

Fig. 5. Comparison of Cry j 1-induced cytokine production and proliferation among specific immunotherapy (SIT)-treated and SIT-untreated Japanese cedar pollinosis (JCP) patients. Peripheral blood mononuclear cells (PBMCs) were incubated in the presence or absence of Cry j 1 for 72 h, after which the levels of IL-5 (a), IL-10 (b) and TGF- β (c) were determined within the supernatant by ELISA. Changes in production were determined by measuring the differences between the levels observed following Cry j 1 stimulation and those observed in the absence of stimulation. In addition, Cry j 1-induced PBMC proliferation was measured by BrdU incorporation (d). *P*-values were determined using the Mann-Whitney's *U*-test. SIT represents the patients treated with SIT. Non-SIT represents SIT-untreated group of patients.

($P=0.007$, Fig. 5a). On the other hand, IL-10 or TGF- β production in response to Cry j 1 was not detected in either group (Figs 5b and c). IL-10 production was not detected even after 12 h incubation with Cry j 1. Cry j 1-induced IL-5 production was similar between basophil-depleted PBMC (121.9 ± 77.9 pg/mL) and control PBMC (128.9 ± 59.6 pg/mL, $P=0.624$), suggesting that basophil responsiveness in IL-5 production is negligible in our culture system. A trend of suppression in Cry j 1-induced proliferation was seen in PBMC from SIT-treated patients as compared with those from SIT-untreated patients ($P=0.057$; Fig. 5d).

Interestingly, the observed Cry j 1-induced changes in BTLA expression were significantly and negatively correlated with IL-5 production ($\rho=-0.747$, $P<0.001$; Fig. 6). However, when we analysed the correlation separately, the correlation was not seen in either SIT-treated ($\rho=-0.243$, $P=0.416$) or SIT-untreated ($\rho=-0.491$, $P=0.141$) group because Cry j 1-induced IL-5 production was lost in most of SIT-treated patients. The changes in Cry j 1-induced IL-5 production were significantly and positively correlated with both naso-ocular symptom scores ($\rho=0.616$, $P=0.024$) and QOL scores ($\rho=0.719$, $P=0.008$). The

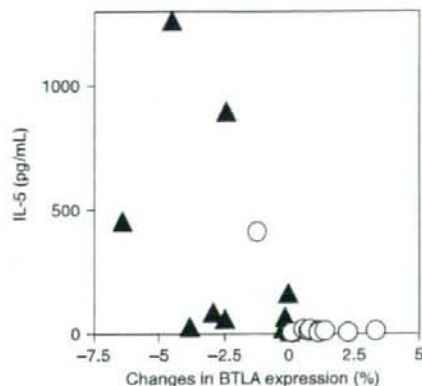


Fig. 6. Relationship between Cry j 1-induced B and T lymphocyte attenuator (BTLA) expression and IL-5 production. Peripheral blood mononuclear cells (PBMCs) were incubated in the presence or absence of Cry j 1 for 72 h, after which the expression of BTLA and IL-5 levels were determined by flow cytometry and ELISA, respectively. Specific immunotherapy (SIT)-treated and SIT-untreated Japanese cedar pollinosis (JCP) patients are represented by the open circles and closed triangles, respectively.

changes in BTLA expression did not correlate with naso-ocular symptom scores ($\rho=-0.063$, $P=0.760$); however, the changes showed a tendency to inversely correlate with QOL scores ($\rho=-0.450$, $P=0.067$).

Effect of B and T lymphocyte attenuator cross-linking on Cry j 1-induced interleukin-5 production

Finally, we sought to determine the *in vitro* role of BTLA in Cry j 1-specific PBMC responses. Immobilized anti-BTLA mAb significantly suppressed Cry j 1-induced IL-5 production by PBMC, compared with control mouse IgG2b ($P=0.016$; Fig. 7a). On the other hand, Cry j 1-induced proliferative responses were not different between immobilized anti-BTLA mAb and the control treatment ($P=0.879$; Fig. 7b).

Discussion

The key finding of the present study was that BTLA expression is down-regulated after Cry j 1 stimulation in patients not treated with SIT, while it is up-regulated in SIT-treated patients. The up-regulation of BTLA in SIT-treated patients was particularly apparent in a CD4⁺ T cell subset. Cry j 1-induced changes in BTLA expression were significantly and inversely correlated with IL-5 production by PBMC. Furthermore, cross-linking of BTLA resulted in inhibition of Cry j 1-induced IL-5 production. These results suggest that both IL-5 production and down-regulation of BTLA in response to allergen are inhibited in SIT-treated patients with JCP.

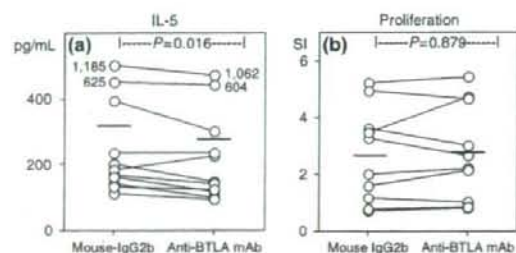


Fig. 7. Effect of cross-linking of B and T lymphocyte attenuator (BTLA) on Cry j 1-induced responses in peripheral blood mononuclear cells (PBMCs). PBMC from patients with Japanese cedar pollinosis (JCP) without immunotherapy were incubated in the presence or absence of Cry j 1, along with either MIH26 or control mouse IgG2b, for 72 h, after which levels of Cry j 1-induced IL-5 (a) and proliferative responses (b) were determined. *P*-values were determined using the Wilcoxon's signed-rank test.

BTLA is a recently identified member of the CD28 family of receptors. Similar to PD-1 and CTLA-4, BTLA contains ITIMs, suggesting that BTLA functions as an inhibitory receptor [17]. In fact, reduced BTLA expression leads to enhanced T cell and B cell responses in mice [17, 25]. However, little is known regarding the potential role of BTLA in human cellular responses. We have previously demonstrated that BTLA ligation inhibits anti-CD3-stimulated proliferation as well as the production of IFN- γ and IL-10 in human CD4⁺ T cells [22]. Another group has reported suppression of anti-CD3-induced proliferation in response to cross-linking of BTLA, and reduced expression of CD25 and production of IL-2 and IL-4 in addition to IFN- γ and IL-10 in human T cells [26]. In the present study, immobilized anti-BTLA mAb that can cross-link BTLA significantly suppressed Cry j 1-induced IL-5 production by PBMC. This result is consistent with previous reports, and demonstrates for the first time that cross-linking of BTLA also inhibits antigen-specific human PBMC responses. On the other hand, BTLA cross-linking did not alter Cry j 1-induced proliferative responses. We have previously shown that BTLA-induced inhibitory signals depend on the strength of TCR signals [22]. These results suggest that BTLA cross-linking selectively affects Cry j 1-induced IL-5 production in our system. Although, to date, no Ab to block human BTLA is available, the definitive experiment would be to block BTLA (with non-cross-linking Abs or Fab fragments) to see if IL-5 production is restored in SIT patients.

Antigen concentration is one of the key factors regulating *in vitro* cellular responses. We previously reported that PBMC showed proliferative responses to Cry j 1 in a dose-dependent manner, and the samples showed positive responses at 10 μ g/mL [20]. Another investigation used Cry j 1 at the concentration of 25–50 μ g/mL with substantial results [27]. Thus, we think that the concentration

of 10 μ g/mL of Cry j 1 is appropriate concentration used in the present study.

Similar to other co-stimulatory molecules, the expression of BTLA is known to change upon activation [22, 25, 26, 28]. The expression of BTLA is up-regulated on T cells and down-regulated on B cells upon activation in mice [25, 28]. In humans, we have demonstrated constitutive BTLA expression on the surface of both CD4 and CD8T cells at high levels, which gradually declines after stimulation with anti-CD3 and anti-CD28 mAb [22]. The relationship between BTLA expression and pathogenesis has been investigated in several human diseases [26, 29, 30]. For example, increased BTLA expression has been demonstrated on CD4⁺ and CD8⁺ T cells within pleural fluid in lung cancer patients [26]. An association between a BTLA gene polymorphism and risk of rheumatoid arthritis has also been reported [29]. The baseline expression of BTLA did not differ significantly among SIT-treated and SIT-untreated patients. However, down-regulation of BTLA was only observed in untreated patients in the present study. Although the precise mechanism by which the expression of BTLA is down-regulated remains unknown, signals through the TCR, as well as cytokines produced by Cry j 1-specific T cells and/or pro-inflammatory cytokines/chemokines secreted by monocytes or B cells may all function to regulate BTLA expression. Identification of mechanism regulating BTLA expression must be made in future investigation.

BTLA was originally cloned from murine Th1 cells, and is predominantly expressed by B cells, followed by T cells and APC in mice [17, 25]. In humans, we have recently reported BTLA expression on CD4⁺ T cells, CD8⁺ T cells and CD19⁺ B cells in freshly isolated human PBMC. Unlike in mice, polarized human Th1 and Th2 cells consist of both BTLA-positive and BTLA-negative populations; however, BTLA expression diminishes with extended length of culture [22]. The present results are consistent with those of previous reports and suggest that BTLA expression persists on human CD4⁺, CD8⁺ and CD19⁺ cells during short-term culture. Furthermore, only CD4⁺ cells bearing BTLA were significantly increased after recall stimulation with Cry j 1, suggesting that SIT selectively enhances BTLA expression on CD4⁺ T cells.

A significant difference in Cry j 1-induced IL-5 production by PBMC was seen among SIT-treated and untreated patients. Inhibition of both local and systemic IL-5 production is known to correlate with clinical efficacy [6, 8]. The present result is consistent with a report by Kakinoki et al. [6] demonstrating that the production of IL-5 following Cry j 1-stimulation by PBMC is significantly reduced in good responders, compared with poor responders to SIT in patients with JCP. On the other hand, Cry j 1-induced IL-10 and TGF- β production was not detected in PBMC from SIT-treated patients. One of the reasons why IL-10 production was not induced in our immunotherapy

is that, although our immunotherapy was clinically effective (Fig. 1) and 2000 JAU/mL of JPC extract is maximal concentration commercially available in Japan, the maintenance dose is relatively low [the mean maintenance dose of the extract was 509.0 JAU which is equivalent to 0.37–1.07 µg of Cry j 1] as compared with other reports [1–5]. Moreover, the observed changes in BTLA expression were significantly and negatively correlated with IL-5 production after Cry j 1-stimulation in the present study. This result confirms the result of our cross-linking study and suggests that BTLA has an inhibitory role with regard to Cry j 1-specific IL-5 production in JCP.

In the present study, the expression of co-inhibitory molecules and cytokine production was determined during the pollen season after at least 2 years of SIT treatment. It is interesting to determine the effect of SIT during the first pollen season after starting this therapy. Our preliminary results showed that, as compared with SIT-untreated patients ($n = 10$), PBMC from the newly SIT-treated patients during the first pollen season after starting cedar immunotherapy ($n = 5$) produced significantly less amount of IL-5 (36.7 ± 49.4 vs. 433.6 ± 581.2 pg/mL, $P = 0.020$). On the other hand, Cry j 1-induced changes in BTLA ($-1.42 \pm 0.80\%$ vs. $-3.42 \pm 0.97\%$, $P = 0.178$), PD-1 ($0.27 \pm 0.38\%$ vs. $0.73 \pm 0.33\%$, $P = 0.390$) and B7-H1 ($0.10 \pm 0.24\%$ vs. $0.01 \pm 0.34\%$, $P = 0.125$) expression as well as Cry j 1-induced IL-10 (2.4 ± 1.5 vs. 0.2 ± 0.2 pg/mL, $P = 0.137$) and TGF- β (0 ± 0 vs. 0 ± 0 pg/mL) production was not statistically different between the groups. These results suggest that period after reaching of maintenance dose is one of the factors regulating BTLA expression. In addition, the direct effect of SIT on expression of co-inhibitory molecules after reaching the maintenance dose, before the pollen season should be determined.

In conclusion, the present study shows an increase in BTLA-bearing CD4⁺ T cells in patients treated with SIT. Furthermore, the alterations in BTLA expression were associated with allergen-specific IL-5 production. These results suggest that protection of BTLA down-regulation is a key mechanism of SIT. T cell co-signalling molecules are potential targets in the treatment of allergic airway disease [31]. The present study adds support to this and further suggests that regulation of BTLA expression may be of therapeutic benefit in the treatment of allergic airway disease.

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CRTH2 Plays an Essential Role in the Pathophysiology of Cry j 1-Induced Pollinosis in Mice¹

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PGD₂ is the major prostanoid produced during the acute phase of allergic reactions. Two PGD₂ receptors have been isolated, DP and CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), but whether they participate in the pathophysiology of allergic diseases remains unclear. We investigated the role of CRTH2 in the initiation of allergic rhinitis in mice. First, we developed a novel murine model of pollinosis, a type of seasonal allergic rhinitis. Additionally, pathophysiological differences in the pollinosis were compared between wild-type and CRTH2 gene-deficient mice. An effect of treatment with ramatroban, a CRTH2/T-prostanoid receptor dual antagonist, was also determined. Repeated intranasal sensitization with Cry j 1, the major allergen of *Cryptomeria japonica* pollen, in the absence of adjuvants significantly exacerbated nasal hyperresponsive symptoms, Cry j 1-specific IgE and IgG1 production, nasal eosinophilia, and Cry j 1-induced *in vitro* production of IL-4 and IL-5 by submandibular lymph node cells. Additionally, CRTH2 mRNA in nasal mucosa was significantly elevated in Cry j 1-sensitized mice. Following repeated intranasal sensitization with Cry j 1, CRTH2 gene-deficient mice had significantly weaker Cry j 1-specific IgE/IgG1 production, nasal eosinophilia, and IL-4 production by submandibular lymph node cells than did wild-type mice. Similar results were found in mice treated with ramatroban. These results suggest that the PGD₂-CRTH2 interaction is elevated following sensitization and plays a proinflammatory role in the pathophysiology of allergic rhinitis, especially pollinosis in mice. *The Journal of Immunology*, 2008, 180: 5680–5688.

Pollinosis, a type of seasonal allergic rhinitis, is the most common allergic respiratory disease and is a global health problem that is increasing in prevalence (1–3). For example, as much as 10–20% of the Japanese population suffers from Japanese cedar pollinosis (JCP).³

Intensive and extensive studies on pollinosis have greatly improved the understanding of its etiology and pathology (4). Mouse models of allergic rhinitis have contributed to these advances. However, these mouse models usually use adjuvants and/or strong Ags to efficiently sensitize animals (5–9). To further examine the pathophysiological mechanism underlying pollinosis, a murine model that naturally mimics human pollinosis by intranasal ad-

ministration of pollen extracts in the absence of adjuvants is needed.

Prostanoids are thought to participate in allergic inflammation (10). PGD₂ is one of the most important of these and it plays roles in allergic respiratory diseases including allergic rhinitis (10–15). For example, nebulized PGD₂ enhances Th2-type inflammatory responses and eosinophilia, leading to the development of airway hyperresponsiveness (14). PGD₂ acts via the D-prostanoid receptor (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (16). The expression patterns and signaling pathways utilized by DP and CRTH2 are different, suggesting that they have distinct roles in allergic responses (16, 17). It appears that signals via DP promote eosinophil survival, whereas signals via CRTH2 mediate shape changes, chemotaxis, and degranulation by eosinophils (16, 18, 19).

The role of CRTH2 in allergic airway inflammation *in vivo* remains controversial (17). CRTH2 has been found to participate in the recruitment of eosinophils from the bone marrow into the bloodstream (19, 20), in eosinophilic airway inflammation (11), and in airway eosinophilia and hyperresponsiveness (21), suggesting that it plays a proinflammatory role *in vivo*. On the other hand, mice deficient for CRTH2 (CRTH2^{-/-}) show eosinophil recruitment and IL-5 production by splenocytes in an asthma model, suggesting that CRTH2 mediates antiinflammatory signals (22). In human nasal mucosa, CRTH2 is expressed in eosinophils and a subset of T cells (23). We have recently reported that there is a close correlation between the number of eosinophils infiltrating into nasal mucosa and the amount of CRTH2, but not DP, in nasal mucosa (12). Also, CRTH2^{-/-} mice have been found to show reduced eosinophil infiltration into skin in a model of chronic allergic skin inflammation (24).

In this study, we established a novel murine model of pollinosis and used it to determine the pathophysiological role of CRTH2 in

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³ Abbreviations used in this paper: JCP, Japanese cedar pollinosis; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DP, D-prostanoid receptor; TP, T-prostanoid receptor; WT, wild type.

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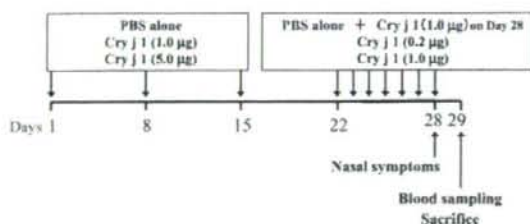


FIGURE 1. Experimental design used to investigate the effect of nasal exposure to Cry j 1 in mice. BALB/c mice (6–9 per group) were sensitized by intranasal administration of either 1.0 or 5.0 µg of Cry j 1 in 10 µl PBS in the absence of adjuvants once a week for 3 wk (on days 1, 8, and 15). One week after the third sensitization, mice were challenged by intranasal administration of one fifth Cry j 1, respectively every day for 7 consecutive days (on days 22 to 27). As a control, mice were treated with PBS, except for the final challenge, where mice were treated with 1.0 µg of Cry j 1. Immediately after the final nasal challenge, nasal symptoms were observed for 10 min, and 16 h after the final nasal challenge, peripheral blood was collected, and the specific Ab content in the serum was measured. After the blood sampling, mice were sacrificed, and the nose and submandibular lymph nodes were obtained for further analysis.

the disease. In this model, intranasal sensitization with Cry j 1, the major allergen of *Cryptomeria japonica* pollen, in the absence of adjuvant induced allergic rhinitis closely resembling human pollinosis. We found that a lack of CRTH2 in the mutant mice greatly reduces allergic pathophysiology in this model.

Materials and Methods

Animals and Ags

BALB/c mice were purchased from Charles River Laboratories Japan and CLEA Japan. Homozygous CRTH2-deficient and wild-type BALB/c mice were obtained as described previously (24). Female mice (7–11 wk old) were used in all the experiments. The mice were maintained in specific pathogen-free conditions at Okayama University and Tokyo Medical and Dental University in accordance with the guidelines set forth by the university committees. All experimental protocols and procedures in the present study were approved by the University Animal Care and Use Committees. Cry j 1 was purified from crude extracts of *C. japonica* pollen as described previously (25). Endotoxin contamination was considered to be negligible due to a negative result in the Endospec ES test (Seikagaku). Ramatroban was obtained from Bayer Yakuhin. Protein concentrations were determined using a bicinchoninic acid assay (Pierce) according to the manufacturer's instructions.

Sensitization of mice

Mice (6–9 animals per group) were sensitized by intranasal application of serial doses of Cry j 1 in 10 µl PBS in the absence of adjuvants using a microsyringe (Hamilton Medical). The low-dose sensitization consisted of a series of administrations of 1.0 µg of Ag once a week for 3 wk (on days 1, 8, and 15), followed by administration of 0.2 µg Ag every day for 7 consecutive days (on days 22 to 28). For high-dose sensitization, 5.0 and 1.0 µg of Cry j 1 were administered once a week for 3 wk (on days 1, 8, and 15) and every day for 7 consecutive days (on days 22 to 28), respectively. As a control, mice were treated with PBS instead of the Ag at all points except for the final challenge, where the mice were administered 1.0 µg of Cry j 1 (Fig. 1). Immediately after the final nasal challenge, the frequencies of sneezing and nasal rubbing were counted in a blinded manner for 10 min. Peripheral blood was collected from the tail vein 16 h after the final nasal challenge, and then sera were prepared by centrifugation at 200 × g, and the levels of Cry j 1-specific Ab in the serum were determined by ELISA. The mice were then sacrificed, and the nose and submandibular lymph nodes were isolated for further immunological and histological analyses.

To determine whether the effect of CRTH2 deficiency is at the level of sensitization or amplification of allergic cascade, outcomes of pollinosis were compared with CRTH2^{-/-} mice sensitized and subsequently challenged with Cry j 1 and nonsensitized CRTH2^{-/-} mice with a single challenge with Cry j 1.

Ramatroban treatment

Ramatroban was suspended in 5% methyl cellulose and administered orally at a dose of 30 mg/kg body weight once a day from 1 day before the first sensitization to the final challenge (day 0 to day 28). Control mice were given 5% methyl cellulose alone.

Ab determination

The levels of Cry j 1-specific IgE, IgG1, and IgG2a were determined by ELISA as previously described (26). The levels of Cry j 1-specific IgE were measured using biotinylated Cry j 1 (Hayashibara Biochemical Laboratories) as a detecting reagent. The titers of Ag-specific Abs were estimated according to the mean OD at 450 nm of serum dilutions of 1/20 for IgE and 1/100 for IgG1 and IgG2a.

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

Submandibular lymph nodules from mice were dispersed and filtered through a 70-µm cell strainer (BD Biosciences) to yield a single-cell suspension. Lymph node cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS (Invitrogen), 100 µg/ml streptomycin, 100 U/ml penicillin, and 20 mM L-glutamine (Sigma-Aldrich). Cells (4×10^5 cells/200 µl) were cultured in the presence or absence of 10 µg/ml Cry j 1 in 96-well flat-bottom plates (BD Biosciences) at 37°C in humidified atmosphere of 5% CO₂ and 95% air. After 72 h of culture, supernatants were harvested. The levels of IL-4, IL-5, and IFN-γ in the culture supernatant were measured using OptEIA sets (BD Biosciences). The levels of IL-13 were measured using DuoSet ELISA development kit (R&D Systems). The detection limits for IL-4, IL-5, IL-13, and IFN-γ in this system were 10, 30, 40, and 60 pg/ml, respectively.

Histological examination

Histological examination was performed as previously described (26). Coronal nasal sections were stained with H&E and Luna solution to detect mononuclear cells and eosinophils, respectively. A blind test was conducted to determine the numbers of infiltrating cells in the posterior part of nasal septum using a high-power (10 × 40) microscopic field.

To determine the infiltration of T cells into nasal mucosa, immunohistochemistry for CD3 was examined. Paraffin-embedded nasal tissues were sectioned into 5-µm slices, deparaffinized, rehydrated and retrieved with microwave. Endogenous peroxidase activity was quenched with 3% H₂O₂, and nonspecific protein binding was blocked with normal rabbit serum (DAKO Japan) for 60 min. After this, the tissue sections were incubated with goat anti-mouse CD3-e polyclonal Ab (sc-1127; Santa Cruz Biotechnology) or control goat IgG Ab (M-20; Santa Cruz Biotechnology) overnight at 4°C. To detect the reaction, N-Histofine Simple Stain MAX PO (G) (Nichirei Biosciences) and diaminobenzidine substrate (DAKO Japan) was used according to the manufacturers' instructions.

Real-time quantitative PCR in nasal mucosa

Mucosal tissues were removed from nasal septum 16 h following the final nasal challenge, immediately soaked in buffer containing guanidine isothiocyanate from the RNeasy Mini Kit (Qiagen), and stored at -80°C until use. Extraction of total cellular RNA, reverse transcription to generate cDNA, and real-time quantitative PCR were performed using a Chromo4 Real-Time PCR detector (Bio-Rad Laboratories, Hercules) and QuantiTect SYBR Green PCR reagents (Qiagen) as described previously (12). The primer sequences for CRTH2 and GAPDH are shown in Table I. Standard curves for both CRTH2 and GAPDH were generated using a PCR fragment of CRTH2 and plasmid DNA of GAPDH as a standard, respectively. Then absolute copy number of CRTH2 and GAPDH for each sample was calculated, and samples were reported with a CRTH2 copy number relative to GAPDH.

Relative amounts of IL-4, IL-5, IL-13, IFN-γ, IL-1β, IL-6, TNF-α, RANTES, and cotaxin mRNA in nasal mucosa were also measured. The primers used are listed in Table I.

Statistical analysis

Statistical significance was determined by nonparametric Mann-Whitney *U* tests. *p* values of <0.05 were considered to indicate statistical significance. Values are shown as means ± SEM.

Table 1. Primary sequences used for real-time PCR amplifications

| | Forward Primer | Reverse Primer | Amplification Size (bp) | Genbank Accession No. |
|---------------|-------------------------|---------------------------|-------------------------|-----------------------|
| IL-4 | CCTCACAGCAACGAAGAACA | CTGCAGCTCCATGAGAACAC | 133 | NM_021283 |
| IL-5 | TCAGCTGTGTCTGGGCCACT | TTATGAGTAGGGACAGGAAGCCTCA | 133 | NM_010558 |
| IL-13 | TGCTTGCCCTGGTGGTCTC | CAGGTCCACACTCCATACC | 151 | NM_008355 |
| IFN- γ | GCGTCATGAATCACACCTG | ACCTGTGGGTTGTGACCTC | 103 | NM_008337 |
| IL-1 β | TCCAGGATGAGGACATGAGCAC | GAACGTACACCAGCAGGTTA | 105 | NM_008361 |
| IL-6 | CCACTTCACAAGTCGGAGGCTTA | GCAAGTGCATCATCGTTGTTTCATC | 112 | NM_031168 |
| TNF- α | ATGAGCACAGAAAGCATGATC | TCCACTTGGTGGTGTGCTACG | 305 | NM_013693 |
| RANTES | AGATCTCTGCAGCTGCCCTCA | GAGCACTTGCTGCTGGTGTAG | 170 | NM_013653 |
| Eotaxin | CAGATGCACCCTGAAAGCCATA | TGCTTTGTGGCATCCCTGGAC | 96 | NM_011330 |
| CRTH2 | TCTCAACCAATCAGCACACC | CCTCCAAGAGTGGACAGAGC | 173 | NM_009962 |
| GAPDH | ACCACAGTCCATGCCATCAC | TCCACCACCTGTGTGCTGTA | 452 | NM_008084 |

Results

Induction of nasal symptoms in Cry j 1-sensitized mice

We first attempted to generate a mouse model mimicking human allergic rhinitis, especially pollinosis, which causes symptoms of nasal symptoms, including sneezing and nasal rubbing, by intranasal administration of Cry j 1. We found a significant and dose-dependent increase in the frequency of sneezing in BALB/c mice sensitized with Cry j 1. Mice that were treated with PBS alone sneezed 1.8 ± 0.3 (mean \pm SEM) times in the 10 min following the final Ag administration, whereas they sneezed 5.8 ± 1.2 times and 15.7 ± 2.7 times when treated with low and high doses of Ag, respectively (Fig. 2A). Similarly, immediately after the final Ag challenge, nasal rubbing was observed more frequently in mice sensitized with a high dose of Cry j 1 than in control mice (37.3 ± 5.8 vs 11.2 ± 2.7 times in 10 min). At a low dose of Cry j 1, there was no significant increase in the frequency of nasal rubbing (Fig. 2B).

Development of Th2-type immune responses in Cry j 1-sensitized mice

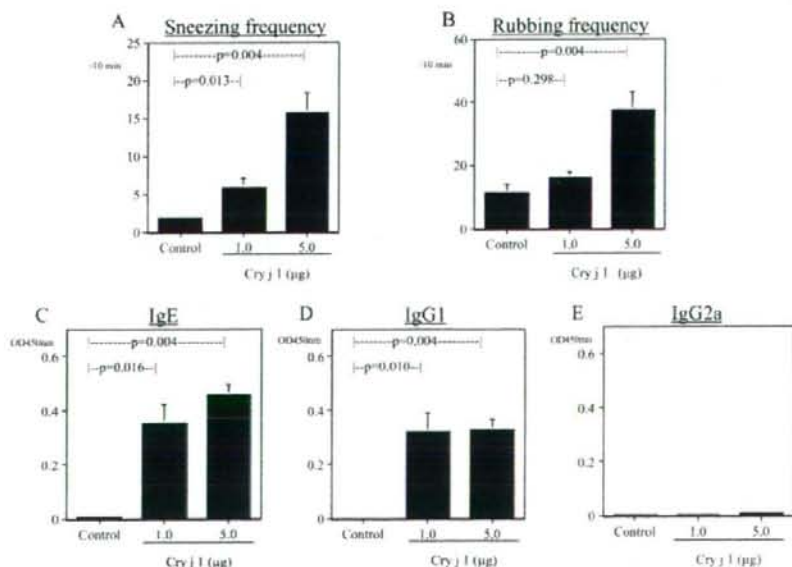
To further characterize the pathogenesis of immune responses caused by Cry j 1, we monitored several parameters associated with pollinosis. Nasal challenge with a low or high dose of Cry j

1 caused a considerable increase in the concentration of Cry j 1-specific IgE in sera when measured 1 day after the final challenge (Fig. 2C). There was also a significant elevation in the concentration of Cry j 1-specific IgG1 (Fig. 2D). The concentration of Cry j 1-specific IgE and IgG1 was not appreciably different at the low and high doses of Cry j 1. Cry j 1, however, had little effect on the level of Cry j 1-specific IgG2a (Fig. 2E).

Eosinophil infiltration into nasal mucosa, another characteristic of pollinosis, is rarely seen in the nasal mucosa in control mice (Fig. 3A). On the contrary, there was a marked accumulation of eosinophils not only in the lamina propria but also in the epithelial layer in mice 1 day after the final challenge (Fig. 3, B and C). Eosinophil numbers per field following intranasal Cry j 1 sensitization/challenge at both low and high doses were significantly higher than in control mice (Fig. 3D). The nasal mucosa of Cry j 1-sensitized mice also showed severe infiltration by mononuclear cells. The nasal septum of mice treated with low and high doses of Cry j 1 contained more mononuclear cells per field (59.8 ± 9.0 ($p = 0.055$) and 80.2 ± 9.1 ($p = 0.016$), respectively) than did control mice (39.8 ± 4.7).

We next examined the *in vitro* production of cytokines in culture by cells isolated from submandibular lymph nodes from mice treated *in vivo* with or without Cry j 1. The amounts of IL-4 and

FIGURE 2. Nasal hyperresponsive symptoms and Ab production in mice following intranasal sensitization and challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of Cry j 1. Nasal allergic symptoms, including the frequency of sneezing (A) and rubbing (B), were determined for the 10 min immediately following the final nasal challenge (day 28). Mean frequencies \pm SEM are shown. Serum samples were obtained 16 h after the final intranasal challenge. Cry j 1-specific IgE (C), IgG1 (D), and IgG2a (E) levels were determined by ELISA. Mean OD values \pm SEM are shown. Results are representative of two independent experiments.



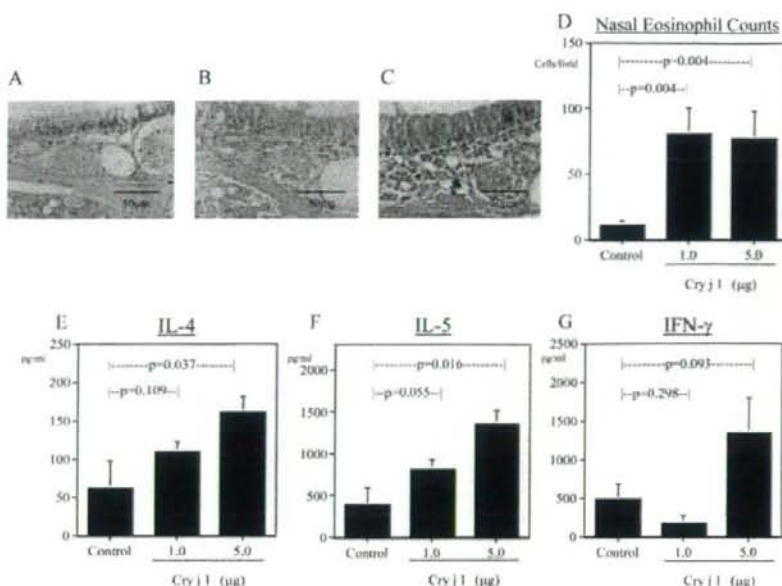


FIGURE 3. Nasal eosinophilia and cytokine production by submandibular lymph node cells following intranasal sensitization and challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of PBS (A), low-dose Cry j 1 (B), or high-dose Cry j 1 (C) according to the schedule shown in Fig. 1. Sixteen hours after the final challenge, nasal sections were collected, fixed, and decalcified, and eosinophils in the nasal mucosa were detected by Luna stain. D, The numbers of eosinophils in the posterior portion of the nasal septum were determined per high-power (10×40) microscopic field. Mean numbers of infiltrating cells per field \pm SEM are shown. Sixteen hours after the final challenge, submandibular lymph node cells were isolated and cultured in the absence or presence of Cry j 1 for 72 h. IL-4 (E), IL-5 (F), and IFN- γ (G) were measured by ELISA. Mean concentrations \pm SEM are shown. Results are representative of two independent experiments.

IL-5 produced by the cells were in proportion to the doses used for in vivo sensitization (Fig. 3, E and F). IFN- γ production was slightly enhanced in lymph node cells from mice treated with a high dose of Cry j 1 compared with control mice, but the increase was not statistically significant (Fig. 3G).

CRTH2 mRNA expression in nasal mucosa of Cry j 1-sensitized mice

We next measured the expression of CRTH2 at sites of nasal inflammation. Control mice treated with PBS expressed a low level of CRTH2 mRNA in the mucosal tissue of the nasal septum. In mice treated with Cry j 1, the level of CRTH2 mRNA was significantly increased (Fig. 4). Thus, we further investigated whether CRTH2 is positively or negatively involved in the pathophysiology of pollinosis using CRTH2 $^{-/-}$ mice.

Impaired pathophysiology of pollinosis in Cry j 1-sensitized CRTH2 $^{-/-}$ mice

A high dose of Cry j 1 was administered to both wild-type (WT) and CRTH2 $^{-/-}$ mice, and the nasal hyperresponsive symptoms were examined immediately after the final nasal challenge. Notably, the number of sneezes in 10 min by the Cry j 1-sensitized mutant mice was significantly lower than by the WT mice (Fig. 5A). Nasal rubbing was also significantly lower in the CRTH2 $^{-/-}$ mice than in the WT mice (Fig. 5B).

The level of Cry j 1-specific IgE in serum samples collected on the day following the final Ag challenge was significantly lower for mutant mice than for WT mice (Fig. 5C). Production of Cry j 1-specific IgG1 was similarly reduced in CRTH2 $^{-/-}$ mice compared with WT mice (Fig. 5D). In contrast, serum levels of Cry j 1-specific IgG2a were the same in the two mouse strains (Fig. 5E).

The number of eosinophils infiltrating into the nasal septum following administration of Cry j 1 was also significantly lower in the CRTH2 $^{-/-}$ mice than in the WT mice (Fig. 6A–C). Although the number of mononuclear cells infiltrating the nasal septum was not significantly different in the mutant and WT mice (Fig. 6D), the number of infiltrating CD3 $^{+}$ cells was significantly reduced in CRTH2 $^{-/-}$ mice as compared with WT mice (Fig. 6E). These

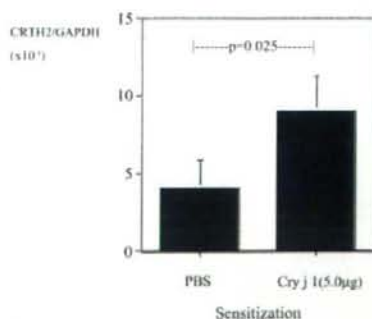
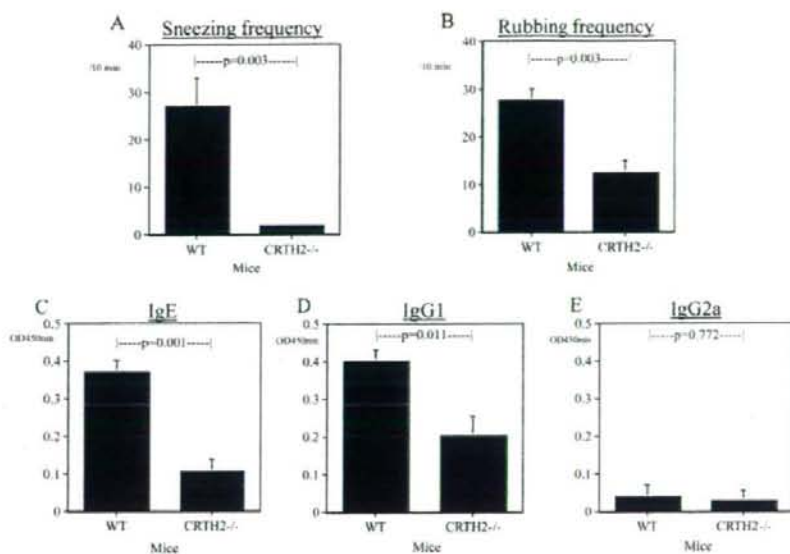


FIGURE 4. Relative amounts of CRTH2 mRNA in nasal mucosa. Mice (6 per group) were sensitized by intranasal administration of 5.0 μ g of Cry j 1 once a week for 3 wk. One week after the third sensitization, mice were challenged by intranasal administration of 1.0 μ g of Cry j 1 each day for 7 consecutive days. Control animals were treated with PBS at all steps except for the final challenge, where they were treated with 1.0 μ g of Cry j 1. Sixteen hours after the final challenge with Cry j 1, mucosal tissues were removed from the nasal septum. The CRTH2 mRNA levels were estimated using real-time quantitative PCR. Results are the mean amounts of mRNA \pm SEM.

FIGURE 5. Nasal symptoms and Ab production in WT and CRTH2^{-/-} mice following the final nasal challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of Cry j 1. Sneezing (A) and rubbing (B) frequency were measured for 10 min following the final nasal challenge (day 28). Mean frequencies \pm SEM are shown. Sixteen hours after the final nasal challenge, blood was sampled from mice, and levels of serum Cry j 1-specific IgE (C), IgG1 (D), and IgG2a (E) were determined by ELISA. Mean ODs \pm SEM are shown. Results are representative of two independent experiments.



results suggest that CRTH2 deficiency affects infiltration of not only eosinophils but also T cells.

To clarify the link between CRTH2 deficiency and the relief of allergic symptoms, we further investigated cytokine production *in vitro* by cells from submandibular lymph nodes obtained the day after the final Ag challenge. The amount of IL-4 was 5-fold lower in CRTH2^{-/-} mice than in WT mice (Fig. 6F). Additionally, there was a slight reduction in the amount of IL-5 (Fig. 6G) and a slight

increase in the amount of INF- γ (Fig. 6I) in the cells from CRTH2^{-/-} mice, but the differences were not significant. On the contrary, the levels of IL-13 were significantly higher in CRTH2^{-/-} mice as compared with WT mice (Fig. 6H).

Additionally, mRNA levels of Th2 cytokines (IL-4, IL-5, and IL-13), Th1 cytokine (INF- γ), proinflammatory cytokines (IL-1 β , IL-6 and TNF- α), and eosinophil-chemotactic chemokines (RANTES and eotaxin) in nasal mucosa were determined. The

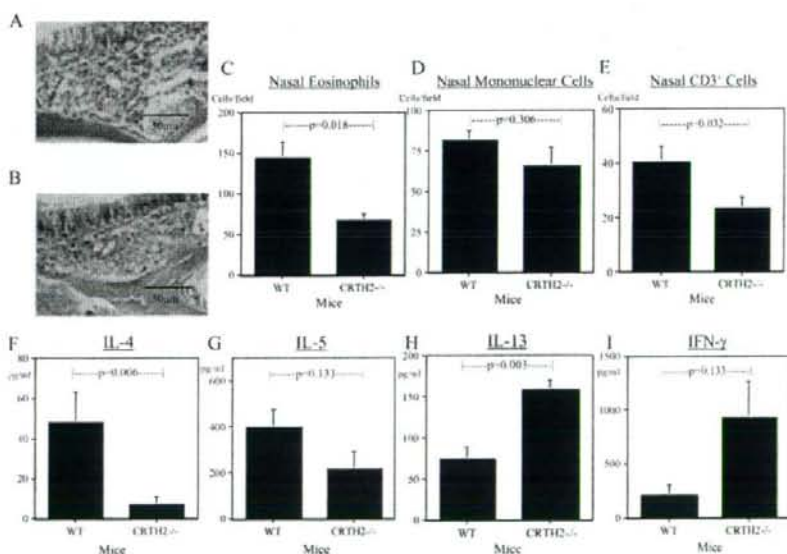
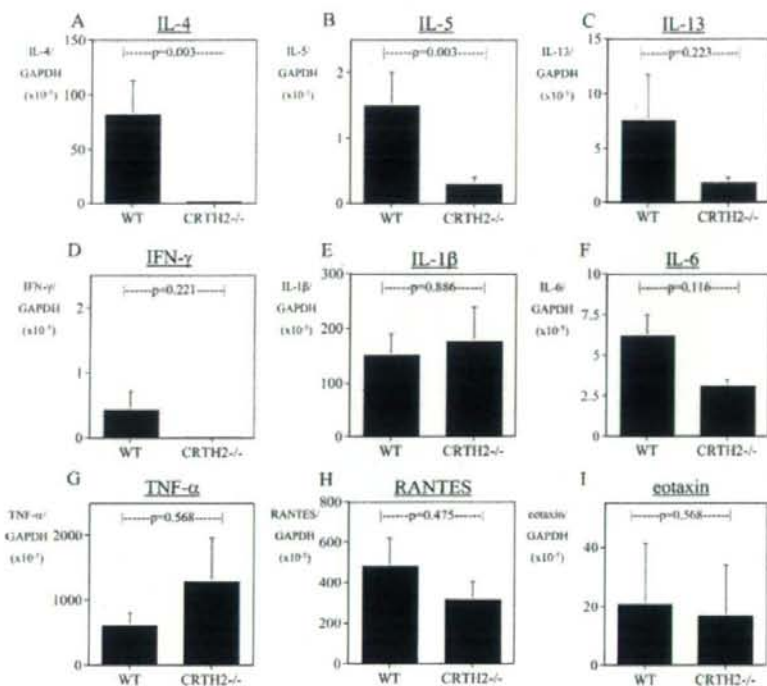


FIGURE 6. Histological changes and cytokine production by submandibular lymphocytes following nasal challenge with Cry j 1 in WT and CRTH2^{-/-} mice. WT (A) and CRTH2^{-/-} (B) mice were sensitized and challenged by intranasal administration of Cry j 1. Sixteen hours following the final nasal challenge with Cry j 1, nasal sections were collected, fixed, and decalcified, and eosinophils in nasal mucosa were detected by Luna stain. C, The number of eosinophils in the posterior portion of the nasal septum was determined per high-power (10 \times 40) microscopic field. Mean numbers of infiltrating eosinophils per field \pm SEM are shown. Numbers of mononuclear cells (D) and CD3⁺ cell (E) in the nasal septum were also determined. Sixteen hours after the final challenge with Cry j 1, submandibular lymph node cells were isolated and cultured with Cry j 1 for 72 h. IL-4 (F), IL-5 (G), IL-13 (H), and IFN- γ (I) were measured by ELISA. Mean concentrations \pm SEM are shown. Results are representative of two independent experiments.

FIGURE 7. Relative amounts of cytokines/chemokines mRNA in nasal mucosa following nasal challenge with Cry j 1 in WT and CRTH2^{-/-} mice. Sixteen hours after the final nasal challenge with Cry j 1, mucosal tissues were removed from nasal septum. Relative amounts of IL-4 (A), IL-5 (B), IL-13 (C), IFN- γ (D), IL-1 β (E), IL-6 (F), TNF- α (G), RANTES (H), and eotaxin (I) mRNA were compared between WT and CRTH2^{-/-} mice. Results are the mean amounts of mRNA \pm SEM.



levels of IL-4 and IL-5 mRNA were significantly lower in CRTH2^{-/-} mice as compared with WT mice, whereas the levels of other cytokines/chemokines were similar between CRTH2^{-/-}

and WT mice (Fig. 7). These results suggest that reduced nasal eosinophilia in CRTH2 deficiency is associated with reduced levels of IL-5 but not RANTES or eotaxin in this model. Additionally,

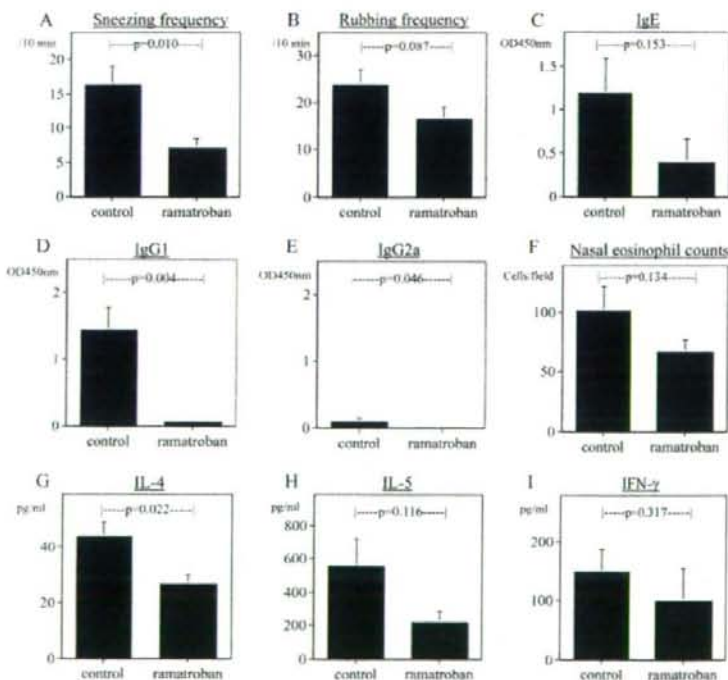


FIGURE 8. Effects of ramatroban on murine JCP. Ramatroban (30 mg/kg body weight), suspended in 5% methyl cellulose, was given orally once a day from 1 day before the first sensitization to the final challenge (day 0 to day 28). Control mice were given 5% methyl cellulose alone. After the final intranasal challenge, the frequencies of sneezing (A) and rubbing (B) were counted, and serum levels of Cry j 1-specific IgE (C), IgG1 (D), IgG2a (E), and nasal eosinophil count (F), as well as Cry j 1-induced IL-4 (G), IL-5 (H) and IFN- γ (I) were determined as described in *Materials and Methods*. Results are expressed as means \pm SEM.

it is suggested that CRTH2-mediated pathway may induce pathology without regulating local production of these proinflammatory cytokines.

Outcomes of pollinosis were compared between sensitized/challenged CRTH2^{-/-} mice and nonsensitized/single-challenged CRTH2^{-/-} mice. The levels of Cry j 1-specific IgE (0.159 ± 0.044 vs 0 ± 0 OD at 450 nm; $p = 0.003$), Cry j 1-specific IgG1 (0.638 ± 0.163 vs 0 ± 0 OD at 450 nm; $p = 0.004$), nasal eosinophilia (66.4 ± 8.2 vs 6.6 ± 1.1 cells/field; $p = 0.005$), and IL-4 production by submandibular lymph node cell (72.8 ± 31.1 vs 6.7 ± 3.8 pg/ml; $p = 0.004$) were significantly higher in sensitized and subsequently challenged CRTH2^{-/-} mice as compared with nonsensitized and single-challenged CRTH2^{-/-} mice. However, the frequencies of sneezing (1.7 ± 0.5 vs 0.6 ± 0.2 times in 10 min; $p = 0.088$) and rubbing (12.3 ± 2.6 vs 10.2 ± 3.1 times in 10 min; $p = 0.516$) were similar between two groups, suggesting that CRTH2 is particularly essential for the development of nasal symptoms.

Effect of ramatroban on Cry j 1-induced pollinosis

As seen in CRTH2-deficient mice, treatment with ramatroban significantly reduced several indicators of pollinosis including sneezing, Cry j 1-specific IgG1 production, and Cry j 1-induced IL-4 production by submandibular lymph node cells as compared with the control treatment (Fig. 8, A, D, and G). Although the differences did not reach to the statistical level, other parameters such as nasal rubbing, Cry j 1-specific IgE production, nasal eosinophilia, and Cry j 1-induced IL-5 production were also reduced by the treatment with ramatroban (Fig. 8, B, C, F, and H).

Discussion

In the present study, we analyzed the pathophysiological effects of nasal exposure to Cry j 1 in BALB/c mice. Mice sensitized with Cry j 1 without adjuvants showed not only allergic symptoms such as sneezing and rubbing but also produced Cry j 1-specific IgE and IgG1 and displayed nasal eosinophilia. Additionally, submandibular lymph node cells isolated from these mice produced IL-4 and IL-5 in recall response to Cry j 1. These results suggest that intranasal sensitization with Cry j 1 induces pollinosis in BALB/c mice.

To investigate the initiation of allergic rhinitis *in vivo*, administration of Ags via the natural route (i.e., through the nostril) is desirable. In fact, it is known that administration of Ags through different routes results in different degrees of IgE production (27, 28). Also, murine models of allergic rhinitis have been generated by intranasal or aerosol-mediated sensitization (8, 29), but these models generally employ adjuvants such as cholera toxin, which have immunoregulatory effects that may distort the physical sensitization (30, 31). Therefore, we and others have established murine models of allergic rhinitis by intranasal sensitization with Ags including *Schistosoma mansoni* egg Ag, phospholipase A₂ from honeybee venom, extracts of *Aspergillus fumigatus*, OVA, and trimellitic anhydride in the absence of adjuvants (5–7, 9, 32). We think that our current model is the first in which murine pollinosis was induced by intranasal sensitization with pollen allergen in the absence of an adjuvant. This model may be useful not only for understanding the pathophysiology of pollinosis but also for developing and/or testing new therapies for allergic rhinitis, especially JCP.

BALB/c mice sensitized with Cry j 1 showed an increase in the expression of CRTH2 mRNA in the nasal septum compared with control mice. This agrees with our recent report demonstrating that the amount of CRTH2 mRNA in nasal mucosa is significantly higher in patients with allergic rhinitis than in control subjects not

showing hypertrophy of inferior turbinates (12). These results suggest that the expression of CRTH2 may play a role in the pathogenesis of allergic rhinitis both in humans and in mice. In fact, it is known that the expression of CRTH2 in eosinophils and CD4⁺ T cells is elevated in atopic patients (33–35). CRTH2 is expressed by eosinophils and a subset of CD3⁺ T cells in nasal mucosa, especially in patients with allergic rhinitis (23). Because a mAb against murine CRTH2 that can be used for immunohistochemistry is not currently available, we could not investigate the phenotype of cells expressing CRTH2 in mice.

The pathophysiology of allergic rhinitis was clearly impaired in CRTH2^{-/-} mice. Following repeated intranasal sensitization and nasal challenge with Cry j 1, CRTH2^{-/-} mice displayed reduced nasal symptoms, production of Cry j 1-specific IgE and IgG1, and nasal eosinophilia compared with WT mice. Additionally, submandibular lymph node cells from Cry j 1-sensitized CRTH2^{-/-} mice produced significantly less IL-4 and IL-5 in response to Cry j 1 than those from WT mice. We think that the present results are the first demonstration of the *in vivo* role of CRTH2 in the initiation of Th2 responses in the upper airway.

We also found that Cry j 1-specific IgE and IgG1 but not IgG2a production was impaired in CRTH2^{-/-} mice. Ag-specific IgE/IgG1 and IgG2a production is known to be positively regulated by Th2 and Th1 responses, respectively, in mice (36). Thus, our results indicate that signals mediated by CRTH2 selectively enhance Th2-type Ab production. The decreased production of IL-4 by submandibular lymph node cells from CRTH2^{-/-} mice in response to Cry j 1 restimulation supports this result because IL-4 plays a critical role in IgE synthesis *in vivo* (37). Although whether CRTH2 activation directly leads to IL-4 production in mice remains unclear, recent investigations have demonstrated that PGD₂ causes the preferential induction of IL-4 production by Th2 cells in humans by binding to CRTH2 (38, 39). Additionally, our recent report showing that CRTH2 signals up-regulate CD40L in resting human Th2 cells supports our conclusions because the engagement of CD40 by CD40L is also essential for IgE isotype switching (39, 40).

After intranasal sensitization with Cry j 1, CRTH2^{-/-} mice developed a weaker eosinophilia than did WT BALB/c mice. This suggests that CRTH2 mediates local eosinophil recruitment in this model, which agrees with reports showing that CRTH2 activation leads to changes in eosinophil shape, chemotaxis, and degranulation *in vitro* (16, 18, 41). Additionally, recent investigations have revealed that CRTH2 plays a proinflammatory role in eosinophil chemotaxis into inflamed tissue *in vivo* (11, 12, 21, 24, 42). On the other hand, submandibular lymph node cells from WT and CRTH2^{-/-} mice produced similar amount of IL-5 after intranasal sensitization with Cry j 1. It is well known that IL-5 plays a critical role in eosinophilic inflammation, especially in mice (43). Although little is known about whether CRTH2 activation enhances IL-5 production in mice, CRTH2 activation on Th2 cells is known to induce IL-5 production in humans (38, 39). One explanation of why nasal eosinophilia was reduced in CRTH2^{-/-} mice irrespective of IL-5 production is that cognate interaction between PGD₂ and CRTH2 on eosinophils may have an additive effect on local eosinophil recruitment, primarily due to the action of IL-5. In fact, in a mouse model of asthma, nebulized DK-PGD₂, a CRTH2 agonist, exacerbates eosinophilic lung inflammation without changes in IL-5 content in lung (21).

CRTH2^{-/-} mice displayed a significantly lower frequency of both sneezing and nasal rubbing after the nasal challenge compared with the WT mice. Several molecules, including IL-5, CD80/CD86, H1, and CD39, have been shown to contribute to these symptoms via different mechanisms (38, 44–46). The present result suggests that activation of CRTH2 is also involved

in the symptoms of nasal hyperreactivity. In humans, nasal challenge with PGD₂ induces a sustained nasal obstruction but not sneezing or rhinorrhea (47). Whether murine mast cells express CRTH2 is not well known, and further investigations are needed to determine whether the effect of CRTH2 on nasal hyperreactivity is due to the control of Th2 responses or to a direct effect on mast cells.

Treatment with ramatroban, a CRTH2/TP dual antagonist, induced a reduction in several indicators of JCP such as sneezing, Cry j 1-specific IgG1 production, and Cry j 1-induced IL-4 production. It is known that ramatroban suppresses allergic responses including nasal signs both in vivo and in vitro (11, 24, 46, 48). For example, ramatroban significantly inhibited sneezing and nasal rubbing induced by Ag in actively sensitized C57BL/6 mice and guinea pigs (46, 48). Our present results are consistent with these reports and support the findings seen in CRTH2^{-/-} mice that suggest a proinflammatory role of CRTH2 in allergic rhinitis. On the other hand, treatment with ramatroban was less effective than CRTH2 deficiency in all parameters of investigation. One of the possible reasons is that ramatroban antagonizes not only CRTH2 but also TP. Since it is not fully elucidated whether signals through TP, especially in mice, are proinflammatory or antiinflammatory in allergic rhinitis, simultaneous blockage with TP may affect changes of the outcomes induced by CRTH2 antagonism.

In conclusion, we developed a novel model of murine allergic rhinitis that mimics pollinosis. Additionally, we found that CRTH2 plays an essential role in the initiation of allergic rhinitis in mice. These results suggest that this murine model will be useful for elucidating the pathophysiology of allergic rhinitis, especially JCP. These observations may provide a basis for developing therapeutic approaches for managing allergic rhinitis, specifically by inhibiting PGD₂-CRTH2 interactions in the nose of individuals with allergic rhinitis.

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Disclosures

The authors have no financial conflicts of interest.

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Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis

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Summary

Background IL-33, an IL-1-like cytokine, is a ligand for IL1RL1, which is an important effector molecule of type 2 T helper responses. Although IL-33/IL1RL1 interaction has been suggested to be important in induction of allergic airway inflammation, serum levels of IL-33 and the genetic influences of the polymorphisms of IL-33 in human allergic diseases are unclear.

Objective The aim of this study was to examine whether the serum IL-33 level and polymorphisms in IL-33 are associated with Japanese cedar (JC) pollinosis, the most common form of allergic rhinitis, and a major public health problem, in Japan.

Methods We performed linkage disequilibrium (LD) mapping of the gene using the HapMap database, and two selected tag single nucleotide polymorphisms were genotyped. We conducted an association study of IL-33 (JC pollinosis, $n = 170$; normal controls, $n = 100$) and measured the IL-33 levels in sera of the 270 subjects by ELISA.

Results Serum levels of IL-33 were significantly higher in patients with JC pollinosis ($P = 0.0018$) than in controls. In genetic association analysis, we found a positive association between the polymorphism and JC pollinosis ($P = 0.048$).

Conclusion Our results support a role for IL-33 in the pathogenesis of JC pollinosis.

Keywords association, IL-33, JC pollinosis, polymorphism, serum level

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Introduction

Allergic diseases are increasing world-wide, and Japanese cedar (JC) pollinosis, which is a disease of allergic rhinitis and allergic conjunctivitis caused by exposure to JC pollen, is one of the most common allergic diseases in Japan [1]. The increase of JC pollinosis in Japan has become a social problem, with a prevalence of > 16% [1]. Allergic rhinitis is the result of an inflammatory reaction triggered by type 2 T helper (Th2) cell-mediated immune responses against allergens [2]. Th2 cytokines induce B cells to produce high amounts of IgG4 and IgE in humans, and promote the growth and differentiation of mast cells and eosinophils [3, 4]. IL1RL1 belongs to the IL-1 receptor

family and functions as an important effector molecule of Th2 responses [5–8]. IL-33, an IL-1-like cytokine, has been identified as a ligand for IL1RL1, and can be detected in epithelial cells from the bronchi and small airways, which indicates a possible role in the regulation of mucosal function [9]. It activates NF- κ B and mitogen-activated protein kinases, and drives production of Th2-associated cytokines from *in vitro* polarized Th2 cells via IL1RL1 [9]. *In vivo* analysis has demonstrated that IL-33 strongly induces gene expression of Th2-associated cytokines such as IL-4, IL-5 and IL-13, and IL-33-treated mice have significantly higher serum levels of IgE. In addition, IL-33 induces pathological changes in mucosal organs such as the lung, resulting in hypertrophied epithelial lining of the airways with large amount of mucus,

and those changes are primarily restricted to the bronchi and larger bronchioles [9].

Although very little work has been done with IL-33, intensive studies of IL-33 receptor IL1RL1 have shown its regulatory functions in the development and effector phases of Th2 responses [10]. The *IL1RL1* gene encodes a soluble-secreted protein, IL1RL1, and a transmembrane protein, ST2L [11]. In murine models of allergic airway inflammation, increases in endogenous IL1RL1 protein after allergen exposure modulate Th2-mediated airway inflammation [12], and blockade of the binding of the ligand for ST2L using a recombinant IgG fusion protein inhibits allergic inflammation [6, 7]. Other studies have reported that ST2L is a reliable selective marker of both murine and human Th2 lymphocytes in allergic airway inflammation [7, 13]. Moreover, a study has revealed that soluble IL1RL1 acts as a negative regulator of Th2 cytokine production via IL-33 signalling in allergic airway inflammation. In asthmatic patients, serum levels of soluble IL1RL1 are markedly elevated during acute attacks and the magnitude of the elevation correlates with the reduction of pulmonary functions and increased levels of serum IL-5 [14]. These findings imply that IL-33 is a good candidate for involvement in JC pollinosis, an allergen-induced upper airway inflammation.

A large number of association studies using polymorphic markers have been performed to discover genetic components in the pathogenesis of allergic diseases [15–17]. Recently, we have reported that functional single nucleotide polymorphisms (SNPs) in the *IL1RL1* distal promoter region are associated with atopic dermatitis. The genetic variants regulate *IL1RL1* expression, and immunohistochemical staining of a skin biopsy specimen from an atopic dermatitis patient showed *IL1RL1* staining in keratinocytes as well as in cells infiltrating the dermal layer [18]. However, there have been no genetic association studies with IL-33.

In this study, to test whether genetic variations of IL-33 contribute to susceptibility to JC pollinosis, we first selected a genetic polymorphism of IL-33 using HapMap linkage disequilibrium (LD) data and conducted association studies. In addition, we examined the associations between serum IL-33 levels and JC pollinosis and serum total IgE levels.

Methods

Study subjects

All subjects were recruited from residents of Eiheiji-cho, in Fukui prefecture, in the central area of Japan between May and June 2006. Because these participants were workers of the Fukui University hospital and students of nursing and medical colleges in Fukui, the number of females was higher than that of males. Specific IgE

to seven aeroallergens, *Cryptomeria japonica*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Candida albicans*, *Aspergillus fumigatus*, *Dactylis glomerata* and *Ambrosia*, were measured with a Pharmacia CAP System (Pharmacia CAP, Uppsala, Sweden) (Table 1). Positive sensitization refers to an allergen-specific serum IgE level >0.7 (CAP RAST score of 2). Diagnosis of JC pollinosis was confirmed by symptoms of allergic rhinoconjunctivitis during the JC pollinosis season and positive serum-specific IgE towards JC pollinosis. A total of 170 patients with JC pollinosis were recruited (Table 1). One hundred healthy subjects who had never had symptoms of allergic rhinitis and showed no sensitization to any of the seven aeroallergens were recruited as controls (Table 1). We recruited 29 subjects with infectious rhinitis who were diagnosed by otolaryngologists and showed no sensitization to any of the seven aeroallergens. All individuals were unrelated Japanese and gave written informed consent to participate in the study according to the rules of the process committees at the School of Medicine, University of Fukui, the Nippon Medical School and The Institute of Physical and Chemical Research.

Selection of polymorphisms for genotyping

Genomic DNA was prepared from peripheral blood samples, using standard protocols. There were 22 SNPs in the *IL-33* gene with a minor allele frequency (MAF) of >10% in the HapMap Japanese data set (URL: <http://www.hapmap.org/index.html.en>) (Table 2). Pairwise LD was calculated as r^2 by using the Haploview 3.2 program (<http://www.broad.mit.edu/mpg/haploview/>). Genotyping of SNPs was performed by the TaqMan™ allele-specific amplification (TaqMan-ASA) method (Applied Biosystems, Foster City, CA, USA). rs1929992 was genotyped by Custom TaqMan® SNP Genotyping Assay Service with primers 5'-GGAAAAAACACATTTTCCCCCAA-3' and 5'-AAACCATCTAACTACTACTAAAATGTATAAAGTGTAGAATTAT-3'. The probes used were VIC-TCATGGTCAAAATATTGAAAT and FAM-ATGGTCAAAATGTTGAAAT. rs10975519 was genotyped by TaqMan(R) Pre-Designed SNP Genotyping Assays, C_2762153_10.

Reagents for human interleukin-33

Recombinant human IL-33 (rhIL-33) and a rabbit-neutralizing anti-hIL-33 IgG antibody were made by Hokudo Co., Ltd. (Sapporo, Japan). Briefly, rhIL-33 (mature form) was amplified from human lung cDNA (BioChain Institute, Hayward, CA, USA) as a template, and subcloned into pET28a vector (Novagen, Madison, WI, USA). BL21 (DE3) RIL was transformed and the expressed recombinant protein was purified with Ni-NTA resin. Endotoxin was removed by filtration through Zetapor (Cuno, Meriden, CT, USA). For establishment of a polyclonal antibody to hIL-33, rabbits

were immunized with rhIL-33 (200 µg/body) with CFA, and boosted with rhIL-33 (200 µg/body) with IFA three times every 2 weeks. Seven weeks later, serum was collected and the antibody was purified using a Protein-A sepharose column. This IgG antibody (R2) was further purified with an rhIL-33 sepharose column and was biotinylated with NHS-biotin (Sigma, St Louis, MO, USA) in our laboratory. This purified anti-hIL-33 antibody could completely neutralize 50 ng/mL of IL-33 at the concentration of 10 µg/mL *in vitro*.

Table 1. Characteristics of the patients with Japanese cedar (JC) pollinosis and controls

| Characteristics | Case | Control |
|--|-------------|--------------|
| Age (year, median with range) | 30 (20–49) | 32.5 (20–49) |
| Total subjects and sex (% male subjects) | 170 (14) | 100 (9.0) |
| Serum total IgE (IU/mL, mean±SEM) | 280.2±879.2 | 42.9±51.5 |
| Atopic sensitization (RAST) (number (%)) | | |
| Japanese cedar pollen positive | 170 (100) | 0 (0) |
| <i>Dermatophagoides pteronyssinus</i> positive | 80 (47.1) | 0 (0) |
| <i>Dermatophagoides farinae</i> positive | 78 (45.9) | 0 (0) |
| <i>Candida albicans</i> positive | 10 (5.9) | 0 (0) |
| <i>Aspergillus</i> positive | 3 (1.8) | 0 (0) |
| <i>Dactylis glomerata</i> positive | 61 (35.9) | 0 (0) |
| <i>Ambrosia</i> positive | 23 (13.5) | 0 (0) |

Enzyme-linked immunosorbent assay of serum levels of interleukin-33

To elucidate the biological roles of the IL-33 gene, we constructed an ELISA system to quantify human IL-33 protein in sera of subjects with JC pollinosis and controls. A 96-well plate (Costar, Cambridge, MA, USA) was coated with the anti-hIL-33 IgG antibody (R2) and blocked with StartingBlock™ blocking buffer (PIERCE, Rockford, IL, USA). Human IL-33 was detected with the biotinylated-anti-IL-33 antibody and streptavidin-HRP. The ELISA system was specific for hIL-33 and did not cross-react with other cytokines tested, which included IL-1β, IL-2, IL-4, IL-12, IL-18, TNF-α, IFN-γ and GM-CSF. Serum samples were collected, and then they were stored at -80 °C until measurement. IL-33 was assayed by ELISA with reference standard curves using known amounts of hIL-33. The lower limit of ELISA sensitivity for serum IL-33 was 30 pg/mL. A value of 0 was assigned to results that were below the assay's lower limit of detection for non-parametric statistical calculations in Fig. 2.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit

Table 2. Locations and allele frequencies of polymorphisms in IL-33 based on the HapMap JPT data set

| SNP* | Location | Amino acid | MAF (%) [†] | NCBI [‡] |
|------------------------|--------------------|------------|----------------------|-------------------|
| -5345 G/A | 5'-Flanking region | | 0.477 | rs928414 |
| -5194 T/G | 5'-Flanking region | | 0.477 | rs4237164 |
| -4432 G/A | 5'-Flanking region | | 0.477 | rs10975509 |
| -1611 C/T | 5'-Flanking region | | 0.466 | rs7025417 |
| -1037 T/C | Intron 1 | | 0.467 | rs10975511 |
| 1256 C/T | Intron 1 | | 0.455 | rs4742170 |
| 2241 C/G | Intron 1 | | 0.455 | rs7019575 |
| 4450 G/A | Intron 1 | | 0.455 | rs10975514 |
| 5999 G/A | Intron 1 | | 0.443 | rs10975516 |
| 9318 C/A | Intron 2 | | 0.443 | rs1317230 |
| 9813 G/T | Intron 3 | | 0.455 | rs1330383 |
| 9894 T/C [†] | Intron 3 | | 0.455 | rs1929992 |
| 11607 T/C | Intron 4 | | 0.432 | rs1113573 |
| 11877 C/T [†] | Exon 5 | Tyr163Tyr | 0.433 | rs10975519 |
| 12016 G/C | Intron 5 | | 0.422 | rs10975520 |
| 12514 T/C | Intron 5 | | 0.427 | rs7044343 |
| 13206 A/G | Intron 6 | | 0.487 | rs7871381 |
| 13316 C/A | Intron 6 | | 0.371 | rs1412421 |
| 13625 G/A | Intron 6 | | 0.422 | rs7047921 |
| 14187 G/T | Intron 6 | | 0.420 | rs1332290 |
| 14598 G/A | Exon 7 | 3'-UTR | 0.409 | rs1048274 |
| 23562 G/C | 3'-Flanking region | | 0.455 | rs10815397 |

*Numbering according to the genomic sequence of IL-33 (AL353741.16) and position 1 is the A of the initiation codon. Major allele/minor allele.

[†]SNPs were genotyped in this study.

[‡]Minor allele frequencies

[§]NCBI, number from the dbSNP of NCBI (<http://www.ncbi.nlm.nih.gov/SNP/>).

SNP, single nucleotide polymorphisms; MAF, minor allele frequency.

test at each locus. We then compared differences in allele frequencies and genotype distribution of the polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom, and calculated odds ratios (ORs) with 95% confidence intervals (CIs). Serum total IgE and IL-33 levels were analysed as quantitative levels, and we investigated associations between these levels and genetic variations. Log-transformed individual serum IgE levels were analysed by one-way ANOVA. When the data for IL-33 levels were not distributed normally after log-transformation, they were analysed using non-parametric equivalents and summarized using the median. Multiple comparisons were first analysed by the Kruskal–Wallis test and then by individual testing by the Mann–Whitney *U*-test if significant. Correlations were analysed by Spearman's test. A *P* value of less than 0.05 was considered statistically significant.

Results

Linkage disequilibrium of the IL-33 gene

A total of 22 polymorphisms with a frequency >0.10 in IL-33 were contained in the public databases available at the NCBI dbSNP website (<http://www.ncbi.nlm.nih.gov/SNP/>) (Table 2). Two variants including a synonymous substitution (Tyr163Tyr) were in the exons, and four variants were in the 5'-flanking region of the IL-33

gene. Pairwise LD among the 22 SNPs was measured by different parameters, r^2 using the Haploview 3.2 program (<http://www.broad.mit.edu/mpg/haploview/>) (Fig. 1), and all the 22 SNPs were in strong LD ($r^2 > 0.75$). We finally selected polymorphism rs1929992 and rs10975519 (Tyr163Tyr) for association studies using tagger in the Haploview 3.2 program, and these two SNPs captured 22 of 22 alleles with a mean r^2 of 0.95 ($r^2 > 0.91$).

Association between polymorphisms in the IL-33 gene and susceptibility of Japanese cedar pollinosis

The locus was in Hardy–Weinberg equilibrium in the entire group. To test the association between the SNP and JC pollinosis, we compared differences in the allele frequency and genotype distribution of each polymorphism between case and control subjects by using contingency chi-square tests with one degree of freedom. ORs with 95% CIs were also calculated. In the population genotyped in this study, the MAF of rs1929992 ($C = 0.49$) was higher than those in the HapMap JPT data set ($C = 0.46$). We found a significant association between rs1929992 (T > C) and JC pollinosis (TT + TC vs. CC: OR, 1.82; 95% CI, 1.00–3.31; $P = 0.048$) (Table 3). The serum total IgE level was analysed as a quantitative level, and we investigated the association between this level and genetic variation. However, we could not find any association between the SNP and serum IgE level in this study ($P = 0.46$ by ANOVA).

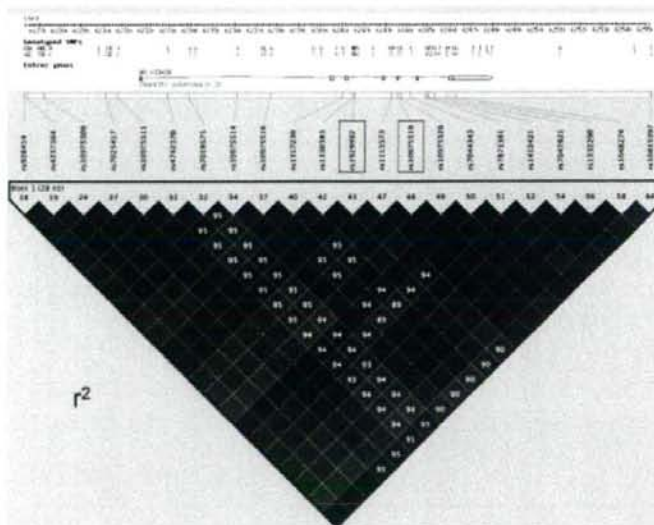


Fig. 1. Pairwise linkage disequilibrium between 22 SNPs as measured by r^2 estimated by the Haploview 3.2 program using the HapMap JPT data set. The boxed polymorphisms, rs1929992 and rs10975519, were genotyped in this study.

Patients with Japanese cedar pollinosis display higher interleukin-33 serum levels than healthy control subjects

To evaluate whether dysregulation at the IL-33 protein level might be a characteristic feature of JC pollinosis, we conducted ELISA assays of sera of patients with JC pollinosis ($n = 170$) and healthy control subjects ($n = 100$). Patients with JC pollinosis exhibited significantly higher serum levels of the IL-33 protein ($P = 0.0018$) (Fig. 2). The median serum IL-33 concentration of JC pollinosis patients was 549 pg/mL, compared with 361.8 pg/mL for controls. In addition, we examined the serum IL-33 level in infectious rhinitis as non-allergic rhinitis. The median serum IL-33 concentration of subjects with infectious

rhinitis was 241.3 pg/mL. There was no significant difference of the serum IL-33 level between healthy control subjects and those with infectious rhinitis. Although total serum IgE and IL-33 levels were analysed as quantitative phenotypes, there was no significant association between the total serum IgE level and serum IL-33 level ($P = 0.095$ by Spearman's test). We also examined whether the *IL-33* genotype affected the serum level of IL-33, but we could not find any significant association between the genotype and serum IL-33 level ($P = 0.58$ by the Kruskal-Wallis test).

Discussion

To determine the role of the *IL-33* gene in the pathogenesis of JC pollinosis, we conducted an association study using the sequence variation of the *IL-33* gene and compared serum IL-33 levels between subjects with JC pollinosis and controls. We found a significant association between JC pollinosis susceptibility and IL-33 polymorphism and higher serum IL-33 levels in subjects with JC pollinosis. Although IL-33 has been thought to play an important role in allergic diseases, this is the first study providing evidence for its involvement in such a disease. We consider the results to be hypothesis generating as the findings in this study need to be confirmed in another population with a larger size.

Recent studies have reported important roles of non-lymphoid cell-derived cytokines such as IL-33 and TSLP in the induction of Th2 differentiation [9, 19]. IL-33 is highly expressed in normal human bronchial epithelial cells and airway smooth muscle cells [9]. It induces Th2-type responses and Th2-associated cytokines IL-4, IL-5 and IL-13 by signalling through IL1RL1 [9, 20]. A recent study has shown that IL-33 induces IL-13 production by mast cells independently of IgE-FcεRI signals in mice. These findings suggest important roles for IL-33 in mast cell- and Th2 cytokine-associated immune disorders [21].

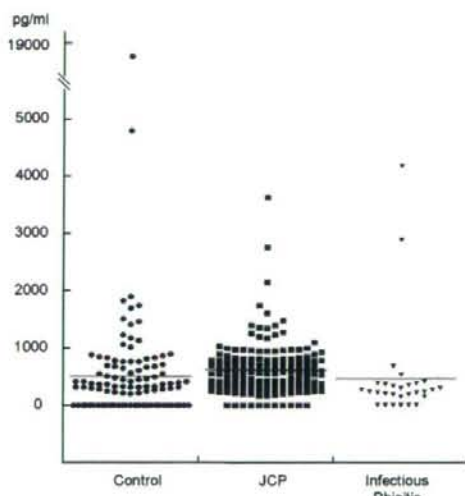


Fig. 2. Comparison of serum IL-33 levels among controls, patients with JC pollinosis and those with infectious rhinitis. Horizontal bars indicate the median value of each group. JCP, Japanese Cedar pollinosis.

Table 3. Association between polymorphisms of *IL-33* and Japanese cedar (JC) pollinosis

| Genotype | Cases ($n = 170$) | Controls ($n = 100$) | Allele | Cases ($n = 170$) | Controls ($n = 100$) | Genotype P | Dominant* P | Recessive† P | Allelic‡ P |
|------------|------------------------|---------------------------|--------|------------------------|---------------------------|--------------|---------------|----------------|--------------|
| rs1929992 | | | | | | | | | |
| TT | 44 (26.0) | 32 (32.3) | T | 162 (47.9) | 112 (56.6) | 0.13 | 0.27 | 0.048 | 0.053 |
| TC | 74 (43.8) | 48 (48.4) | C | 176 (52.1) | 86 (43.4) | | | | |
| CC | 51 (30.2) | 19 (19.2) | | | | | | | |
| rs10975519 | | | | | | | | | |
| CC | 52 (30.6) | 36 (36.0) | C | 177 (52.1) | 119 (59.5) | 0.20 | 0.36 | 0.074 | 0.093 |
| CT | 73 (42.9) | 47 (47.0) | T | 163 (47.9) | 81 (40.5) | | | | |
| TT | 45 (26.5) | 17 (17.0) | | | | | | | |

*Dominant model (TT vs. CC+TC in rs1929992, CC vs. CT+TT in rs10975519).

†Recessive model (TT+TC vs. CC in rs1929992, CC+CT vs. TT in rs10975519).

‡Allelic model (T allele vs. C allele in rs1929992, C allele vs. T allele in rs10975519).

Structurally, IL-33 is related to IL-18, and intensive studies of the relationship between allergic inflammation and IL-18 have been conducted. IL-18 was the first cytokine demonstrated to activate T cells to produce abundant IFN- γ without T cell receptor (TCR) engagement [22]. Furthermore, genetic association studies of the *IL-18* gene have provided evidence for an association with atopic diseases [23–26]. Verhaeghe et al. reported the up-regulation of IL-18 in nasal secretions in allergic rhinitis and the persistence of elevated IL-18 concentrations until after the season [27]. Increased IL-33 concentrations were observed in subjects with JC pollinosis in the present study; however, there was no significant difference in the serum IL-33 level between controls and subjects with infectious rhinitis. Up-regulation of the IL-33 level appears to be characteristic of JC pollinosis. Further analyses of the involvement and interactions of those structurally similar cytokines in allergic inflammation should also be conducted.

Recent reports have shown that IL1RL1 is a reliable marker of Th2 lymphocytes in allergic airway inflammation [7, 13, 28]. Elevated levels of the soluble form of IL1RL1 in the circulation of patients with asthma with acute exacerbation have been reported [14]. The study has also shown that a differential rise of serum IL1RL1 level that correlates well with the severity of asthma exacerbation [14]. In a murine model of allergic airway inflammation, serum murine (m) IL1RL1 protein levels increased after allergen exposure, and pre-treatment with soluble mIL1RL1 protein significantly inhibited the Th2 cytokine production [12]. Other studies have shown that administration of either a monoclonal antibody against IL1RL1 or a recombinant IL1RL1 fusion protein attenuates eosinophilic inflammation of the airways and suppresses IL-4 and IL-5 production *in vivo* following adoptive transfer of Th2 cells [6, 7]. These findings suggest that blocking IL1RL1 pathways would be therapeutically efficacious as a new treatment for allergic diseases, and expression of soluble IL1RL1 could serve as a physiological mechanism to down-regulate Th2-driven immunopathology [10]. In this study, we did not measure the serum soluble IL1RL1 levels, and further examination of the relationship between serum IL-33 and soluble IL1RL1 is needed to clarify their functions in Th2 inflammation. The genetic factors of the *IL-33* gene or serum IL-33 level might provide valuable information for selecting appropriate therapeutic options.

We showed here a significant association between susceptibility to JC pollinosis and a polymorphism. In this study, we selected polymorphisms using HapMap information, and did not examine the functional effects of polymorphisms in strong LD with the related variant. Previous studies have shown that polymorphisms in exons often contribute to their transcript stability [29, 30]. Variants rs10975519 (Tyr163Tyr) and rs1048274 in the

exon might affect the expression level or mRNA stability of the *IL-33* gene. In addition, four genetic variations were in the 5'-flanking region, which is often involved in transcriptional regulation of the gene. Several transcription factors are involved in asthmatic inflammation, including NF- κ B, activator protein-1 (AP-1), nuclear factor of activated T cells (NF-AT), cyclic AMP response element-binding protein (CREB) and signal transduction-activated transcription factors (STAT) [31]. Using the TRAFAC system, we surveyed whether SNPs in the 5' region of the *IL-33* gene create transcription factor binding sites. However, we could not find any SNP that changed the affinity of those transcription factors. The functions of these linked polymorphisms remain to be elucidated. Demonstrating the alteration of gene functions as the result of polymorphisms is necessary to further validate the involvement of the *IL-33* gene in the pathogenesis of JC pollinosis. Furthermore, there were gender differences in the population in this study, and several studies have suggested that sex affects the asthma phenotype, possibly via hormone-related events [32, 33]. If there is a sex-related difference in the association of IL-33 with JC pollinosis, looking at females only might be informative.

Our data strongly support the important role of IL-33 in JC pollinosis. Further investigation of the connections between genotypes and the functional role of IL-33 during allergic events may provide additional targets for therapeutic interventions and would be helpful to clarify the aetiology of allergic diseases.

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