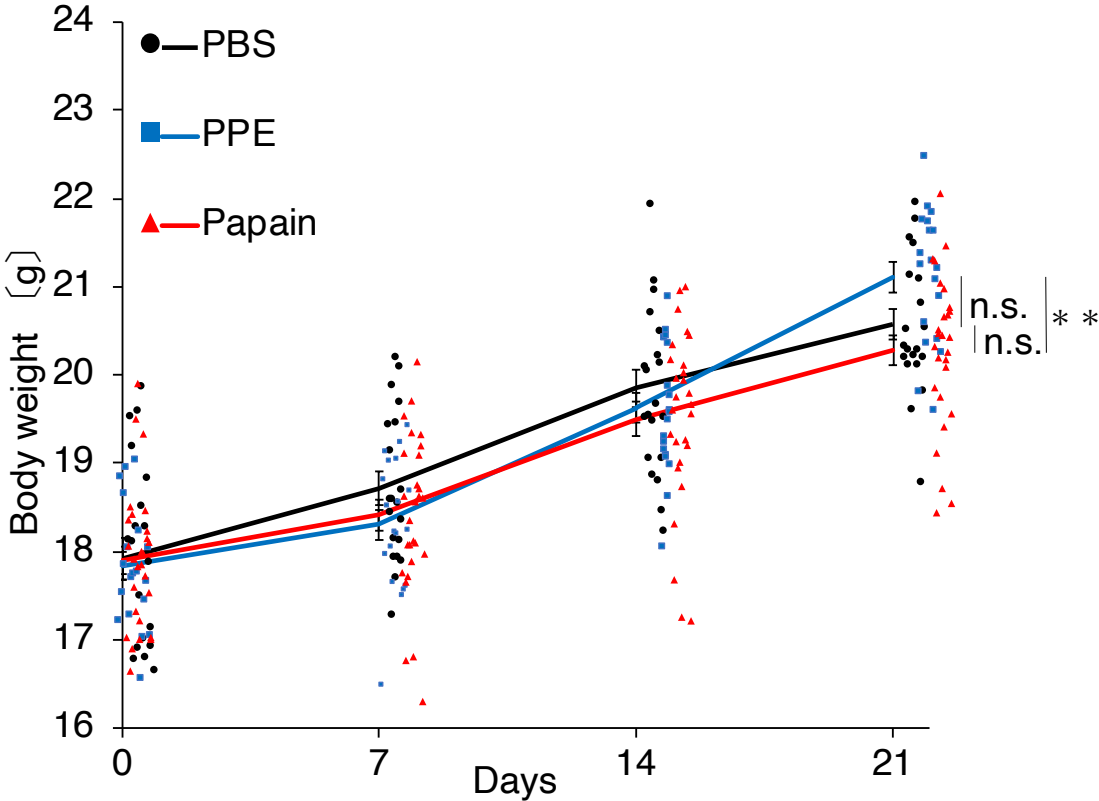


Figure S2

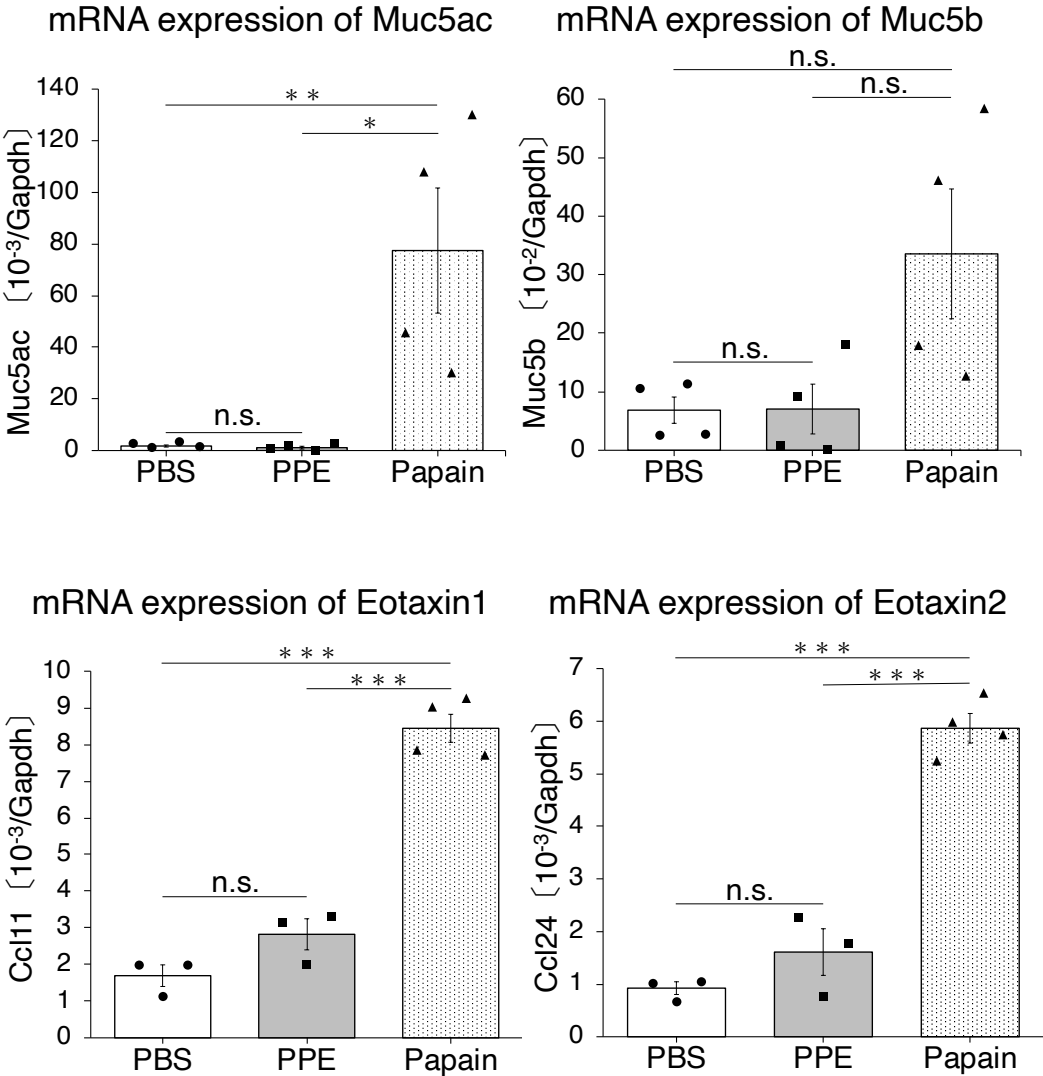
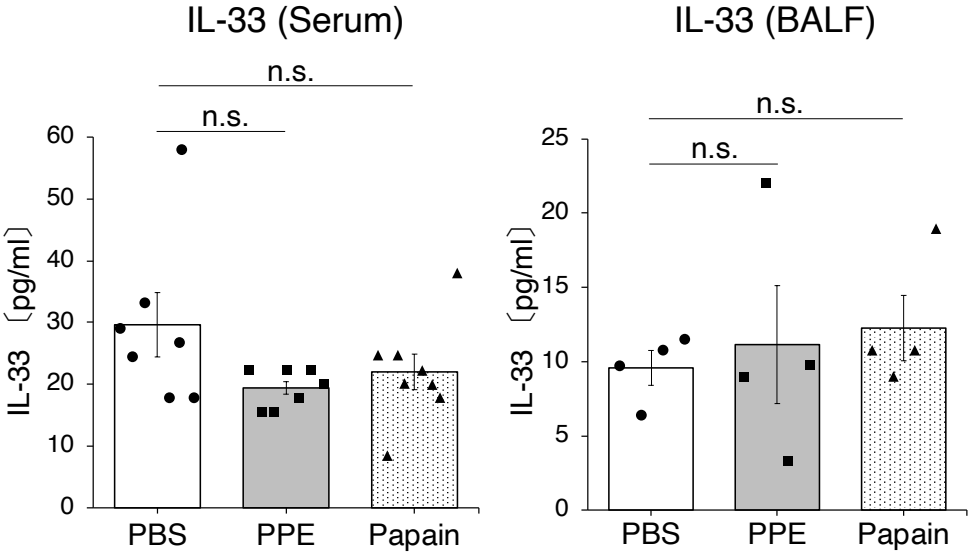


Figure S3



Appendix S1

Materials and Methods

Mice. Specific pathogen-free 5–6-week-old female C57BL/6N mice were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). All animal experiments were performed with the approval of the Animal Ethics Committee of the University of Tokyo (P17-006).

Models of airway inflammation. Mice were anaesthetised using 4.0% isoflurane (Mylan-Pfizer, Pennsylvania, USA). A COPD model was induced by treating mice intratracheally with 0.25 U of PPE (Merck KGaA, Darmstadt, Germany) in 50 µl of PBS on day 0. In the ACO model, mice were treated intratracheally with 50 µg of papain (Merck KGaA) in 50 µl of PBS on days 0, 7, 14, and 21. In each model, control mice were treated with the PBS vehicle. Papain or PPE was administered in aerosol form using Micro Sprayer Aerosolizer (Penn Century, Pennsylvania, USA). To generate acute exacerbation models, 100 µg of poly(I:C) (Tocris Bioscience, Bristol, UK) was intratracheally administered on days 22, 23, and 24 following PPE or papain treatment. Mice were sacrificed and analysed on days 25 or 56.

Bronchoalveolar lavage fluid analysis. To obtain the BALF, the trachea was catheterised, and the lungs were washed with 1 ml of saline three times. The BALF was centrifuged, pellets were dissolved in PBS, and the cells were counted. Differential cell counts were performed on cytopsin preparations stained with May-Grünwald-Giemsa.

Histopathological analysis. The lungs were inflated with 10% neutral buffered formalin for fixation, paraffin embedded, and cut into 5-µm sections. For histopathological assessments, the lung sections were stained with HE or AB–PAS. The mean linear intercept was calculated to assess the severity of pulmonary emphysema.¹ The degree of mucus secretion was assessed by examining the AB–PAS-stained sections.

Respiratory mechanics. The respiratory mechanics were analysed using a computer-controlled small-animal ventilator (flexiVent, Scireq, Quebec, Canada). Mice were weighed and euthanised with sodium pentobarbital (200 mg/kg, Kyoritsu Pharmaceutical, Nara, Japan) via intraperitoneal injection. Animals were tracheotomised, cannulated with a blunt 20-g needle, and connected to flexiVent. The inspiratory capacity, the airway resistance (R), and compliance (C) in a single-compartment model (snapshot method) were measured using the flexiVent software.

Determination of airway hyper-responsiveness. Mice were anaesthetised with an intraperitoneal injection of 2,2,2-tribromoethanol (300 mg/kg, Tokyo Chemical Industry, Tokyo, Japan), paralysed with pancuronium (3 mg/kg, Sigma-Aldrich, Missouri, USA), and intubated with a catheter after tracheotomy. Mice were placed on the flexiVent to measure airway resistance. To evaluate the airway hyper-responsiveness, we performed the methacholine challenge test.² Increasing concentrations of aerosolised methacholine (Sigma-Aldrich; 0, 50, 100, and 200 mg/ml) were administered.

ELISA and quantitative real-time PCR of lung mRNA. ELISAs for IL-33 (R & D Systems, Minnesota, USA), IgE (BioLegend, California, USA), and NGAL (R & D Systems) were performed as per the manufacturer's instructions. For papain-specific IgE assays, the coating buffer was substituted with 30 µg/ml of the papain solution. RNA was extracted from whole lung homogenates and reverse transcribed, and quantitative RT-PCR was performed. The expression of the genes of interest was calculated according to the comparative threshold cycle method by using Gapdh as the internal control. Following primer sequences were used.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Gapdh	CCCACTCTTCCACCTTCGATG	AGGTCCACCACCCTGTTGCT
Muc5ac	ACCACTTTCTCCTTCTCCACAC	AACAGGGCTCTTCACAGACAATA
Muc5b	AGGAAGACCAGTGTGTTTGTC	GTCCTCATTGAAGAAGGGCTG
Ccl11 (Eotaxin1)	AGCTAGTCGGGAGAGCCTAC	AAGGAAGTGACCGTGAGCAG
Ccl24 (Eotaxin2)	TCCCAAACATGCTGATGGGT	AAATCCTCCGTTTCTGCCAAC

For the amplification, we used 40 cycles at 95 °C for 5 s, annealing temperature (55 °C–60 °C) for 10

s and 72 °C for 21 s.

Antibody array detection of cytokine levels in the BALF. Cytokine levels in the BALF samples were measured in quadruplicates using the Quantibody mouse cytokine array kit (QAM-CYT-1-2, RayBiotech, Georgia, USA), in which multiple cytokines/chemokines were quantitatively detected. The assay was performed according to the manufacturer's protocol and the data were analysed with a laser scanner for microarray (GenePix 4400A, Molecular Devices, California, USA).

Statistics. The two-tailed Student *t* test was used for comparisons between two groups, whereas the one-way or two-way ANOVA was used for comparisons among multiple groups with the Holm–Bonferroni post hoc test for significance (JMP Pro 14.2.0, SAS Institute, North Carolina, USA). The level of statistical significance was set at $p < 0.05$. Data were presented as the mean $\pm$ SEM.

Supplemental Table S1

	PBS	PPE	Papain	PPE+Poly(I:C)	Papain+Poly(I:C)
GM-CSF	18.06 ($\pm$ 2.395)	15.08 ($\pm$ 1.970)	15.45 ($\pm$ 0.8398)	18.29 ($\pm$ 2.709)	13.64 ($\pm$ 2.463)
IFN- γ	25.36 ($\pm$ 6.080)	33.81 ($\pm$ 7.248)	26.08 ($\pm$ 4.268)	50.42 ($\pm$ 20.57)	30.16 ($\pm$ 5.597)
IL-1 α	1.602 ($\pm$ 1.055)	3.063 ($\pm$ 1.134)	3.717 ($\pm$ 0.7083)	9.052 ($\pm$ 0.8196)	10.26 ($\pm$ 1.238)
IL-1 β	15.27 ($\pm$ 4.282)	23.17 ($\pm$ 7.681)	14.20 ($\pm$ 1.665)	ND	ND
IL-2	17.71 ($\pm$ 2.586)	24.55 ($\pm$ 10.19)	10.72 ($\pm$ 3.905)	8.454 ($\pm$ 5.865)	8.915 ($\pm$ 1.934)
IL-3	2.168 ($\pm$ 0.2027)	2.085 ($\pm$ 0.2380)	2.032 ($\pm$ 0.1490)	2.160 ($\pm$ 0.1912)	1.863 ($\pm$ 0.09033)
IL-4	2.448 ($\pm$ 1.495)	1.968 ($\pm$ 0.8217)	2.401 ($\pm$ 1.317)	ND	4.411 ($\pm$ 0.4189)
IL-5	ND	ND	26.12 ($\pm$ 12.46)	ND	ND
IL-6	ND	ND	362.1 ($\pm$ 244.7)	1848 ($\pm$ 371.3)	731.2 ($\pm$ 298.0)
IL-9	95.93 ($\pm$ 38.63)	93.81 ($\pm$ 45.73)	92.39 ($\pm$ 40.30)	ND	ND
IL-10	ND	ND	ND	ND	ND
IL-12	7.216 ($\pm$ 2.439)	5.941 ($\pm$ 3.815)	5.554 ($\pm$ 2.330)	8.554 ($\pm$ 3.454)	4.997 ($\pm$ 1.615)
IL-13	ND	ND	173.6 ($\pm$ 82.79)	ND	291.1 ($\pm$ 116.8)
IL-17	4.496 ($\pm$ 1.583)	11.28 ($\pm$ 5.999)	9.658 ($\pm$ 0.8300)	ND	ND
KC	ND	ND	17.27 ($\pm$ 9.034)	14.21 ($\pm$ 6.342)	9.165 ($\pm$ 2.619)
MCP-1	20.75 ($\pm$ 2.047)	21.99 ($\pm$ 0.8488)	47.85 ($\pm$ 13.85)	610.5 ($\pm$ 43.70)	914.4 ($\pm$ 316.9)
M-CSF	ND	ND	13.03 ($\pm$ 6.842)	1.818 ($\pm$ 1.119)	3.344 ($\pm$ 0.7033)
RANTES	2.008 ($\pm$ 0.6949)	3.225 ($\pm$ 0.7369)	18.34 ($\pm$ 9.646)	456.9 ($\pm$ 110.8)	316.6 ($\pm$ 79.96)
TNF- α	14.72 ($\pm$ 6.985)	22.50 ($\pm$ 15.38)	37.25 ($\pm$ 9.076)	137.5 ($\pm$ 13.27)	52.06 ($\pm$ 13.90)
VEGF	35.15 ($\pm$ 4.788)	39.52 ($\pm$ 5.512)	48.96 ($\pm$ 7.898)	58.59 ($\pm$ 13.02)	10.70 ($\pm$ 2.302)

(pg/ml)

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