Inhibition of Both Cyclooxygenase-1 and -2 Promotes Epicutaneous Th2 and Th17 Sensitization and Allergic Airway Inflammation on Subsequent Airway Exposure to Protease Allergen in Mice

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Keywords

Protease allergen · Nonsteroidal anti-inflammatory drugs · Cyclooxygenases · Epicutaneous sensitization · Airway challenge

Abstract

Introduction: Epicutaneous (e.c.) allergen exposure is an important route of sensitization toward allergic diseases in the atopic march. Allergen sources such as house dust mites contain proteases that involve in the pathogenesis of allergy. Prostanoids produced via pathways downstream of cyclooxygenases (COXs) regulate immune responses. Here, we demonstrate effects of COX inhibition with nonsteroidal anti-inflammatory drugs (NSAIDs) on e.c. sensitization to protease allergen and subsequent airway inflammation in mice. **Methods:** Mice were treated with NSAIDs during e.c. sensitization to a model protease allergen, papain, and/or subsequent intranasal challenge with low-dose papain. Serum antibodies, cytokine production in antigen-restimulated skin or bronchial draining lymph node (DLN) cells, and airway in-

flammation were analyzed. **Results:** In e.c. sensitization, treatment with a nonspecific COX inhibitor, indomethacin, promoted serum total and papain-specific IgE response and Th2 and Th17 cytokine production in skin DLN cells. After intranasal challenge, treatment with indomethacin promoted allergic airway inflammation and Th2 and Th17 cytokine production in bronchial DLN cells, which depended mostly or largely on COX inhibition during e.c. sensitization or intranasal challenge, respectively. Co-treatment with COX-1-selective and COX-2-selective inhibitors promoted the skin and bronchial DLN cell Th cytokine responses and airway inflammation more efficiently than treatment with either selective inhibitor. **Conclusion:** The results suggest that the overall effects of COX downstream prostanoids are suppressive for development and expansion of not only Th2 but also, unexpectedly, Th17 upon exposure to protease allergens via skin or airways and allergic airway inflammation.

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Introduction

Allergen sources such as house dust mites, fungi, and pollen produce or contain proteases, which are frequently allergens themselves. The proteolytic activity of allergens involves in the pathogenesis of allergies [1–4]. The papaya fruit-derived occupational protease allergen, papain, and house dust mite major allergens Der f 1 and Der p 1 belong to the same family of cysteine proteases [5, 6]. Papain has been used as a model protease allergen that mimics those contained in allergen sources. Recent studies using murine models of airway inflammation [7–10] or sensitization via skin [11–14] demonstrated that the protease activity of papain is important to induce the airway and skin inflammation and serum IgE/IgG1 responses.

Clinically, development of allergic diseases such as asthma, rhinitis, and food allergy after earlier epicutaneous (e.c.) presensitization to allergens is known as the atopic march, a natural history of allergic diseases [15, 16]. e.c. administration of protease allergen in mice disrupts skin barrier function and stimulates epidermal, neuronal, and immune responses [1, 3, 4, 11, 12, 17, 18]. Our recent study showed that e.c. presensitization to papain contributed to the onset of allergic airway inflammation, which was triggered by a subsequent airway challenge with low-dose papain [10, 12, 19].

Cyclooxygenase (COX)-1 and COX-2 are the enzymes involved in the conversion of arachidonic acid to prostanooids [20, 21]. COX inhibition by treatment with non-steroidal anti-inflammatory drugs (NSAIDs) provokes respiratory reactions in patients suffering from NSAID-exacerbated respiratory disease [22]. Inhibition or deficiency of COXs exacerbates allergic asthma in murine models where chicken egg ovalbumin (OVA) has been used as an experimental model antigen for immunization [23, 24]. In e.c. sensitization models to OVA or hapten, COX inhibition exacerbates Th2-driven skin inflammation [25, 26] but limits Th17/Th22-driven inflammation [27].

Recently, we reported that COX inhibition promoted airway inflammation in adaptive-type and innate-type asthma models with i.n. papain administration and an innate-type model with i.n. IL-33 administration [28]. However, whether COX inhibition affects e.c. sensitization to protease allergens and subsequent airway inflammation is still unknown. In the present study, we examine the effect of COX inhibition on e.c. papain sensitization and the onset of allergic airway inflammation triggered by subsequent i.n. antigen challenge in mice.

Materials and Methods

Mice

Female 7- to 11-week-old C57BL/6J mice (Sankyo Lab Japan, Ibaraki, Japan) were maintained in a specific pathogen-free animal facility at Juntendo University and used in accordance with the guidelines of the institutional committee on animal experiments after institutional approval of experiments.

Treatment with NSAIDs

Indomethacin (Sigma, St. Louis, MO, USA), SC560 (COX-1-selective inhibitor) [23, 29] (Abcam, Cambridge, UK), and rofecoxib (COX-2-selective inhibitor) [30] (Tokyo Chemical Industry, Tokyo, Japan) were administered in drinking water (15 µg/mL, which is the half of the concentration used by Peebles et al. [23] or 30 µg/mL) starting 2 days before the first e.c. or i.n. administration of papain.

e.c. Sensitization

A volume of 12.5 µL of papain solution (Calbiochem, San Diego, CA, USA) (10, 1 or 0.1 mg/mL in PBS containing 0.5% (v/v) Tween 20; 125, 12.5, or 0.125 µg/side) was applied to each sides of the surface of the both ears of lightly anesthetized mice (500, 50, or 0.5 µg/50 µL/mouse) followed by being held with a finger wearing a plastic glove for 2 s in the present study, which was performed differently from some of our previous studies [11, 12, 31]. This procedure was repeated twice per week for 2 weeks for a total of 4 times. Aliquots of papain solution were stored at –80°C and thawed just before use.

i.n. Challenge and Bronchial Alveolar Lavage

e.c. sensitized mice with light anesthetization were i.n. challenged with low-dose papain resolved in PBS (2.5 µg/50 µL/animal) after the last e.c. administration twice with a 4-day interval. Sera and bronchial alveolar lavage fluids were collected 4 days after the last i.n. administration and analyzed, as described previously [10, 28].

Restimulation of Draining Lymph Node Cells

Skin draining lymph nodes (DLNs; cervical LNs) or bronchial DLNs (mediastinal LNs) were collected. DLN cells were restimulated with medium alone or E-64 (Peptide Institute, Osaka, Japan)-treated papain (E-64-papain) in the presence or absence of recombinant IL-33 (10 ng/mL; BioLegend, San Diego, CA, USA) in 96-well culture plates for 96 h, as described previously [10, 19]. We used a covalent complex between papain and the protease inhibitor E-64 as the antigen for restimulation to avoid potential protease activity-dependent effect.

ELISA

Serum total IgE and papain-specific IgE and IgG1 were measured, as described previously [10]. Cytokine and chemokine concentrations were measured with ELISA kits (R&D Systems), except for IL-9 (BioLegend).

Statistical Analysis

A one-way ANOVA with Tukey's post hoc test, the Kruskal-Wallis test with Dunn's 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 statistically significant.

Fig. 1. Treatment with indomethacin promoted e.c. papain Th2 and Th17 sensitization. The animal groups administered with indomethacin or vehicle were e.c. sensitized to papain (0.1, 1, or 10 mg/mL) via intact ear skin. **a** Time line. **b** Antibody responses. **c** Skin DLN cell cytokine responses restimulated in vitro. Data are indicated as the mean $\pm$ SD of 5 mice per group (**b**) and 3 wells of

pooled DLN cells (**c**) and are representative of 3 independent experiments with similar results. **c** Detection limits are indicated by dotted lines. * $p < 0.05$ by the t test (**b**) or ANOVA (**c**: vs. no indomethacin, among the 8 groups). # $p < 0.05$ by ANOVA (**b**: vs. 0.1 mg/mL, among the 3 animal groups; **c**: vs. medium, among the 8 groups). DLN, draining lymph node; e.c., epicutaneous.

Results

Treatment with Indomethacin Promoted e.c. Papain Th2 and Th17 Sensitization

For e.c. sensitization, papain solutions with different concentrations were applied to ear skin of mice (Fig. 1a). Indomethacin was administered in drinking water. e.c. administration of 10 mg/mL of papain induced higher levels of serum total IgE and papain-specific IgE and IgG1 than that of lower concentrations of papain (Fig. 1b). COX inhibition by treatment with indomethacin enhanced serum total IgE and papain-specific IgE but not IgG1 levels in mice e.c. administered with 10 mg/mL of papain. In the model, mice were e.c. sensitized to papain

via intact ear lobe skin, which caused less severe skin inflammation than other models with e.c. sensitization via skin tape-stripped or treated with detergent [12, 19], and no significant difference of ear thickness was observed between mice with and without the indomethacin treatment (unpublished observations). Skin DLN cells from mice e.c. sensitized with 10 mg/mL of papain were restimulated with the antigen for cytokine production by papain-specific Th cells in the presence or absence of IL-33 (Fig. 1c), which is indispensable to papain-induced allergic airway inflammation [7, 12, 19]. Antigen-restimulated skin DLN cells from indomethacin-treated mice with e.c. papain sensitization produced higher levels of Th2 (IL-4, IL-5, and IL-13) and Th17 (IL-17A) cytokines

Fig. 2. Treatment with indomethacin promoted allergic airway inflammation in mice, which were e.c. papain sensitized and subsequently i.n. challenged with low-dose papain. The animal groups administered with indomethacin or vehicle were e.c. sensitized (0.1, 1 or 10 mg/mL papain) via intact ear skin and subsequently i.n. challenged (2.5 μ g papain). **a** Time line. **b** Airway inflammation. **c** Antibody responses. Data are indicated as the mean $\pm$ SD

of 5 mice per group and are representative of 3 independent experiments with similar results. * $p < 0.05$ by the t test or Mann-Whitney U test (**b, c**). # $p < 0.05$ (vs. 0.1 mg/mL among the 3 animal groups) by ANOVA (**b**) and the Kruskal-Wallis test (**c**). \$ $p < 0.05$ by the t test or Mann-Whitney U test (**c**: vs. the Fig. 1b data obtained before i.n. challenge). e.c., epicutaneous. BAL, bronchial alveolar lavage

Fig. 3. COX inhibition during the e.c. sensitization or i.n. challenge phase contributed to the enhancement of airway inflammation modestly or largely, respectively. Mice were administered with indomethacin or vehicle during e.c. sensitization, i.n. challenge, or both phases. All the animal groups were e.c. sensitized to 10 mg/mL papain via intact ear skin and subsequently i.n. challenged (2.5 µg papain). **a** Time line. **b** Airway inflammation. **c** Th2-attracting

chemokines in BAL fluid. **d** Antibody responses. 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 < 0.05 by ANOVA among the 4 animal groups. # p < 0.05 by the Mann-Whitney U test (vs. vehicle). \$ p < 0.05 by the Mann-Whitney U test (**d**: vs. indomethacin during e.c. sensitization). COX, cyclooxygenase; e.c., epicutaneous; BAL, bronchial alveolar lavage.

with a synergistic increase in the presence of IL-33 and showed a tendency of an increase in production of a Th17/Th22 cytokine, IL-22. These results indicate that COX inhibition promotes e.c. papain sensitization for papain-specific IgE, IgG1, Th2, and Th17.

Treatment with Indomethacin Promoted Allergic Airway Inflammation in e.c. Presensitized Mice on Subsequent i.n. Challenge with Low-Dose Papain

Next, we examined induction of airway inflammation by i.n. challenge with low-dose papain in mice (Fig. 2a). Mice that were e.c. presensitized with 1 mg/mL of papain showed the most severe allergic airway inflammation (Fig. 2b). COX inhibition promoted lung eosinophilia and infiltration of lymphocytes upon the i.n. challenge in mice e.c. presensitized with papain. The i.n. challenge enhanced serum levels of papain-specific IgE and IgG1 and/

or total IgE in mice, which were e.c. presensitized to 10 mg/mL of papain with the indomethacin treatment or 1 mg/mL of papain with or without the indomethacin treatment (Fig. 2c), in comparison to the levels before the i.n. challenge (Fig. 1b).

Before the i.n. challenge, indomethacin treatment of mice e.c. sensitized to 10 mg/mL but not lower concentrations of papain promoted induction of serum papain-specific IgE (Fig. 1b). After the i.n. challenge, without the indomethacin treatment, mice e.c. presensitized to 10 mg/mL of papain showed less allergic airway inflammation than those e.c. presensitized to 1 mg/mL of papain (Fig. 2b). Focusing on these issues, next, we examined whether COX inhibition during e.c. sensitization to 10 mg/mL of papain or that during the effector phase with i.n. challenge contributed to the enhancement of airway inflammation (Fig. 3).

Fig. 4. COX inhibition during the e.c. sensitization or i.n. challenge phases contributed to the promotion of Th2/Th9/Th17 expansion in bronchial DLNs after the i.n. challenge modestly or largely, respectively. Mice were administered with indomethacin or vehicle during e.c. sensitization, i.n. challenge, or both phases. All the animal groups were e.c. sensitized to 10 mg/mL papain via intact ear skin and subsequently i.n. challenged (2.5 µg papain). **a** Time line. **b** Bronchial DLN cell cytokine responses restimulated in vitro.

Data are indicated as the mean ± SD of 4 wells of pooled DLN cells from 5 or 4 mice per group and are representative of 3 independent experiments with similar results. Detection limits are indicated by dotted lines. * $p < 0.05$ by ANOVA among the 4 animal groups (vs. no indomethacin). # $p < 0.05$ by ANOVA among the 4 animal groups (vs. medium). COX, cyclooxygenase; DLN, draining lymph node; e.c., epicutaneous.

COX Inhibition during the e.c. Sensitization or i.n. Challenge Phase Contributed to the Enhancement of Airway Inflammation Modestly or Largely, Respectively

Water containing indomethacin or vehicle was fed to mice during e.c. sensitization, i.n. challenge, or both (Fig. 3a). The group with COX inhibition throughout the experiment showed the most severe allergic airway inflammation (Fig. 3b) and the highest levels of Th2-attracting chemokine release in the lung (Fig. 3c) and serum total and papain-specific IgE (Fig. 3d), being followed by the group with COX inhibition during the i.n. challenge phase only.

COX Inhibition during the e.c. Sensitization or i.n. Challenge Phase Contributed to the Promotion of Th2/Th9/Th17 Responses in Bronchial DLNs after the i.n. Challenge Modestly or Largely, Respectively

Using mice with the same in vivo treatment (Fig. 3a), we analyzed cytokine responses upon in vitro restimulation of bronchial DLN cells with the antigen in the presence or absence of IL-33 (Fig. 4a). On the DLN cell restimulation with the antigen plus IL-33, 2 groups with COX inhibition during the i.n. challenge showed higher levels of production of Th2 (IL-5 and IL-13) and Th9 (IL-9) cytokines than the other 2 groups without COX inhibition during the i.n. challenge (Fig. 4b). The results shown in Figures 3 and 4 indicat-

Fig. 5. Co-treatment with COX-1-selective and COX-2-selective inhibitors promoted Th2/Th17 differentiation in e.c. papain sensitization. Mice were administered with COX-inhibitor(s) or vehicle during e.c. sensitization. All the animal groups were e.c. sensitized to 10 mg/mL papain via intact ear skin. **a** Time line. **b** Skin DLN cell cytokine responses restimulated in vitro. Data are indi-

cated as the mean $\pm$ SD of 3 wells of pooled DLN cells from 3 or 4 mice and are representative of 3 independent experiments with similar results. Detection limits are indicated by dotted lines. $*p < 0.05$ by *t* test (vs. no inhibitors). COX-1 inhibitor, SC560. COX-2 inhibitor, rofecoxib. COX, cyclooxygenase; DLN, draining lymph node; e.c., epicutaneous.

ed that the enhancement of allergic airway inflammation and Th2 and Th9 differentiation by COX inhibition was largely dependent on COX inhibition during the effector phase.

We considered that the COX inhibition during e.c. sensitization had a modest contribution to the effector phase responses because the group with COX inhibition throughout the experiment showed the most significant in vivo responses (Fig. 3) and solely showed increased IL-5, IL-5/IL-13/IL-9, and IL-17A production on restimulation of bronchial DLN cells with the antigen alone, IL-33 alone, and the antigen plus IL-33, respectively (Fig. 4). However, as COX inhibition during the e.c. sensitization phase alone did not enhance the effector phase responses, the contribution seemed to be limited.

Co-Treatment with COX-1-Selective and COX-2-Selective Inhibitors Promoted Th2 and Th17 Differentiation in the e.c. Sensitization Phase

Indomethacin is a nonspecific COX inhibitor, which inhibits both COX-1 and COX-2. To clarify whether inhibition of COX-1 or COX-2 contributes to the promotion of e.c. sensitization and subsequent airway inflammation, we examined effects of treatment with COX-1-selective and/or COX-2-selective inhibitors (Fig. 5a, 6a). Antigen-restimulated skin DLN cells from e.c. sensitized mice treated with both the 2 inhibitors produced higher levels of Th2 and Th17 cytokines (Fig. 5b). The enhancement was reproducible in the group co-treated with the 2 inhibitors. Two groups treated with either one showed no enhancement, as shown in Figure 5b, but occasionally showed enhanced responses in other independent experiments (unpublished data). The results indicate that inhibition of both of the COX-1 and COX-2 activities is necessary for effective promotion of Th2 and Th17 differentiation in e.c. papain sensitization.

Fig. 6. Co-treatment with COX-1-selective and COX-2-selective inhibitors promoted airway inflammation and Th2 responses in the effector phase of i.n. challenge. Mice were treated with COX inhibitor(s) or vehicle during the i.n. challenge phase. All the animal groups were e.c. sensitized to 10 mg/mL papain via intact ear skin and subsequently i.n. challenged (2.5 µg papain). **a** Time line. **b** Airway inflammation. **c** Th2-attracting chemokines in BAL fluid. **d** Bronchial DLN cell cytokine responses restimulated in vitro.

Data are indicated as the mean $\pm$ SD of 5 mice per group (**b, c**) or that of 3 wells of pooled DLN cells from 3 mice (**d**), and are representative of 3 independent experiments with similar results. **d** Detection limits are indicated by dotted lines. * $p < 0.05$ by the Mann-Whitney U test (**b, c**: vs. no inhibitors) or ANOVA (**d**: vs. combination of the 2 inhibitors). COX-1 inhibitor, SC560. COX-2 inhibitor, rofecoxib; COX, cyclooxygenase; DLN, draining lymph node; e.c., epicutaneous; BAL, bronchial alveolar lavage.

Co-Treatment with COX-1-Selective and COX-2-Selective Inhibitors Promoted Airway Inflammation and Th2 Responses in the Effector Phase of i.n. Challenge after the e.c. Presensitization

The group treated with both the COX-1-selective and COX-2-selective inhibitors during the i.n. challenge showed the most severe airway inflammation (Fig. 6a, b) and the highest levels of release of Th2 attracting chemokines in the lung (Fig. 6c, significantly for CCL22 and tendency for CCL17). Antigen-restimulated bronchial DLN cells from i.n. challenged mice treated with both the 2 inhibitors produced the highest levels of Th2 cytokines, IL-5, and IL-13 (Fig. 6d). The enhancement of the effector phase responses was reproducible in the group co-treated with the 2 inhibitors. Two groups treated with either one showed no enhancement, as shown in Figure 6b–d, but the group treated with the COX-1-selective inhibitor alone occasionally showed enhancement of some of the responses in other independent experiments (unpub-

lished data). The results indicate that inhibition of both of the COX-1 and COX-2 activities during i.n. challenge with low-dose papain is necessary for effective promotion of airway inflammation and airway Th2 responses in e.c. presensitized mice.

Discussion

In the present study, we demonstrated that NSAID treatment promoted responses in the protease allergen-induced e.c. sensitization and subsequent airway inflammation. Treatment with indomethacin that inhibits both COX-1 and COX-2 activities promoted e.c. papain sensitization (Fig. 1) and effector phase responses on subsequent i.n. challenge with low-dose papain (Fig. 2), which does not induce responses in mice without e.c. presensitization [10]. COX inhibition during the i.n. challenge largely contributed to the enhancement of the effector

phase responses and that during the e.c. sensitization also, but modestly, contributed (Fig. 3, 4). Co-treatment with COX-1-selective and COX-2-selective inhibitors was effective for the promotion of e.c. sensitization (Fig. 5) and effector phase responses (Fig. 6). Thus, the inhibition of both COX-1 and COX-2 promoted Th responses for not only Th2 but also, unexpectedly, Th17 upon antigen exposure via skin or airways and allergic airway inflammation in the e.c.-i.n. papain model.

We used papain as a model of protease allergen, which could be more relevant to natural exposure to protease-containing airborne allergens than OVA because the proteolytic activity of allergens involves in the pathogenesis of allergies [1–4]. In the effector phase responses on the i.n. challenge after the e.c. presensitization, COX inhibition promoted airway inflammation, serum papain-specific and total IgE production, and bronchial DLN cell Th2, Th9, and Th17 cytokine responses (Fig. 2–4). Co-stimulation with the antigen and IL-33 of bronchial DLN cells synergistically promoted the cytokine responses (Fig. 4), supporting the IL-33-dependency of the papain-induced allergic airway inflammation [7, 12, 19] and suggesting contribution of IL-33-responsive Th2 and ILC2 [10, 28]. COX inhibition during i.n. challenge largely contributed to the enhancement of the effector phase responses, although that during both the e.c. sensitization and i.n. challenge periods showed the most severe responses, indicating a modest contribution of COX inhibition during e.c. sensitization (Fig. 3, 4). Both Th2 and ILC2 contribute to the adaptive i.n. papain model without e.c. presensitization [7, 9, 28], and Th2 seems to contribute to the e.c.-i.n. papain model, in which antigen-specific adaptive immunity should be established during the e.c. sensitization [10]. Therefore, the prostaglandin (PG) E₂-EP2/EP4, PGI₂-IP, and/or PGD₂-DP1 pathways [20] could be considered as candidate pathways responsible to the prostanoid-mediated suppression of the effector phase of the e.c.-i.n. papain model.

COX inhibition promoted serum total and papain-specific IgE response and Th2 and Th17 cytokine production in skin DLN cells upon e.c. papain sensitization via intact ear skin (Fig. 1). Costimulation with the antigen and IL-33 of skin DLN cells synergistically promoted the cytokine responses (Fig. 1c). Although we cannot exclude the possibility that IL-33 enhanced the *in vitro* Th recall responses via indirect pathways such as stimulation of antigen-presenting cells, the results may suggest that IL-33-responsive Th cells were differentiated during the e.c. sensitization.

The Th2 subset appears to be a key factor for inflammation in allergic diseases; however, contribution of other subsets such as Th9, Th17, and Th22 has been suggested. In OVA asthma models, treatment with each of COX-1-selective and COX-2-selective inhibitors or each of COX-1^{-/-} and COX-2^{-/-} mice showed exacerbated allergic airway responses [23, 24, 32]. COX-1^{-/-} mice showed increased Th2 cytokine responses [32], while COX-2^{-/-} mice showed increased Th9 and attenuated Th17 cytokine responses [33, 34]. In an e.c. OVA sensitization model via shaved and tape-stripped back skin, treatment with a COX-2-selective inhibitor showed exacerbated skin inflammation, increased serum OVA-specific IgE and IgG1, and increased IL-4 and attenuated IFN- γ responses in spleen cells [25]. Thus, the indomethacin promotion of Th2/Th9 response and/or IgE production is common between the papain model (Fig. 1–4) and other models [23–26, 32, 33]. In contrast, the indomethacin promotion of Th17 response in the papain model (Fig. 1, 4) is apparently different from results obtained in the OVA asthma model [34] and the hapten-induced contact dermatitis model [27]. Mechanisms for the unexpected differential results for Th17 responses could be due to unknown mechanisms dependent on the allergen protease activity, which has potential to disrupt skin/airway barrier function and stimulate epidermal/epithelial, neuronal, and innate/adaptive immune responses [1–4, 7–14, 17, 18, 35–40], the route of exposure, and/or the strategy how to evaluate the contribution of COXs (chemical inhibitors or deficient mice). Generally, PGE₂ has been considered to upregulate Th17 responses [41]; however, some studies showed PGE₂-mediated suppression and NSAID-induced promotion of Th17 responses in different models [42, 43].

Molecular species of prostanoids and their receptors are multiple, and they play a variety of roles in different contexts [21]. In e.c. sensitization models to OVA or hapten, COX inhibition exacerbates allergic skin inflammation [25, 26], while deficiency of CRTH2, a receptor for PGD₂, attenuated it [44]. PG signaling has regulatory roles in not only T-cell-mediated allergic inflammation but also innate-type responses in ILC2 and epithelial cells including keratinocytes [20, 26, 28, 45, 46], and NSAIDs decrease epithelial barrier integrity in gastrointestinal epithelial cells [47]. Further mechanisms downstream of COXs in the papain model should be investigated in future studies.

In the present papain model, co-treatment with COX-1-selective and COX-2-selective inhibitors promoted the responses in both the e.c. sensitization (Fig. 5) and airway

effector phase responses (Fig. 6). As mentioned in the Results section, treatment with either one of the 2 selective inhibitors occasionally showed enhancement of the responses (unpublished data). Therefore, we speculate that in our present model, prostanoid production mediated by each of COX-1 and COX-2 is barely sufficient to negatively regulate the responses and/or that downstream prostanoid actions are not the same between the 2 COXs due to possible differences in prostanoid producer cell types, production sites, timing, amounts, target cell types, and so on. We cannot exclude possibilities that relatively long half-lives in blood of the 3 NSAIDs used in the present study and/or their potential nonspecific effects independent of COX inhibition partially contributed to the results.

Very recently, we demonstrated that prophylactic e.c. administration of papain with or without protease activity prevented papain-induced Th2-mediated airway inflammation [31]. Without COX inhibition, mice e.c. pre-sensitized to 10 mg/mL of papain showed markedly less airway inflammation on i.n. challenge than those e.c. pre-sensitized to 1 mg/mL of papain, also suggesting induction of tolerance by e.c. presensitization with 10 mg/mL of papain against the onset of airway inflammation (Fig. 2b) [12]. Interestingly, the COX inhibition canceled the tolerance (Fig. 3, 4), the mechanism of which is yet to be investigated. In the OVA asthma model, treatment with indomethacin during the intraperitoneal immunization abrogates PGI₂-mediated airway tolerance, which was induced by OVA inhalation prior to the immunization [48].

In conclusion, we demonstrated that COX inhibition promoted the Th differentiation or expansion upon antigen exposure via skin (Th2 and Th17) or airways (Th2, Th9, and/or Th17), respectively, and allergic airway inflammation in the protease allergen model. The protease allergen model may assist in elucidating the roles and mechanisms of prostanoid-mediated regulation of e.c.

sensitization and the onset of effector phase responses induced by protease-containing natural allergen sources.

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Statement of Ethics

The animal experiments were approved by the committee on animal experiments of Juntendo University School of Medicine (approval numbers: 2020244, 310039, 3000066, 290001, 280143, and 270113) and conducted according to the guidelines of the committee.

Conflict of Interest Statement

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

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Author Contributions

P.S. and T.T. wrote the manuscript. T.T. organized the study. P.S., T.T., S.K., and N.M. performed the experiments, analyzed the data, and/or interpreted the data. P.S., T.T., T.Y., Y.S., K.O., S.I., and H.O. contributed to the study design. All the authors read and approved the final manuscript.

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