Cyclooxygenase inhibition in mice heightens adaptive- and innate-type responses against inhaled protease allergen and IL-33

Short title: NSAID heightens adaptive and innate immunity against inhaled protease

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**Abbreviations**

AERD: aspirin-exacerbated respiratory disease;
BALF: bronchial alveolar lavage fluid;
COX: cyclooxygenase;
NSAID: nonsteroidal anti-inflammatory drug;
OVA: ovalbumin;
PG: prostaglandin;
rIL-33: recombinant IL-33;
ST2: a subunit of the IL-33 receptor
TSLP: thymic stromal lymphopoietin
Cyclooxygenases (COXs) are responsible for the formation of prostanoids such as prostaglandins (PGs) and thromboxanes. Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity, provoke aspirin-exacerbated respiratory disease (AERD), and inhibition or deficiency of COXs exacerbates allergic asthma in murine models where ovalbumin (OVA) is used for immunization, although the mechanism has not been fully elucidated. Environmental allergen sources, such as house dust mites, insects, fungi, and pollen, contain proteases, which are frequently allergens. In the present study, we used the protease papain as a model allergen, which is more relevant to natural allergen exposure than OVA, and examined whether COX inhibition promotes adaptive-type (Fig. 1: the adaptive papain model) and innate-type responses (Fig. 2A, B: the innate papain model; Fig. 2C–E: the innate IL-33 model) in IL-33-mediated protease-dependent models (for further information, see Supporting Information).

Upon inhalation of 10 µg papain, mice treated with the NSAID indomethacin (Fig. 1A–D and Fig. E1A–C) or aspirin (Fig. E2) had severe allergic airway inflammation compared to that of the control group. Upon inhalation of 5 µg papain, which does not induce airway inflammation without NSAID treatment, indomethacin-treated mice had increased responses including airway mucus production and airway hyperresponsiveness to methacholine (Fig. 1E–I and Fig. E1D, E). Genes for IL-33, a subunit of the IL-33 receptor (ST2), and thymic stromal lymphopoietin (TSLP) are susceptible loci associated with allergic diseases including asthma, and nasal polyps from AERD patients show increased levels of IL-33 and TSLP. Next, we evaluated whether epithelial cytokines IL-33 and TSLP were involved in the immune response upon COX inhibition. Exacerbation by COX inhibition was markedly reduced in IL-33-deficient and TSLP receptor-deficient mice (Fig. 1J and Fig. E3), which suggests involvement of IL-33 and TSLP.

Tissue damage due to proteolytic activity of allergens induces the release and activation of IL-33. Airway inflammation induced by COX inhibition in papain-inhaled mice was associated
with infiltration of approximately equivalent numbers of ST2⁺ Th cells and ST2⁺ ILC2 and release of IL-5 and Th2-attracting chemokines into bronchial alveolar lavage fluid (BALF) (Fig. 1K, L and Figs. E4 and E5). COX inhibition in papain-inhaled mice promoted differentiation of antigen-specific T cells in bronchial draining lymph nodes to produce Th2 (IL-4, IL-5, IL-13, and amphiregulin), Th9 (IL-9), Th17 (IL-17A), and Th1 cytokines (IFN-γ) on antigen restimulation; costimulation with recombinant IL-33 (rIL-33) promoted production of IL-5, IL-13, amphiregulin, IL-9, and IFN-γ, whereas stimulation with rIL-33 alone induced the production of IL-5, IL-13, IL-9, and IFN-γ (Fig. 1M, N). Th cells including ST2⁺ Th cells mediated cytokine production in an antigen-dependent or IL-33-dependent manner, but we cannot exclude the contribution of innate cells, such as ILC2, which may be contained in the lymph node samples, to IL-33-dependent cytokine production. These results suggest that COX inhibition exacerbates the model via the “antigen plus IL-33-Th axis”¹⁰ and IL-33-ILC2 axis.⁸

Next, we examined whether COX inhibition affected the models in a way that was dependent on innate immunity involving the IL-33-ILC2 axis (Fig. 2 and Fig. E6). Indomethacin-treated Rag2-deficient mice showed exacerbation of airway eosinophilia upon 20 µg papain inhalation. Indomethacin-treated naive wild-type mice showed exacerbation of airway eosinophilia associated with an increase of Th2 cytokines and Th2-attracting chemokines upon low-dose (20 ng) inhalation of rIL-33. COX inhibition promoted the innate-type allergic response against inhaled papain or rIL-33.

In the OVA-alum models, inhibition or deficiency of COXs results in exacerbated airway inflammation mainly composed of eosinophils, airway mucus production, airway hyperresponsiveness, and increases Th2 and Th9 but attenuates Th17 differentiation and increases IgE in BALF (see Supplementary References).³ ⁴ However, in the adaptive papain model, indomethacin-treatment promoted differentiation of Th cells producing not only Th2 and Th9 but also Th17 and Th1 cytokines and airway inflammation mainly composed of eosinophils and
monocytes/macrophages. The experimental protocols were largely different between the two models. In the adaptive papain model, mice were sensitized and challenged with intranasal administration of papain with protease activity (5–10 μg; concentration: 0.1–0.2 mg/mL; days 0 and 8); while in typical OVA-alum models, mice were intraperitoneally sensitized with OVA (20 or 10 μg) with alum (day 0 and/or day 1) and challenged with 30–40 min inhalation of aerosols containing OVA (concentration: 10 mg/mL; 4–8 consecutive days from day 14). The intranasal sensitization without alum and lower antigen concentration for airway challenge are features of the adaptive papain model.

PG signaling has suppressive roles in allergic airway inflammation models such as the OVA-alum models (see Supplementary References). PGE$_2$ or PGI$_2$ signaling suppresses ILC2 responsiveness in vitro, and PGD$_2$ signaling promotes ILC2 responsiveness. NSAIDs decrease epithelial barrier integrity (see Supplementary References). The mechanism of eicosanoids downstream of COX in the models in the present study remains unclear. Leukotrienes, which are generated from arachidonic acid by the 5-lipoxigenase-mediated pathway but not the COX-mediated pathway, are upregulated in AERD. However, levels of leukotrienes were relatively low and no significant change was observed in our assay system (data not shown), but we cannot exclude the potential contribution of locally generated leukotrienes to the responses.

In conclusion, we found that NSAID treatment heightened both the adaptive-type and innate-type allergic responses against inhalation of papain or rIL-33. We demonstrated that COX inhibition by NSAID treatment promoted airway inflammation, airway mucus production, airway hyperresponsiveness, antigen-specific Th development, and IgE production in the adaptive papain model and exacerbated allergic airway inflammation in the innate papain model and innate IL-33 model. This study suggested that NSAID administration makes mice, which have been sensitized or not to allergens, more sensitive to stimulation with exogenous proteases or IL-33-inducing stimuli via involvement in IL-33-responsive Th cells and ILC2. Additionally, the models described in the present study may assist in elucidating the roles and mechanisms of prostanoid-mediated regulation.
of adaptive- and innate-types of allergic airway diseases.

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Author contributions. NM and T. Takai wrote the manuscript with help from the other authors. T. Takai organized the study. NM, T. Takai, SK, PS, MO, T. Takeshige, MS, MH, KM, and SH performed the experiments and/or analyzed the data. NM, T. Takai, SK, NH, SN, KS, TO, TY, HO, KO, and SI contributed to the study design and/or interpretation of the data. All the authors read and approved the final manuscript.

COI disclosure. The authors have no conflict of interest in relation to this work.
References


**Figure Legends**

**Fig. 1. Cyclooxygenase inhibition promotes allergic airway inflammation in the adaptive papain model.**  
M, N: Draining lymph node (DLN) cell cytokine production (M: restimulated with the antigen). *P < 0.05 (N: vs no indomethacin) by a Student's t-test or Mann-Whitney U test. †P < 0.05 (N: vs Medium) by ANOVA. i.n., intranasal administration. Indomethacin, drinking water containing indomethacin.  
TSLPR, TSLP receptor. ns, not significantly different.

**Fig. 2. Cyclooxygenase inhibition promotes innate-type allergic inflammation induced by inhalation of papain or rIL-33.**  
Papain or rIL-33 was intranasally administered to mice with or without indomethacin treatment. A, B: Timeline and airway eosinophilia upon high-dose papain inhalation in Rag2−/− mice, which lack adaptive immune cells. C–E: Timeline of airway eosinophilia and BALF for IL-5, IL-13 and Th2-attracting chemokines upon rIL-33 inhalation in naive wild-type mice. *P < 0.05 by a Student's t-test. i.n., intranasal administration. Indomethacin, drinking water containing indomethacin.
Supporting Information

Methods

Mice. Female 7–11-wk-old C57BL/6J mice (Sankyo Lab Japan, Ibaraki, Japan), IL-33−/− (C57BL/6N background), thymic stromal lymphopoietin (TSLP) receptor (TSLPR)−/−, and Rag2−/− mice (C57BL/6J background) were maintained in a specific pathogen-free animal facility at Juntendo University and were used according to the guidelines of the Institutional Committee on animal experiments.

NSAID administration. Indomethacin (Sigma, St. Louis, MO) (15 µg/mL), which is half the concentration used by Peebles et al.3,4, or aspirin (Sigma) (0.18 mg/mL) was administered in the drinking water starting 2 days before the first intranasal (i.n.) administration of papain or recombinant mouse IL-33 (rIL-33) [day (-2)]. Stock solutions were prepared by dissolving indomethacin (120 mg) or aspirin (1.44 g) in 40 mL of 99.5% ethanol. One milliliter of the stock solution of indomethacin or aspirin or 1 mL of 99.5% ethanol as the control was added to 200 mL of drinking water. The bottles were exchanged two or three times per week.

i.n. administration. Mice were intranasally (i.n.) administered papain (Calbiochem, San Diego, CA) dissolved in 50 µL PBS on day 0 and day 8 or administered rIL-33 (20 ng/50 µL) (BioLegend, San Diego, CA) on day 1, 2, and 3. Sera, mediastinal draining lymph nodes, and bronchial alveolar lavage fluid (BALF) were collected one, four, or more than four days after the last papain inhalation and the day after the last rIL-33 inhalation and analyzed as described below.

Recovery of BALF. Mice were terminally anesthetized, the tracheas were cannulated, and internal airspaces were lavaged with the tracheas were cannulated, and internal airspaces were lavaged with 20 gILC2s and CD4+ T cells in BALF samples by flow cytometry. Flow cytometry of BAL cells. Some studies detected ILC2s and CD4+ T cells in BALF samples by flow cytometry. The schematic gating strategy is shown in Fig. E4. Fc receptors (FcR) were blocked with purified anti-mouse CD16/32 (clone: 2.4G2, TONBO biosciences) in 10% normal rat serum. After blocking the FcR, cells were stained with mAbs (see below). Lineage CD25−CD44+ ILCs were analyzed as follows: BAL cells were stained and analyzed using the following Abs: PE-Cy7-conjugated anti-mouse lineage markers [CD3 (clone 145-2C11), CD11b (clone M1/70), CD16/CD32 (clone 2.4G2), TER-119 (clone TER-119) (BD Pharmingen, New Jersey, CA), CD11c (clone N418), B220 (clone RA3-6B2), FcεRIε (MAR-1) (BioLegend), CD49b (DX5), and CD14 (clone Sa2-8) (eBioscience, Santa Clara, CA)], allophycocyanin-conjugated anti-mouse CD25 (clone PC61), FITC-conjugated anti-mouse CD44 (clone IM7) (BioLegend), biotinylated anti-mouse T1/ST2 (clone DJ8) (MD Bioproducts, Zürich, Switzerland), and streptavidin-PE (BD Pharmingen). CD44+CD62L+CD4+ cells were analyzed as follows: BAL cells were stained and analyzed using the following Abs: PE-Cy7-conjugated anti-mouse CD44 (clone IM7), PE-conjugated anti-mouse CD4 (clone RM4-5), streptavidin-APC, FITC-conjugated anti-mouse CD62L (clone MEL-14) (BioLegend). Normal rat serum (MILLIPORE) was inactivated by heating at 56 °C for 30 minutes. Acquisition and analyses were performed using a FACS Verse cell sorter (BD Biosciences) and FLOWJO software (Tomy, Tokyo, Japan). Threshold fluorescence levels were determined by fluorescence levels of the following isotype control antibodies: FITC-conjugated rat IgG2a and rat IgG2b, PE-conjugated rat IgG2b and streptavidin, PE-Cy7-conjugated rat IgM (BD Biosciences), PE- Cy7-conjugated Armenian hamster IgG, rat IgG2a, rat IgG2b, APC-conjugated rat IgG1 and streptavidin, and biotinylated rat IgG1 (BioLegend). Zombie violet fixable viability kit (BioLegend) was used to detect dead cells. In Fig. 1K, 1.8–3.8×10^4, 1.8–7.7×10^4, and 5.0–10.1×10^5 BAL cells from vehicle drinking/PBS i.n., vehicle drinking/papain i.n., and indomethacin drinking/papain i.n. groups, respectively, were used for staining of ST2+ ILC2s and ST2+ CD4+ T cells.

Airway responsiveness. Mice were anesthetized with pentobarbital and xylazine and then intubated with metal 18-gauge catheters via a tracheotomy. The needle was then immediately connected to the flexiVent™ (SCIREQ, Montreal Qc, Canada). The mice were mechanically ventilated with an average breathing frequency of 150 breaths/min. After measurement of baseline resistance (saline inhalation), the mice were challenged with increasing concentrations (0, 6, 12, 24, and 48 mg/mL) of methacholine aerosol generated with an in-line nebulizer and administered directly through the ventilator for 5 s.

Histology. Lungs were removed from mice that were not subjected to the bronchial lavage procedure. Lungs were inflated with intratracheal instillation of 20% buffered formalin (pH7.4) and fixed for more than 24 hours. The fixed tissues were embedded in paraffin, sliced, and subjected to periodic acid-Schiff staining.

Restimulation of draining lymph node cells. Draining lymph node cells were collected from mice 24 hours (Fig. 1M) or 4 days (Fig. 1N) after the last i.n. administration. Draining lymph node cells were placed in RPMI1640 medium with 10% FCS, 10 mM HEPES, 0.0004% 2-mercaptoethanol, and antibiotics and were grinded aseptically. Blood erythrocytes were lysed with ACK lysis buffer. 100 µg/mL antigen (Fig. 1M) or medium, 25 µg/mL antigen, rIL-33 (10 ng/mL), or both (Fig. 1N)
and lymph node cells ($5 \times 10^5$ cells/200 µL/well) were incubated in flat-bottomed 96-well culture plates at 37 °C. After 72 hours (Fig. 1M) or 92 hours (Fig. 1N), plates were centrifuged at 300 × g for 5 min, and culture supernatant was recovered. We used a covalent complex between papain and the protease inhibitor E-64** as the antigen for restimulation of lymph node cells to avoid potential protease activity-dependent effects.

Cytokine/chemokine ELISA. Cytokine and chemokine concentrations were measured with ELISA kits (Duo Set; R&D Systems). For IL-9 ELISA, the kit was purchased from BioLegend. Lower detection limits were indicated for standard, and then loaded on an Oasis HLB cartridge with a fixed amount of methanol previously measured by HPLC-electrospray ionization tandem mass spectrometry (MS/MS). A chiralpak AD-RD column (Crawford Scientific, Strathaven, Scotland) was used for chiral separation.

Measurement of lipid mediators. Lipid mediators were measured by a reversed-phase mass spectrometry (MS)-based quantitation technique as described previously. BALFs were diluted with an equal amount of methanol containing 0.1% formic acid, mixed with deuterium-labeled eicosanoids as an internal standard, and then loaded on an Oasis HLB cartridge (Waters, Milford, MA, USA). The column was washed with 1 mL of water, 1 mL of 15% methanol, and 1 mL of petroleum ether and then eluted with 0.2 mL of methanol containing 0.1% formic acid. Eicosanoids were quantified by reverse-phase HPLC-electrospray ionization tandem mass spectrometry (MS/MS). A chiralpak AD-RD column (Crawford Scientific, Strathaven, Scotland) was used for chiral separation.

Statistical analysis. A one-way ANOVA with the Tukey post hoc test, the Mann-Whitney U test (two-tailed), or Student’s t-test (two-tailed) was used as indicated in the figure legends. A value of $p < 0.05$ was regarded as significant. Data were representative of two independent experiments with similar results. In Fig. 1, *$P < 0.05$ by a Student's t-test (Fig. 1, B–D, F–J) or Mann-Whitney U test (Fig. 1, K–M).

Numbers of mice. The numbers of mice (n) used in each of the experimental groups were n = 4–8 (Fig. 1B, C), n = 5 (Fig. 1D), n = 4–6 (Fig. 1F, G), n = 4 (Fig. 1I), n = 4 or 5 (Fig. 1J), n = 6 or 7 (Fig. 1K), n = 3–7 (Fig. 1L, M); n = 2–4 (Fig. 2B, C), n = 3 (Fig. 2D, E); n = 3–8 (Fig. E2A, B), and n = 4 or 5 (Fig. E2C). In Fig. 1H, representative data of a mouse from each group are shown. In Fig. 1K, the draining lymph node cells from three mice for each of the three groups were pooled and stimulated in four wells.

Discussion

IL-33 and TSLP. IL-33 and TSLP are epithelial cytokines, which are important in both the innate and adaptive allergic responses. Genome-wide association studies revealed that IL-33, ST2, and TSLP genes are susceptible loci associated with allergic diseases including asthma. Nasal polyps from AERD patients show increased levels of IL-33 and the active form of TSLP. Upon inhalation of 5 µg papain, indomethacin drinking slightly exacerbated airway inflammation in TSLP−/− mice, but airway inflammation was less severe than in wild-type mice (Fig. 1L and Fig. E3A). Because IL-33−/− mice with or without indomethacin drinking showed no airway eosinophilia and IgE/IgG1 response upon 5 µg papain inhalation (unpublished data), we examined higher doses of inhalation (20 µg and 40 µg).

ST2+ Th cells and ILC2. In the model without COX inhibition, the IL-33-ILC2 axis is critical to Th2 differentiation, airway recruitment of Th2 cells, and airway eosinophilia; but ST2+ Th cells are involved in other murine models and IL-33-induced IL-5 and/or IL-13 production of Th cells occurs in nasal polyps of patients with eosinophilic chronic rhinosinusitis.

Innate models. Rag2−/− mice with or without indomethacin drinking showed no airway eosinophilia and IgE/IgG1 response upon 5 µg papain inhalation (unpublished data). We examined higher-dose inhalation (20 µg and 40 µg). The dose of rIL-33 administered in the present study (20 ng) (Fig. 2, C–E and Fig. 5B) was less than that administered to induce airway eosinophilia without indomethacin treatment in previous reports (500 ng or 100 ng).

Comparison between the adaptive i.n. papain model and OVA-alum models. In OVA-alum models, inhibition or deficiency of COX enzymes results in exacerbated airway inflammation mainly composed of eosinophils, airway mucus production, airway hyperresponsiveness, and increased Th2 and Th9, but attenuated Th17 differentiation and increased BALF IgE.

Possible roles of eicosanoids downstream of COX. The mechanisms behind the negative regulation by eicosanoids downstream of COX, for example COX-1 and COX-2, cellular sources, and prostanoid-receptor pathway(s) should be investigated in adaptive and innate i.n. papain models and the innate i.n. rIL-33 model in future studies. PGE2, PGD2, and PGI2 signaling has suppressive roles in the OVA-alum and i.n. house dust mite extract models and i.n. house dust mite extract models. Recent studies demonstrated that PGE2-EP2/EP4 and PGI1-IP signaling negatively and PGD2-CRTH2 signaling positively regulates ILC2 responsiveness to IL-33 to produce Th2 cytokines in vitro. Lipoxin A4 suppresses ILC2 responsiveness, but not eicosanoids downstream of COX. NSAIDs decrease epithelial barrier integrity.
Supplementary References


**Supplementary Figure Legends**

**Fig. E1.** Airway inflammation, papain-specific IgG1 responses, and BALF prostanoids in the adaptive papain model with or without COX inhibition. Fig. E1 is supplementary to Fig. 1, A–G. A–C: Airway inflammation (A), serum papain-specific IgG1 response (B) and BALF prostanoids (C) upon 10 µg papain inhalation. D, E: Airway inflammation (D) and serum papain-specific IgG1 response (E) upon 5 µg papain inhalation. *P < 0.05 vs without indomethacin* by a Student’s t-test, *P < 0.05 by ANOVA.

**Fig. E2.** COX inhibition by aspirin promotes papain-induced allergic inflammation on days 14–18 upon 10 µg papain inhalation. A: Airway inflammation. B: Serum antibody responses. C: BALF prostanoids. *P < 0.05 vs without indomethacin* by a Student’s t-test.

**Fig. E3.** Airway inflammation and papain-specific antibody responses in TSLPR<sup>−/−</sup> and IL-33<sup>−/−</sup> mice in the adaptive papain model with or without COX inhibition. Fig. E3 is supplementary to Fig. 1, J, A, B: TSLPR<sup>−/−</sup> mice. C, D: IL-33<sup>−/−</sup> mice. *P < 0.05 vs (without indomethacin) by a Student’s t-test. *P < 0.05 by ANOVA.

**Fig. E4.** Gating strategy for flow cytometry of ST2<sup>+</sup> ILC2s and ST2<sup>−</sup> CD4<sup>+</sup> T cells. BALF samples were collected on day 9 and BAL cells were analyzed by flow cytometry. A: Gating strategy for total BAL cells, lymphocytes, and frequency of Zombie violet<sup>+</sup> dead cells among total BAL cells. B: Gating strategy for ST2<sup>+</sup> ILC2s and levels of non-specific staining with isotype control antibodies and autofluorescence. C: Gating strategy for ST2<sup>+</sup> CD4<sup>+</sup> CD62L CD44<sup>+</sup> T cells and levels of non-specific staining with isotype control antibodies and autofluorescence. Dot plots and histograms are representatives of 8 (A) or 4 (B and C) samples. Frequencies are shown as means ± SD of 8 (A) or 4 (B and C) samples.

**Fig. E5.** Cytokines/chemokines in BALF in the adaptive papain model with or without COX inhibition. Fig. E5 is supplementary to Fig. 1, L. *P < 0.05 vs without indomethacin* by Mann-Whitney U test and *P < 0.05 by ANOVA.

**Fig. E6.** Airway inflammation in innate models with or without COX inhibition. Fig. E6 is supplementary to Fig. 2, A–D. A: The innate papain model in Rag2<sup>−/−</sup> mice. B: The innate rIL-33 model in naive wild-type mice. *P < 0.05 vs (without indomethacin) by a Student’s t-test, *P < 0.05 by ANOVA.
Figure E4