Role of prostaglandin D$_2$ and DP$_1$ receptor on Japanese cedar pollen-induced allergic rhinitis in mice


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Nonstandard abbreviations: IgE, immunoglobulin E; PGD₂, prostaglandin D₂; JC, Japanese cedar; Th2, T helper 2; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; IFN-γ, interferon gamma; i.n., intranasal; NALF, nasal lavage fluid;
MK-0524,

(-)-(3R)-4-(4-chlorobenzyl)-7-fluoro-5-(methylsulfonyl)-1,2,3,4-tetrahydrocyclopenta[b]indol-3-yl]acetic acid; asapiprant, 2-[2-(oxazol-2-yl)-5-[4-[[propan-2-yl]oxy]benzenesulfonyl]]piperazin-1-yl]phenoxy] acetic acid; OC000459, (5-fluoro-2-methyl-3-quinolin-2-ylmethylindo-1-yl)-acetic acid; BW245C, (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid; DK-PGD₂,

13-14-dihydro-15-keto-PGD₂

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Abstract

Although we previously demonstrated the contribution of the DP₁ receptor in nasal obstruction using animals sensitized with ovalbumin in the presence of adjuvant, the contribution of the DP₁ receptor in sneezing is unclear. Here, we developed a mouse model of Japanese cedar (JC: Cryptomeria Japonica) pollinosis to evaluate the symptoms of sneezing. To achieve this, we used JC pollen crude extract in the absence of adjuvant to sensitize mice to develop a model to closer to the pathophysiology of human JC pollinosis. The immunological and pharmacological features of this model are highly similar to those observed in JC pollinosis in humans. Using this model we found that DP₁ receptor antagonists suppressed JC pollen extract-induced sneezing, and that a DP₁ receptor agonist induced sneezing. Moreover, JC pollen extract-induced sneezing was diminished in DP₁ receptor KO mice. In conclusion, we developed a novel mouse model of allergic rhinitis that closely mimics human JC pollinosis. A strong contribution of DP₁ receptor signaling to sneeze was demonstrated using this model, suggesting that DP₁ receptor antagonists could suppress sneezing as well as nasal obstruction, and therefore they could be a new therapeutic option for allergic rhinitis.
Introduction.

Japanese cedar (JC: Cryptomeria japonica) pollen is one of the most common sources of allergens that elicit seasonal allergic rhinitis in Japan. About 30% of Japanese suffer from JC pollinosis, and JC pollinosis patients exhibit several symptoms such as sneezing, pruritus, rhinorrhea, and nasal obstruction, which greatly affect daily activity, work productivity, learning, sleep, and the quality of life in patients. In addition, the prevalence of JC pollinosis has increased more than two-fold in the last decade, especially in younger individuals (Osawa et al., 2012), and therefore JC pollinosis is considered a national affliction.

Cry j 1 and Cry j 2 have been well characterized as the major allergen components of JC pollen. Previous investigations used an animal model of JC pollinosis sensitized with Cry j 1 or Cry j 2 (Murasugi et al., 2005; Nomiya et al., 2008). However JC pollinosis patient sera contain distinguishable IgE-binding patterns inlaid with 4–87 spots from a total of 131 IgE-binding protein spots detected by two-dimensional immunoelectrophoresis analysis (Fujimura et al., 2004), suggesting a large variation of IgE-binding patterns and that numerous JC allergens other than Cry j 1 and Cry j 2 might contribute to the pathophysiology of JC pollinosis.

Our first aim of this study was to develop a novel mouse model mimicking human JC pollinosis. For this purpose, we sensitized mouse with JC pollen crude extract in the absence of adjuvant and we improved our understanding of the pathophysiological mechanisms of JC pollinosis with this model. In addition we examined the effect of therapeutic agents for the treatment of allergic rhinitis to characterize this model.

It is well known that allergic rhinitis, including JC pollinosis, is caused by inflammatory mediators released from mast cells. Among them histamine and leukotrienes are the notable mediators that cause
several symptoms in patients with allergic rhinitis (Scadding et al., 2008). Prostaglandin D2 (PGD2) is another mediator produced by mast cells (Lewis et al., 1982; Peters et al., 1982). PGD2 and its metabolites have been proposed to be selective markers of mast cell activation in vivo after provocation with allergen (O'Sullivan et al., 1996; Bochenek et al., 2003; Dahlen and Kumlin, 2004). PGD2 has been detected at high concentrations in the nasal mucosa from allergic subjects challenged with allergen (Naclerio et al., 1983). In addition, PGD2 contributes to several allergic responses in both the upper and lower airways (Fujitani et al., 2002; Harris et al., 2002; Honda et al., 2003; Shiraishi et al., 2005; Okano et al., 2006a; Okano et al., 2006b; Pettipher, 2008).

The actions of PGD2 are mediated through two different receptors, the DP1 receptor (also called DP) (Boie et al., 1995) and the chemoattractant receptor homologous molecule expressed on T helper type 2 cells (CRTH2; also called DP2) (Hirai et al., 2001). Our previous studies demonstrated that a DP1 receptor antagonist, S-5751, inhibited nasal obstruction in guinea pigs sensitized with ovalbumin (Arimura et al., 2001; Takahashi et al., 2012). Although it is thought that the DP1 receptor functions by dilating the sinusoid vessels and mediating nasal obstruction, other contributions of this receptor in nasal symptoms is unclear. In the present study, we investigated the contribution of DP1 receptor to sneezing using a newly developed mouse model and DP1 receptor knock out (KO) mice.
**Materials and Methods.**

**Animals.**

Female, 6-week-old BALB/c mice (Japan SLC, Hamamatsu, Japan) were used for the development of the JC pollinosis model and to evaluate the efficacy of therapeutic medicines in allergic rhinitis. The animals were housed in a room maintained at 23 °C ± 1 °C with a 12-h light/dark cycle. Food and water were given *ad libitum*. These procedures were approved by the Institutional Animal Care and Use Committee of Shionogi Research Laboratories, Osaka, Japan.

DP<sub>1</sub> receptor KO mice (Matsuoka et al., 2000) were backcrossed for 10 generations into the BALB/c background. Homozygous DP<sub>1</sub> receptor KO mice and wild-type BALB/c mice were used to evaluate the role of the DP<sub>1</sub> receptor in JC pollinosis. The animals were housed at 23 °C ± 3 °C with a 12-h light/dark cycle. Food and water were given *ad libitum*. These procedures were approved by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. (Osaka, Japan), which has been accredited by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Animals were randomized by weight prior to the start of the experiments.

**Compounds and antigen.**

MK-0524

\((-\text{-})\)\{3R\}-4-(4-chlorobenzyl)-7-fluoro-5-(methylsulfonyl)-1,2,3,4-tetrahydrocyclopenta[b]indol-3-yl\}acetic acid, asapiprant

\((2\{-2\text{-}(\text{oxazol-2-yl})\}-5\{-4\{-[(\text{propan-2-yl})\text{oxy}]\text{benzenesulfonyl}\}][\text{piperazin-1-yl}\text{phen oxy}]\text{acetic acid})\)
(Takahashi et al., 2015) and OC000459 ((5-fluoro-2-methyl-3-quinolin-2-ylmethylindo-1-yl)-acetic acid) (Horak et al., 2012; Pettipher et al., 2012) were synthesized in our laboratories. Cetirizine dihydrochloride was purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), fluticasone propionate (FP) was from Glaxo SmithKline K.K. (Tokyo, Japan). These drugs were administrated once before 30 min of final challenge at JC model (Fig. 1). BW245C and 13-14-dihydro-15-keto-PGD$_2$ (DK-PGD$_2$) were from the Cayman Chemical Company (MI, USA). JC pollen extract was purchased from Wako Filter Technology Ltd. (Tokyo, Japan). The batch to batch variability of JC pollen extract was monitored and controlled by the quantity of Cry j 1 by ELISA and SDS-PAGE.

**JC pollen extract-induced allergic rhinitis model in mice.**

To develop a JC pollinosis mouse model, JC pollen extract (3 mg/ml in phosphate buffered saline [PBS]) was administered intranasally at 10 µL per nostril on days 0, 7, 14, 21, 22, 23, 24, 27, 28, 29 and 30 in the absence of adjuvant (Fig. 1). Administration of JC pollen extract was performed in non-anesthesia conditions to prevent JC pollen extract from reaching the lower airways (McCusker et al., 2002; Nakaya et al., 2006). After the challenge on day 30, mice were placed in an acrylic cage immediately, and the number of sneezes was counted for 30 min. After the sneezing counts, mice were euthanized by intraperitoneal injection of pentobarbital (60 mg/kg), and serum, nasal lavage fluid (NALF), the tissue of nasal mucosa, and submandibular lymph nodes were obtained. To determine the effect of agonists for DP$_1$ receptor or CRTH2, BW245C or DK-PGD$_2$ (dissolved in PBS with 1% ethanol) was challenged intranasally at 10 µL per nostril instead of final challenge with JC pollen extract on day 30. At this agonists challenge study, PBS with 1% ethanol was challenged as a control.
Determination of total IgE concentrations in mouse serum.

The serum total IgE concentrations were measured using enzyme-linked immunosorbent assay (ELISA) as previously described (Nagai et al., 2000; Komai et al., 2003; Balaha et al., 2012). Briefly, total serum IgE was measured by coating flat-bottomed 96-well microtiter plates (Immuno-Plate I 96-F, Nunc, Roskilde, Denmark) with monoclonal rat anti-mouse IgE heavy chain (MCA419, Serotec Co., Ltd., Oxford, UK) at a concentration of 5 μg/ml. After blocking with 1% bovine serum albumin (BSA, Merck Millipore, Darmstadt, Germany), a standard and serum dilutions were incubated for 1 h followed by peroxidase-labeled polyclonal anti-mouse IgE goat IgG antibody (Nordic immunological laboratories, Tilburg, The Netherlands). Sequentially diluted monoclonal anti-DNP IgE (SPE-7, Sigma, St. Louis, MO, USA) was used as a standard.

Determination of NALF

NALF was collected by washing the nose 3 times with PBS from the choana to external naris (PBS with 0.1% BSA (Sigma), 0.5 mM EDTA (Nacalai Tesque Inc., Tokyo, Japan). The number of nucleated cells in the NALF was counted after Türk solution staining. A differential count for eosinophils was made on a smear prepared with a cytocentrifuge (Cytospin 3, Shandon, Cheshire, UK) and stained with Diff-Quick solution (Sysmex Corporation, Hyogo, Japan).

Histology and Immunohistochemistry.

Collected nasal mucosa was fixed with 10% FormalinNeutral Buffer Solution (containing 4% formaldehyde, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and followed by histological and immunohistochemical staining. Histological examination was performed with Luna staining for eosinophil counts. The protein expression of DP1 receptor and H1 receptor were detected by cross-sections stained...
according to following protocol. Four-µm-thick sections mounted on slides were then dewaxed in xylene, rinsed with distilled H₂O, and transferred to a microwave-resistant plastic staining jar containing 10 mM citrate buffer. The fully covered slides were then placed in a microwave oven and were then allowed to cool down at 20 °C. The slides were transferred to a blocking solution containing normal horse serum in PBS (Vector Laboratories, Inc., Burlingame, CA, USA), and afterwards washed in PBS. Finally, the slides were incubated overnight at 4 °C with polyclonal antibody against DP₁ receptor (dilution 1:100; rabbit, Abcam, UK) or H₁ receptor (dilution 1:200; goat, LifeSpan BioSciences, Inc., WA, USA). After washing with PBS, the samples were incubated with a biotinylated goat anti-rabbit IgG secondary antibody and biotinylated rabbit anti-goat IgG secondary antibody Kit (Vectastain ABC Kit, Vector Laboratories, Inc.). Then the sections were visualized using a DAB substrate Kit (Thermo Fisher Scientific, Waltham, MA, USA).

In vitro culture of submandibular lymph node cells and cytokine production.

Submandibular lymph nodes from mice were dispersed and filtered through a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA) to yield a single-cell suspension. Lymph node cells were suspended in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FBS (Equitech-Bio Inc, Kerrville, TX, USA), 100 U/ml Penicillin-Streptomycin (Gibco, Grand Island, NY, USA), 10 mM HEPES (Gibco), 2-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), MEM Non-Essential Amino Acids Solution (Invitrogen), and 20 mM L-glutamine (Invitrogen). Submandibular lymph node cells (1 × 10⁶ cells/1 ml) were cultured in the presence of 0.3 mg JC pollen extract in 24-well flat-bottom plates (Corning, Tewksbury, MA, USA) at 37 °C in a humidified atmosphere of 5% CO₂. After 96 h of culture, supernatants were harvested. The levels of interleukin (IL)-4, IL-5, IL-13 and interferon (IFN)-γ in the
culture supernatant were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according
to the manufacturer's protocol.

**Statistical Analysis.**

All data are expressed as the mean ± S.E.M. Statistical analysis was performed using Windows SAS
program. *P* values below 0.05 were considered statistically significant. Statistical analysis was performed by
Student’s *t*-test for two-group analysis or Dunnett's test for multiple group comparisons.
Results

**JC pollen-induced Allergic Rhinitis Model in Mice.**

First, we developed a mouse model that mimicked human allergic JC pollinosis, which causes symptoms of sneezing. The mice repeatedly exposed by JC pollen extract began to sneeze from day 22 and reached the plateau at around day 27 (Data not shown). At day 30, JC treated mice significantly increased numbers of sneezed at 25.1 ± 2.4 times, (mean ± S.E.M., N=10), whereas PBS treated mice sneezed 7.3 ± 1.1 times (N=10) in the 30 min following the final antigen challenge (Fig. 2A). To characterize further the immune responses caused by JC pollen extract in this model, we monitored IgE secretion in serum, infiltration of eosinophils in NALF, and cytokine production by submandibular lymph node cells. Nasal challenge with JC pollen extract significantly increased total IgE (1688.7 ± 421.5 ng/ml, N=10) at day 30 compared with PBS challenge (328.9 ± 48.3 ng/ml, N=10) (Fig. 2B). The number of eosinophils after challenge with JC pollen extract were significantly increased in NALF (0.417 ± 0.116 × 10^5 cells/ml, N=6) compared with PBS challenge (0.040 ± 0.015 × 10^5 cells/ml, N=6) (Fig. 2C). After the final challenge, submandibular lymph node cells were isolated and cultured with JC pollen extract at 0.3 mg/ml for 96 h. The amounts of IL-4, IL-5 and IL-13 after incubation of submandibular lymph node cells were significantly increased by the repeated treatment of JC pollen extract when compared with control treatment (Fig. 2D-F). However, IFN-γ secretion was not affected by the administration of JC pollen extract (data not shown). In addition to observe the difference in local inflammatory site after repeated allergen challenge, we conducted the histological and immunohistochemical investigation. Nasal eosinophils were detected in the nasal mucosa after the administration of JC pollen extract (Fig. 3A). Although the expression of DP1 receptor (Fig. 3B) and H₁
receptor (Fig. 3C) were observed in nasal mucosa after repeated administration with JC pollen extract, no clear difference between treatment of JC pollen extract and control was observed. In DP₁ expression was accepted by a nasal mucosa epithelium cells, a glandulae nasales and a blood vessel endothelium. In H₁ expression was accepted in a nasal mucosa epithelium cells and a glandulae nasales.

To determine the pharmacological profile of this model, we examined the effect of H₁ receptor antagonists and nasal steroids on sneezing in the JC pollinosis model. Single oral administration of cetirizine suppressed the number of sneezes in a dose-dependent manner, and significant inhibition was observed at 30 mg/kg ($P < 0.05, N=8$). By contrast, single nasal administration of adequate dose of fluticasone propionate did not affect sneezing (Fig. 4).

**Contribution of DP₁ receptor in the JC pollinosis model.**

We examined the effects of DP₁ receptor antagonists, MK-0524 and asapiprant, in the JC pollinosis model. Single oral administration of MK-0524 and asapiprant exhibited a dose-dependent suppression of the number of sneezes. Significant inhibition was achieved with 3 mg/kg of MK-0524 ($N=6$) and 0.3 mg/kg of asapiprant ($N=6$). On the other hand, 30 mg/kg of OC000459, a CRTH2 antagonist, did not reduce the number of sneezes ($23.67 \pm 3.19, N=6$) compared to vehicle ($28.67 \pm 3.57, N=6$) (Fig. 5).

We next investigated whether DP₁ receptor or CRTH2 agonists caused sneezing in the JC pollinosis model. Nasal administration of BW245C, a selective DP₁ receptor agonist, at 0.1% ($N=6$) on day 30 significantly induced sneezing compared to PBS-1% ethanol challenge at JC model (repeated JC pollen extract challenge for day 29, $N=6$) (Fig. 6). By contrast, 13-14-dihydro-15-keto-PGD₂ (DK-PGD₂), a selective CRTH2 receptor agonist, at 0.1% ($N=6$) did not induce sneezing (Fig. 6).
To confirm the contribution of DP1 receptor to sneezing, we compared the number of sneezes between DP1 receptor KO mice and wild-type mice. As shown in Fig. 7, in WT mice, sneezing induced by JC pollen extract challenge was significantly increased (25.6 ± 2.4, N=10) compared with that induced by PBS challenge (8.1 ± 1.5, N=10). However, DP1 receptor KO mice did not show an increase in sneezing by JC pollen extract challenge (8.0 ± 1.7, N=10) compared with PBS challenge (8.4 ± 2.0, N=10) (Fig. 7).
Discussion.

Here we developed a mouse model that mimics the clinical features of JC pollinosis in humans. For this purpose, we changed two points in the sensitization steps from methods previously reported. One was to use crude allergen extract instead of a single allergen, and another was not to use adjuvant for sensitization. We measured the content of Cry j 1 and Cry j 2 in the JC pollen crude extract used in the present study. The JC pollen extract contained 3.4% Cry j 1 and 0.9% Cry j 2 protein (data not shown), and the remaining extract contained > 95% proteins other than Cry j 1 and Cry j 2. JC pollen contains a great variety of IgE-binding patterns (Fujimura et al., 2004) and numerous JC allergen components other than Cry j 1 and Cry j 2, which might contribute to the pathology of JC pollinosis. Adjuvants such as alum and cholera toxin are usually used for the sensitization of animal models of allergic diseases. These may affect immune responses, resulting in features distinct from pathological conditions in humans. Our JC model succeeded in inducing sneezing, IgE production in serum, nasal eosinophilia and Th2 cytokine synthesis in the absence of adjuvants. We suspect that protease activities in JC pollen extract (Gunawan et al., 2008; Ibrahim et al., 2010a; Ibrahim et al., 2010b) might help the sensitization. Indeed protease allergens were reported to promote IgE secretion (Gough et al., 1999) and to induce inflammatory effects in the epithelium (King et al., 1998). As a result, our newly developed model using JC pollen extract in the absence of adjuvant shows a high similarity to the pathophysiology of JC pollinosis in humans.

To verify the usefulness of this model, we performed a pharmacological investigation to examine the effect of cetirizine and fluticasone propionate on sneezing. Cetirizine, an H₁ receptor antagonist, showed a significant suppressive effect on sneezing in this JC pollinosis model, similar to that in humans, whereas a
single nasal administration of fluticasone propionate had no effect. We used 5 µg of fluticasone propionate, equal to the concentration used in humans. In a preliminary study, we tried other concentrations of fluticasone propionate, but no suppressant effect was observed (data not shown). Single administration of nasal steroid is not potent to the allergic responses immediately after its administration as seen in clinical practice (Derendorf and Meltzer, 2008). Of note, 10 days of repeated dosing of fluticasone propionate had a significant suppressive effect in this model (data not shown). These results suggest that our model reflects well the efficacy of the existing medicine in human. That is, our model is useful for the evaluation of therapeutic medicines for JC induced sneezing.

Next, we examined the effect of MK-0524 and asapiprant on JC pollen extract-induced sneezing in this model, to determine the potential contribution of DP1 receptor in the pathology of JC pollinosis. Both antagonists of the DP1 receptor inhibited sneezing, whereas a suppressive effect was not observed with OC000459, a CRTH2 receptor antagonist. Considering previous reports, the onset of the effect of CRTH2 antagonist might be slow. Repetitive dosing of OC000459 for 8 days was required to obtain the maximum effect (Horak et al., 2012). Ramatroban showed a significant effect on sneezing after administration for 29 days (Nomiya et al., 2008). Another CRTH2 antagonist, AM211 inhibited sneezing induced by OVA after repeated administration for 5 days (Bain et al., 2011). These results suggest that DP1 receptor antagonists quickly exert a suppressive effect on sneezing likely via H1 receptor antagonists, which might differentiate them from CRTH2 antagonists.

The contribution of the DP1 receptor on sneezing was confirmed by the disappearance of induction of sneezing by JC pollen extract challenge in DP1 receptor KO mice, and by a significant increase in sneezing
after stimulation with a DP1 receptor agonist BW245C in WT mice. Sneezing in mice began immediately after administration of JC pollen extract or BW245C, and peaked at 10 min. The mechanism of DP1 receptor-mediated sneezing is not clear. Our previous report showed that DP1 receptor mediated Th2 inflammation resulting in the induction of airway hyperresponsiveness (Matsuoka et al., 2000), and the induction of nasal obstruction via dilation of the sinusoid vessels (Takahashi et al., 2012). In addition, using our JC model, we showed that the secretion of total IgE was diminished in DP1 receptor KO mice (Supplemental Data). We evaluated Th2 cytokine production at in vitro culture of submandibular lymph node cells and eosinophils in NALF after treatment with asapiprant, and they were not affected by the drug (Data not shown). These mechanisms via DP1 receptor targeting inflammatory cells might be distinct from those observed in the present study targeting neuronal cells. Ebersberger et al. showed that DP1 receptor activation increased the amplitude of TTX-R Na+ currents in dorsal root ganglion neurons (Ebersberger et al., 2011), suggesting that DP1 receptor activation might modulate sensory neurons. The symptoms of sneezing are considered to be induced through the activation of sensory neurons (Taylor-Clark et al., 2005). Therefore, activation of DP1 receptor might induce sneezing by mediating sensory neuron signaling. A more detailed mechanism of the relationship between DP1 receptor and sensory neurons will be investigated in our future studies.

We suggested DP1 receptors are important in sneezing. However, NSAIDs did not effective in allergic rhinitis. We think that clinical ineffectiveness of NSAIDs is not by mechanism to suppress PGD2 but by another mechanism exacerbate allergic reactions. NSAIDs leads to the secretion of cysteinyl leukotrienes (CysLTs), reflecting the overexpression of leukotriene C4 synthase (enhanced 5-lipoxigenase pathway).
Furthermore, NSAIDs activated eosinophils and mast cells directly (Steinke et al., 2014). Moreover, we previously demonstrated that DP₁ antagonist, S-5751, did not block inhaled leukotriene D₄-induced bronchoconstriction in sheep (Shichijo et al., 2009).

In conclusion, we report two novel findings in this study. First we developed a novel model of mouse JC pollinosis by using JC pollen crude extract in the absence of adjuvant. From the immunological and pharmacological profiles, this model appears to be similar to the clinical condition observed in human JC pollinosis. The second novel point is that DP₁ receptor plays an important role in the symptoms of sneezing. These results suggest that disturbing PGD₂-DP₁ receptor interactions might be a new therapeutic target for the treatment of allergic rhinitis.
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Authorship Contributions.

Participated in research design: Nakano, Furue, Tomita, and Tanaka.

Conducted experiments: Nakano, Kidani, and Goto.

Contributed new reagents or analytic tools: Nakano, and Goto.

Performed data analysis: Nakano, Goto, and Tomita.

Wrote or contributed to the writing of the manuscript: Nakano, Goto, Tomita, Inagaki, Tanaka and Shichijo.
References.


Figure Legends.

**Figure 1:** Experimental protocol for JC pollinosis model using BALB/c mice.

i.n., intranasal injection; PBS, phosphate-buffered saline; JC, Japanese cedar extract

**Figure 2:** (A) Number of sneezes after repeated allergen challenge in BALB/c mice. Results are represented as the means ± S.E.M. of 8 mice. (B) Serum total IgE levels after repeated allergen challenge in BALB/c mice. Results are represented as the means ± S.E.M. of 7-8 mice. (C) Eosinophils in nasal alveolar fluid were detected after repeated allergen challenge in BALB/c mice. Results are represented as the means ± S.E.M. of 6 mice. After the final challenge, submandibular lymph node cells were isolated and cultured in the JC pollen extract at 0.3 mg/ml for 96 h. IL-4 (D), IL-5 (E), and IL-13 (F) were measured by ELISA. Results are represented as the means ± S.E.M. of 10 mice. **p < 0.01, ***p < 0.001 (vs. PBS, Student’s t-test).

**Figure 3:** Histological and immunohistochemical LUNA stain (A), DP1 (B) and H1 (C) receptor protein stain in nasal mucosa after repeated allergen challenge in BALB/c mice as described in materials and methods. Eosinophils were stained by LUNA agent, and the protein expression of DP1 receptor and H1 receptor were detected using antibodies. Nasal septum was determined per high-power (10 × 40 or 10 × 20) microscopic field. Arrow indicated eosinophils.

**Figure 4:** (A) Effect of histamine H1 receptor antagonist on the number of sneezes after repeated allergen challenge in BALB/c mice. Results are represented as the means ± S.E.M. of 5-8 mice. **p < 0.01 (vs. PBS, Student’s t-test); †p < 0.05 (vs. Vehicle, Dunnett’s multiple comparison test). (B) Effect of fluticasone propionate on the number of sneezes after repeated allergen challenge in BALB/c mice. Results are
represented as the means ± S.E.M. of 4-5 mice. FP, fluticasone propionate; **p < 0.01 (vs. PBS, Student’s t-test).

**Figure 5:** Effect of DP₁ and CRTH2 antagonists on the number of sneezes after repeated allergen challenge in BALB/c mice. Results are represented as the means ± S.E.M. of 6 mice. ***p < 0.001 (vs. PBS, Student’s t-test); †p < 0.05, ††p < 0.01, †††p < 0.001 (vs. Vehicle, Dunnett’s multiple comparison test).

**Figure 6:** The number of sneezes after DP₁ or CRTH2 agonist administration in a JC model in BALB/c mice. Results are represented as the means ± S.E.M. of 6 mice. PBS model, PBS treated mice from day 0 to day 29; JC model, JC treated mice from day 0 to day 29; **p < 0.01 (vs. PBS-1% ethanol with PBS model, Student’s t-test); †††p < 0.001 (vs. PBS-1% ethanol with JC model, Student’s t-test).

**Figure 7:** The number of sneezes after repeated allergen challenge in WT and DP₁ receptor KO mice. Results are represented as the means ± S.E.M. of 10 mice. ***p < 0.001 (vs. WT PBS, Student’s t-test); †††p < 0.001 (vs. WT JC, Student’s t-test).
Female Balb/c mice; 6 weeks

0 7 14 21 22 23 24 27 28 29 30 (day)

Drug administration po or i.n.

PBS or JC 3 mg/ml intranasal challenge
instillation of 10 μl per nostril

Assay

Fig. 1
**Fig. 4**

**Sneezing**

A

- Number/30 min
- PBS
- Vehicle
- 3 mg/kg
- 10 mg/kg
- 30 mg/kg

Cetirizine

B

- Number/30 min
- PBS
- Vehicle
- FP 5 μg
Fig. 5
Fig. 6