



A mechanism of interleukin-25 production from airway epithelial cells induced by Japanese cedar pollen

Hideaki Kouzaki ^{*}, Hirotaka Kikuoka, Koji Matsumoto, Tomohisa Kato, Ichiro Tojima, Shino Shimizu, Takeshi Shimizu

Department of Otorhinolaryngology, Shiga University of Medical Science, Otsu 520-2192, Shiga, Japan

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ABSTRACT

IL-25 likely has vital roles in initiating and activating type-2 immune responses in AR. We hypothesized that the molecules produced IL-25 by allergen-producing organisms such as JC is involved in the pathogenesis of AR. Participants included 13 patients with Japanese cedar pollinosis and 10 HCs. We measured the IL-25 protein concentration in nasal secretions and in culture supernatants of PNECs. NHBE cells were stimulated with pharmacological and immunological agents and JC. The IL-25 concentration in nasal secretions was significantly higher in patients with Japanese cedar pollinosis than in HCs. JC stimulated IL-25 production from PNECs. TNF- α , IL-4, and IL-13 significantly enhanced JC-induced IL-25 production; *their activation by serine proteases was sufficient to enhance IL-25 production. Furthermore, the NADPH oxidase activity, including JC enhanced IL-25 production. A better understanding of JC-induced IL-25 production by epithelial cells may allow the development of novel therapeutic and preventive strategies for Japanese cedar pollinosis.*

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1. Introduction

Japanese cedar pollinosis is the most common seasonal allergic rhinitis (AR) in Japan, and affects one-third of the Japanese population [1]. Japanese cedar (JC) pollen is usually dispersed between February and April, although the pollen count varies annually. Inhalation of pollen leads to sensitization through the mucosal tissues of the upper respiratory tract, followed by the generation of JC-specific immunoglobulin E (IgE) against two major peptide antigens: *Cryptomeria japonica* 1 (Cry J1) and *Cryptomeria japonica* 2 (Cry J2) [2–4]. Several pollen-derived molecules may be involved in the pathophysiology of allergic diseases through sensitization and exacerbation via IgE-independent or IgE-dependent mechanisms [5–9]. More information on the localization, release, and biological activities of pollen-derived molecules is required for understanding the mechanism of sensitization and exacerbation of

AR. Pollen-derived lipids [7,8], proteases [9,10], and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [11,12] may contribute to the initiation and activation of allergic inflammation.

Increasing evidence supports the important roles of interleukin (IL)-25 in the pathophysiology of allergic airway inflammation such as bronchial asthma. IL-25 (also known as IL-17E) is a member of the IL-17 family consisting of structurally related but functionally distinct proteins. IL-17A and IL-17F appear to have important roles in neutrophilic inflammation and autoimmunity. By contrast, IL-25 promotes eosinophilic inflammation and type-2 immune responses [13]. IL-25 is expressed in IgE-activated mast cells, alveolar macrophages, eosinophils, basophils, epithelial cells, and endothelial cells [14]. The exogenous [15,16] or transgenic [17,18] expression of IL-25 induces Th2-type inflammation in mouse airways. IL-25 production is induced in animal bronchial challenge models [19], and its inhibition reduces the associated Th2-type inflammation [20,21]. Thus, IL-25 appears to be necessary and sufficient for the development of Th2-type immune responses in the airways. However, little is known about the role and mechanism of epithelial cell-derived IL-25 production in the pathophysiology of AR.

In the present study, to elucidate the roles of IL-25 production in the pathophysiology of Japanese cedar pollinosis, we examined IL-25 concentration in nasal secretions and in culture supernatants of primary nasal epithelial cells (PNECs). Moreover, to examine what type of molecules, including JC, is involved in the mechanisms of IL-25 production, airway epithelial cells were stimulated with JC-structural components.

Abbreviations: AR, allergic rhinitis; APMSF, 4-amidinophenylmethanesulfonyl fluoride; Cry J1, *Cryptomeria japonica* 1; DPI, diphenyleneiodonium chloride; HC, healthy control; IT, inferior turbinate; JC, Japanese Cedar; NADPH, nicotinamide adenine dinucleotide phosphate; PNECs, primary nasal epithelial cells; NHBEs, human bronchial epithelial cells; poly(I:C), polyinosinic-polycytidylic acid; E-64, *trans*-epoxysuccinyl-L-leucylamide (4-guanidino) butane.

^{*} Corresponding author at: Department of Otorhinolaryngology, Shiga University of Medical Science, Otsu 520-2192, Shiga, Japan

E-mail address: hkhouzaki@belle.shiga-med.ac.jp (H. Kouzaki).

2. Materials and methods

2.1. Study participants

Allergic rhinitis is the presence of typical nasal symptoms during the cedar pollen season with a serum CAP-radioallergosorbent test (CAP-RAST) score for JC of ≥ 0.70 UA/mL (i.e., RAST score ≥ 2) and positive skin prick test for JC. The exclusion criteria were the presence or history of asthma and asthma-like symptoms. No patient included in this study had been treated with systemic corticosteroids or topical corticosteroids for at least 4 weeks before sample collection, although some patients had received antihistaminic agents. The control study participants had no nasal symptoms and no history of atopic disorders and a negative allergen-specific IgE against JC. The PNECs were harvested from the inferior turbinates of patients with Japanese cedar pollinosis or patients without allergy or infection (i.e., healthy controls [HCs]) during septoplasty surgery. Nasal secretions were collected during the peak season of JC exposure. All patients gave written informed consent before sample collection. The study was approved by the institutional review board of Shiga University of Medical Science (Otsu, Japan).

2.2. Reagents

We purchased the following materials: polyinosinic-polycytidylic acid [poly(I:C)] (InvivoGen, San Diego, CA, USA); pepstatin A, *trans*-epoxysuccinyl-L-leucylamide (4-guanidino) butane (E-64), 4-amidinophenylmethanesulfonyl fluoride (APMSF), and diphenyleneiodonium chloride (DPI) (Sigma-Aldrich, St. Louis, MO, USA); recombinant Cry J1 and Cry J2 (Hayashibara, Okayama, Japan); leukotriene B₄ (LTB₄) (Cayman Chemical, Ann Arbor, MI, USA); prostaglandin E₂ (PGE₂) (Tocris Bioscience, Bristol, United Kingdom); VAS-2870 and ENMD-1068 (Enzo, New York, NY, USA); recombinant IL-4, IL-5, IL-13, IFN- γ , and TNF- α (R&D Systems, Minneapolis, MN, USA).

Small interfering RNA (siRNA) for protease-activated receptor (PAR)-1, PAR-2, Toll-like receptor (TLR)2, TLR4, and a control siRNA were obtained from Qiagen (Venlo, the Netherlands). Crude allergen from *Cryptomeria japonica* (Japanese cedar) was purchased from Greer Laboratories (Lenoir, NC, USA). Normal human bronchial epithelial cells and serum-free Bronchial Epithelial Cell Growth Medium (BEGM) were both purchased from Lonza (Walkersville, MD, USA).

2.3. Enzyme immunoassay

The concentration of IL-25 in the nasal secretions and supernatants were measured using immunoassay kits (Uscn Life Science, Inc., Wuhan, China). The albumin value in nasal secretions was measured using immunoassay kits (Bethyl, Montgomery, TX, USA). The limits of detection of the IL-25 assays were 15.6 pg/mL.

2.4. Cell culture

Primary nasal epithelial cells were collected from the inferior turbinates by curettage with a Rhinoprobe (Arlington Scientific, Inc., Springville, UT, USA). The PNECs were maintained and cultured in BEGM. They were seeded (3×10^4 cells/well) in a 24-well tissue culture plate and grown until 80% confluence in the BEGM without steroid. Cultured PNECs were incubated with JC (200 μ g/mL) or with poly(I:C) (10 μ g/mL) for 24 h. The cell-free culture supernatants were thereafter collected and examined by using enzyme-linked immunoassay (ELISA). The NHBE cells were maintained and cultured in BEGM. The cells were used with three passages. The NHBE cells were seeded (3×10^4 cells/well) in a 24-well tissue culture plates and grown until 80% confluence, usually for 3–4 days. These cells were then incubated for 24 h with JC (50–400 μ g/mL), poly(I:C) (10 μ g/mL), Cry J1 (2 μ g/mL), Cry J2 (2 μ g/mL), LTB₄ (10 nM), or PGE₂ (1 μ M). In some experiments, IL-4 (100 ng/mL), IL-5 (100 ng/mL), IL-13 (100 ng/mL), TNF- α

(100 ng/mL), or IFN- γ (100 ng/mL) was added. In some experiments, a stimulus such as JC or poly(I:C) was pretreated with a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor (VAS-2870 or DPI), ENMD-1068 (5 mM), an aspartate protease inhibitor (pepstatin A [10 μ g/mL]), a serine protease inhibitor (APMSF [100 μ M]), a cysteine protease inhibitor, or E-64 (50 μ M) for 30 min at room temperature before adding to the NHBE cells.

At 30%–50% confluence, the NHBE cells were transfected with small interfering RNAs (siRNAs) targeting PAR-1, PAR-2, TLR-2, TLR-4, or control RNA at 5 nM using HiPerFect transfection reagent (Qiagen, Venlo, the Netherlands). The transfected cells were grown for 48 h and then incubated with JC (200 μ g/mL) or poly(I:C) (10 μ g/mL) for 24 h. Target gene knockdown was verified by examining the expression of PAR-1, PAR-2, TLR-2, or TLR-4 mRNA by real-time polymerase chain reaction (RT-PCR) (discussed later).

2.5. RNA isolation and real-time RT-PCR

Total RNA from NHBE cells was purified using the PureLink RNA Mini Kit (Invitrogen, Grand Island, NY, USA). Complementary DNA (cDNA) was synthesized from 1 μ g of a purified RNA sample using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). The reaction was incubated at 45 °C for 60 min and was stopped by heating to 85 °C for 5 min. The RT-PCR reaction contained 1 μ L of cDNA, 10.0 μ L of LightCycler480 Probe Master (Roche), 0.2 μ L of probes, and 0.1 μ L of two primer sets: PAR-1 (forward primer: TCAGAAGATGCCTCCGATA; reverse primer: CACAGATGGGACAAAGAGTGTC); PAR-2 (forward primer: CACATACATGGCAACTGG; reverse primer: GCCATAGAAAAAGCCAATAAGC); TLR2 (forward primer: TGTCATTCTTCTCCTGCTAAGA; reverse primer: CTAGGTAGGACAGAGAATGCCTTT); TLR4 (forward primer: CTCTCCTGCGTGAGACCAG); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control (forward primer: AGCCACATCGCTCAGACAC; reverse primer: GCCCAATACGACCAAATCC).

All primers were purchased from Nihon Gene Research Laboratories (Miyagi, Japan). Reactions were all brought to a final volume of 20 μ L by using sterile water. Amplification and detection of specific products were performed using the LightCycler 480 Real-Time PCR System (Roche). The RT-PCR protocol was as follows: denaturation by a hot start at 95 °C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). Transcription was normalized to the GAPDH transcription in each sample, and the data were expressed relative to the NHBE cells.

2.6. Statistical analysis

All data are reported as the mean $\pm$ the standard error of the mean (SEM) from the indicated number of samples. Two-sided differences between two samples were analyzed using the Mann–Whitney *U* test. Values of $P < 0.05$ were significant.

3. Results

3.1. IL-25 concentration in nasal secretions and in the culture supernatants of the PNECs

The clinical and demographic characteristics of the patients are shown in Table 1. No significant difference existed between the HC and Japanese cedar pollinosis patients with regard to sex and age. However, total IgE and JC-specific IgE in the serum were significantly elevated in patients with Japanese cedar pollinosis (Table 1). IL-25 concentration in the nasal secretions of patients with Japanese cedar pollinosis during the peak pollen season was significantly higher than that of the HCs (Fig. 1A). Japanese cedar stimulated IL-25 production by the cultured PNECs. The TLR3 ligand poly(I:C) was the positive

Table 1
Patients' characteristics.

Groups	Healthy control	Japanese cedar pollinosis
Total	10 (6 M/4F)	13 (7 M/6F)
Age (years)	38.5 (21–55)	36.8 (19–51)
Serum total IgE (IU/ml)	31.4 (3.2–62.5)	125.5 (7.5–313.6)*
Serum specific IgE (UA/ml)	<0.35	32.5 (1.71–89.6)*

F: female, M: male, specific IgE: Japanese cedar pollen-specific IgE; immunoCAP raw value.

* $P < 0.05$.

control because previous research shows it induces IL-25 production [22]. IL-25 was interestingly significantly increased in PNECs from patients with Japanese cedar pollinosis, compared to the control participants, after stimulation with JC but not with poly(I:C) (Fig. 1B).

3.2. The effect of different combinations of cytokines and JC on IL-25 protein production

IL-25 is implicated in type-2 immune responses and in the pathogenesis of allergic diseases. To investigate the mechanisms of IL-25 production by airway epithelial cells, we investigated the effects of exposure to JC on IL-25 production with and without cytokines. When the levels of IL-25 protein in the cell-free supernatants were analyzed, a significant increase in extracellular IL-25 was observed with JC exposure in a dose-dependent manner (Fig. 2A). To test the possible role of the regulation of IL-25 production in airway epithelial cells, the NHBE cells were treated with cytokines such as TNF- α , IL-4, IL-5, IL-13, and IFN- γ . IL-25 protein was detected in the supernatant of TNF- α -treated NHBE, IL-4-treated NHBE, IL-13-treated NHBE, and IFN- γ -treated NHBE (Fig. 2B).

TNF- α is usually expressed at inflammatory sites and may contribute to an increase in Th1 and Th2 cytokine-dependent expression [23]. To examine whether the combination of Th1, Th2, or inflammatory cytokines with cedar enhanced cytokine-dependent IL-25 protein production, the NHBE cells were treated with combinations of TNF- α and cytokines such as IL-4, IL-5, IL-13, and IFN- γ . We found that TNF- α , IL-4, and IL-13 synergistically and significantly enhanced JC-dependent IL-25 protein production (Fig. 2B).

3.3. Protease and NADPH oxidase activity induce IL-25 production in normal human bronchial epithelial cells

Several molecules produced by cedar-producing organisms may be involved in the pathogenesis of allergic diseases through sensitization and exacerbation. Cry J1 and Cry J2 are major allergens of Japanese cedar pollinosis [2–4]. First, we exposed NHBE cells to Cry J1 and Cry J2, as well as to JC. However, Cry J1 and Cry J2 did not induce IL-25 production from the NHBE cells (Fig. 3A). We next examined the effects of LTB4 and PGE2, because pollens have eicosanoid-like substances that

appear to be involved in IgE-independent allergic responses [9]. However, LTB4 and PGE2 did not show any effects on IL-25 production (Fig. 3B). Japanese cedar reportedly has NADPH oxidases activity [12]. When JC was preincubated with the NADPH oxidase inhibitor VAS-2870 or DPI for 30 min before adding it to the NHBE cells, JC-induced IL-25 production was strongly inhibited. In addition, the poly(I:C)-induced IL-25 response was also significantly inhibited by DPI (Fig. 3C).

We next examined the effects of protease inhibitors because pollen also has protease activity. Japanese cedar was preincubated with the aspartate protease inhibitor pepstatin A, the serine protease inhibitor APMSF, or the cysteine protease inhibitor E-64 for 30 min before adding to NHBE cells. APMSF significantly inhibited JC-induced IL-25 production, although pepstatin A and E-64 showed no effect. By contrast, all of these protease inhibitors had no effect on poly(I:C)-induced IL-25 production (Fig. 4A). These results indicate that serine protease activity of JC is involved in JC-induced IL-25 production by epithelial cells.

Airway epithelial cells express protease-activated receptors (PARs): PAR-1, PAR-2, PAR-3, and PAR-4; PAR-2 is activated by trypsin and trypsin-like serine proteases [24,25]. Japanese cedar has serine protease activity [10]. Protease-activated receptor-2 responds to trypsin and trypsin-like serine proteases. Therefore, we hypothesized that protease activity in Japanese cedar pollen also regulates IL-25 production via PAR-2. Pretreating NHBE cells with the selective PAR-2 inhibitor ENMD-1068, significantly inhibited JC-induced IL-25 production, whereas pretreating with ENMD-1608 showed no effects on poly(I:C)-induced IL-25 production (Fig. 4B).

To examine the cellular receptors involved in the NHBE cell response to JC, we used a gene knockdown approach. The NHBE cells were transfected with siRNAs specific for PAR-1, PAR-2, TLR2, or TLR4, or transfected with a control siRNA. Transfection with the specific siRNAs, but not the control siRNA, significantly suppressed the expression of the target mRNA > 80% (Fig. 4C). The knockdown NHBE cells were then stimulated with JC. The JC-induced IL-25 protein was significantly inhibited in the PAR-2 siRNA knockdown cells (Fig. 4D). By contrast, Japanese cedar pollinosis-induced IL-25 protein production was not affected in the PAR-1, TLR2 or TLR4 siRNA knockdown cells (Fig. 4E).

4. Discussion

Allergic rhinitis is an IgE-mediated type 1 hypersensitivity disease caused by allergen exposure [26,27]. The characteristic symptoms of AR are sneezing, rhinorrhea, and nasal congestion caused by IgE-mast cell-mediated early-phase responses of AR. In addition, type 2 cytokine and chemokines mediate the cellular influx into nasal mucosa, typically eosinophilia, which cause the late-phase responses. Several studies have been published about the role of IL-25 in AR.

In the present study, IL-25 concentration in nasal secretions from patients with Japanese cedar pollinosis during the peak pollen season was significantly higher than that of HC. Japanese cedar pollen stimulated

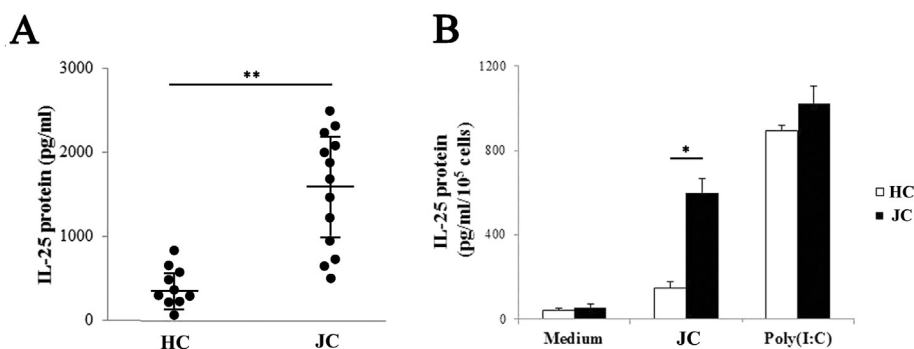


Fig. 1. Interleukin-25 concentration in nasal secretion and in the supernatant of primary nasal epithelial cells. A. Interleukin-25 (IL-25) concentration in nasal secretion. ** $P < 0.01$. B. Primary nasal epithelial cells (PNECs) from healthy controls (HCs) or patients with Japanese cedar pollinosis incubated with medium alone (control), Japanese cedar (JC) pollen (200 µg/mL), or polyinosinic-polycytidylic acid [poly(I:C)] (10 µg/mL) for 24 h ($n = 7$). * $P < 0.05$.

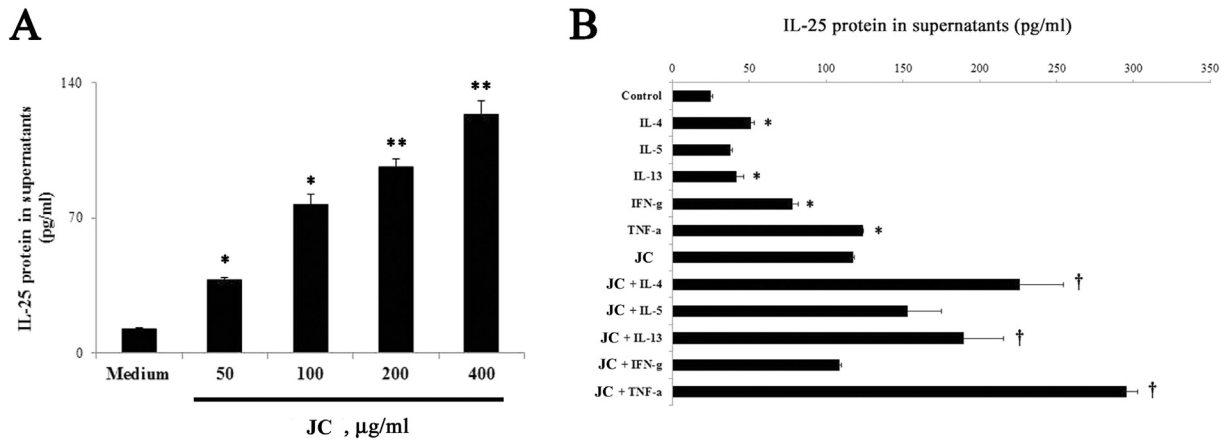


Fig. 2. The effect of different combinations of cytokines and Japanese cedar on interleukin-25 (IL-25) protein production. A. The normal human bronchial epithelial (NHBE) cells incubated with Japanese cedar (JC) pollen (50–400 µg/mL) for 24 h ($n = 5$). * $P < 0.05$ and ** $P < 0.01$. B. Normal human bronchial epithelial cells incubated for 24 h with a medium containing JC (200 µg/mL) with and without interleukin (IL)-4 (100 ng/mL), IL-5 (100 ng/mL), IL-13 (100 ng/mL), interferon (IFN)-γ (100 ng/mL), or tumor necrosis factor (TNF)-α (100 ng/mL). * $P < 0.05$, compared to cells with medium alone. † $P < 0.05$, compared to cells with JC alone ($n = 5$).

IL-25 production from cultured PNECs, and IL-25 production was significantly enhanced in PNECs from Japanese cedar pollinosis patients. This is the first report demonstrating JC-induced IL-25 production by nasal epithelial cells. In a previous study [22], IL-25 was detected in nasal lavages from patients with AR caused by house dust mites, and IL-25 mRNA was upregulated in the nasal mucosa of a mouse model of ovalbumin-specific AR [28]. In addition, several studies have shown a relationship between IL-25 receptor (also called interleukin-17 receptor B [IL-17RB]) and seasonal AR [29,30]. Microarray analysis of CD4-positive blood mononuclear cells from patients with Japanese cedar pollinosis revealed the upregulation of the mRNA expression of IL-17RB [29]. Basophils from patients with seasonal AR expressed the mRNA of IL-17RB (i.e., IL-25R), and allergen exposure enhanced the membrane protein expression of IL-17RB (i.e., IL-25R) on basophils from AR patients. Stimulation of basophils by IL-25 inhibited cellular apoptosis and enhanced IgE-induced degranulation [30]. Some research indicates that IL-17RB is highly expressed on a subset of naive and activated CD4+ invariant natural killer T cells, and that group 2 innate lymphoid cells, which produce large amounts of Th-2 cytokines that are substantially increased by IL-25 stimulation [31,32]. These results indicate that JC-induced epithelial cell-derived IL-25 has important roles in the pathophysiology of AR by activating Th2 type immune reactive cells in the early and late-phase responses.

In the present study, IL-4, IL-13, TNF-α and IFN-γ induced IL-25 production from airway epithelial cells, and IL-4, IL-13, and TNF-α synergistically enhanced JC-induced IL-25 production. However, IFN-γ had no effect on JC-induced IL-25 production. These results indicate that the local cytokine milieu influences epithelial cell-derived IL-25 responses. Similar results have been reported in dsRNA-induced TSLP production

from human keratinocytes. TSLP was enhanced by IL-4, IL-13 and TNF-α, whereas it was inhibited by IFN-γ, TGF-β and IL-17 [33]. Receptors for IL-4 and IL-13 share the same IL-4R α-chain, and it activates transcription factor STAT6 [34], and TNF-α induces transcription responses through NF-κB [35]. STAT6 and NF-κB may be involved in the activation of IL-25 production. Thus, the potential relationships among allergic status and IL-25 expression deserve future clinical studies.

In the present study, JC-induced IL-25 production was significantly inhibited when JC was preincubated with the NADPH oxidase inhibitor VAS-2780 or DPI. Most pollens such as grasses, birch, and ragweed have NADPH oxidase activity on the surface, in the cytoplasm, or in subpollen particles. Japanese cedar pollen has NADPH oxidase activity on the inner surface or in the cytoplasm, which is sloughed off after rupture [12]. Pollen NADPH oxidase activity generates reactive oxygen species (ROS) in the airway epithelium, and may have a prominent role in pathophysiology of allergic inflammation. Pollen NADPH oxidases from ragweed rapidly increases the levels of ROS in lung epithelium, and induces neutrophil recruitment to the airways, independent of the adaptive immune response. Removing pollen NADPH oxidase activity from a challenge material reduced antigen-induced allergic inflammation, which was characterized by reduced antigen-specific IgE levels, eosinophil infiltration, and mucus production in sensitized mice [11]. Co-administration of antioxidants with the ragweed extract challenge also attenuated eosinophil infiltration and mucus production in the airways of sensitized mice by inhibiting pollen NADPH oxidase-induced ROS generation [36].

Cedar pollen contains serine protease (CPA9) and aspartic protease (CPA63) in its cytoplasm [37]. Serine and cysteine proteases such as trypsin and papain stimulate epithelial cells at the mucosal surface

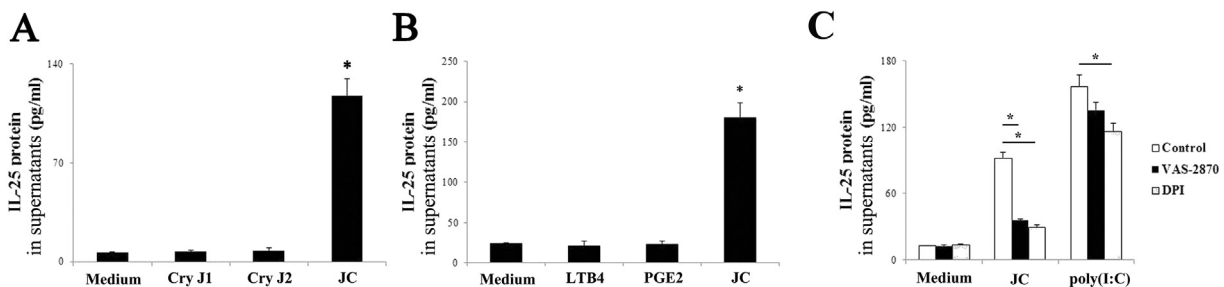


Fig. 3. Allergen spectrum of interleukin-25 (IL-25) production, including Japanese cedar. A. Normal human bronchial epithelial (NHBE) cells incubated for 24 h with 2 µg/mL *Cryptomeria japonica* peptide 1 (Cry J1; 2 µg/mL), *Cryptomeria japonica* peptide 2 (Cry J2; 2 µg/mL) or Japanese cedar (JC; 200 µg/mL). B. Normal human bronchial epithelial cells incubated for 24 h with leukotriene B4 (LTB4; 10 nM), prostaglandin E2 (PGE2; 1 µM) or Japanese cedar (JC; 200 µg/mL). C. Japanese cedar (JC; 200 µg/mL) and polyinosinic-polycytidylic acid [poly(I:C); 10 µg/mL] preincubated for 30 min with the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor VAS-2780 or diphenyleneiodonium chloride (DPI) before adding to the NHBE cells. * $P < 0.05$, compared to cells with medium alone ($n = 5$; for A and B). * $P < 0.05$, compared to cells with untreated JC or poly(I:C) ($n = 5$; for C).

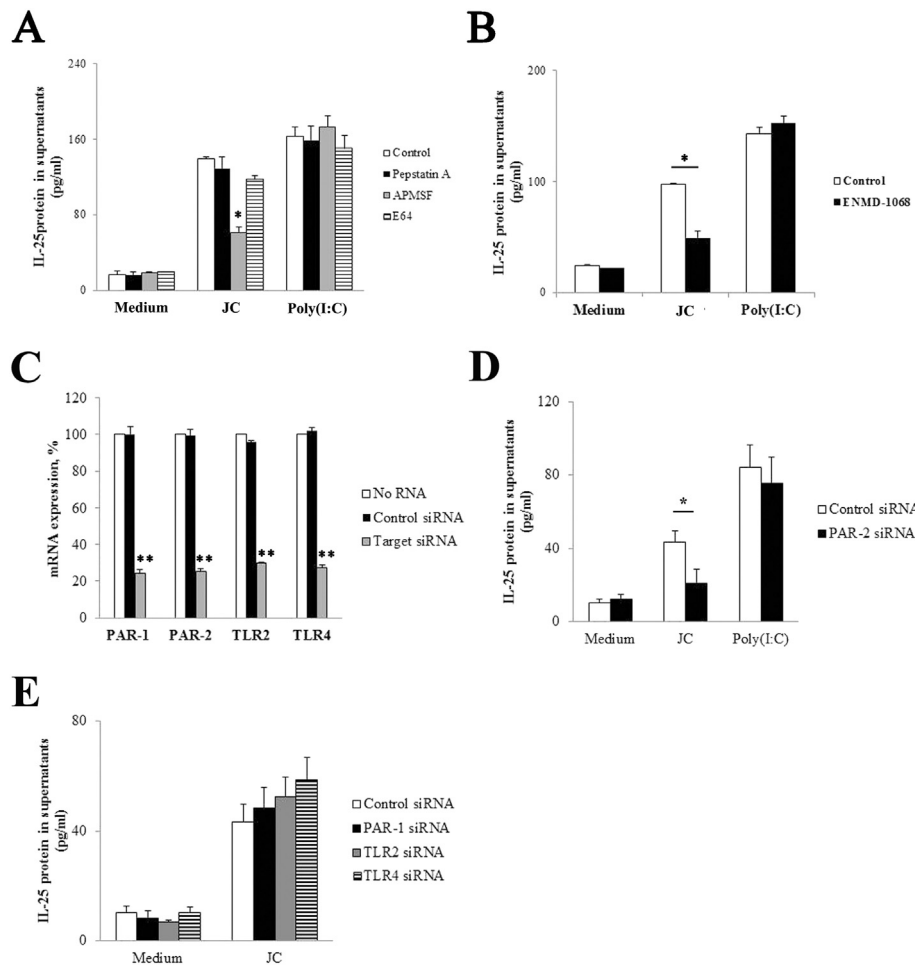


Fig. 4. Protease activity induce interleukin-25 (IL-25) production in normal human bronchial epithelial cells. **A.** Japanese cedar (JC; 200 μ g/mL) and polyinosinic-polycytidylic acid [poly(I:C); 10 μ g/mL] pretreated for 30 min with the following protease inhibitors: the aspartate protease inhibitor pepstatin A; the serine protease inhibitor 4-amidinophenylmethanesulfonyl fluoride (APMSF); or the cysteine protease inhibitor E-64, before adding to normal human bronchial epithelial (NHBE) cells. $^*P < 0.05$, compared to untreated JC ($n = 5$; for A). **B.** Normal human bronchial epithelial cells pretreated for 30 min with the selective protease-activated receptor (PAR)-2 inhibitor ENMD-1068, before incubating them with JC (200 μ g/mL) or poly(I:C) (10 μ g/mL). $^*P < 0.05$, compared to untreated NHBE cells ($n = 5$; for B). NADPH, nicotinamide adenine dinucleotide phosphate. **C.** Normal human bronchial epithelial (NHBE) cells transfected with siRNAs specific for protease-activated receptor (PAR)-1, PAR-2, Toll-like receptor (TLR)2, TLR4, or control small interfering RNA (siRNA). The expression of messenger RNA (mRNA) was determined by using real-time RT-PCR. The data are expressed as the ratio to the mock-transfected cells without siRNA, which is assumed to be 100%. $^*P < 0.05$, and $^{**}P < 0.01$, compared to control cells without siRNA and with the control siRNA ($n = 5$; for C). **D** and **E.** The transfected NHBE cells were incubated for 24 h with JC (200 μ g/mL) or poly(I:C) (10 μ g/mL). $^*P < 0.05$ ($n = 5$).

and induce the release of thymic stromal lymphopoietin (TSLP) or IL-25 via protease-activated receptor 2 (PAR2) activation [24,25]. Furthermore, a recent study [38] showed that allergen-induced ATP secretion activates the production of epithelial NADPH oxidase dual oxidase 1 (DUOX1), which mediates IL-1 α , IL-25, and IL-33 secretion. We previously demonstrated that ATP was involved in IL-25 secretion from epithelial cells [25]. Intrinsic protease activity include allergen was involved in ATP secretion [39]. Therefore, protease dependent mechanisms could be responsible for IL-25 secretion via ATP release and epithelial NADPH oxidase activity. Japanese cedar-induced IL-25 production may be affected by biological processes in epithelial NADPH oxidase and pollen NADPH oxidase (Fig. 3C). Further studies will be necessary to elucidate precisely the molecular mechanisms involved in NADPH oxidase-induced IL-25 production.

In conclusion, we demonstrated that protease activity and NADPH oxidase activity, including JC, are important for IL-25 production via airway epithelial cells, and airway exposure to JC may result in an elevated IL-25 response in allergic individuals, compared to normal individuals. Epithelial cell-derived IL-25 production after exposure to JC can then stimulate target cells, and lead to early and late-phase reactions relevant for rhinitis symptoms and the subsequent development and/or

exacerbation of Th2-type inflammatory responses. The involvement of the PAR pathway in response to protease and NADPH oxidase activity, and JC, may be a key factor that forms an interface between innate and adaptive immune responses. A better understanding of the spectrum of allergens for JC and their relationships with IL-25 immunobiology may allow investigators to develop novel therapeutic and preventive strategies for Japanese cedar pollinosis.

Disclosure statement

All authors declare that they have no conflicts of interest.

Author contributions

The individual author contributions were as follows:
Study design and research: HK.
Study design: SS.
Surgeons: HK, TK, KM, IT.
Study design and overall organization: HK, TS.

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