

DR. TOMOHISA IINUMA (Orcid ID : 0000-0002-9940-5520)

DR. DAIJU SAKURAI (Orcid ID : 0000-0002-5264-8760)

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Original Article

Pathogenicity of memory Th2 cells is linked to stage of allergic rhinitis

Tomohisa Iinuma, MD, PhD,^a Yoshitaka Okamoto, MD, PhD,^a Yuki Morimoto, MD^a

Tomoyuki Arai, MD, PhD,^a Toshioki Sakurai, MD, PhD,^a Syuji Yonekura, MD, PhD,^a Daiju

Sakurai, MD, PhD,^a Kiyoshi Hirahara, MD, PhD,^b and Toshinori Nakayama, MD, PhD,^b

^aDepartment of Otorhinolaryngology, Head and Neck Surgery, Chiba University Graduate

School of Medicine, Chiba, Japan

^bDepartment of Immunology, Chiba University Graduate School of Medicine, Chiba, Japan

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Corresponding author

Yoshitaka Okamoto, MD, PhD,

Department of Otolaryngology, Head and Neck Surgery

Chiba University

1-8-1 Inohana, Chuo-ku, Chiba,

260-8670, Japan

Telephone: +81-43-226-2137

Fax: +81-43-226-3442

E-mail: yokamoto@faculty.chiba-u.jp

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Short title

Pathogenic Th2 cell and stage of allergic rhinitis

ABSTRACT

Background: Allergic rhinitis (AR) consists of three developmental stages that are based on the presence/absence of antigen-specific IgE and symptoms. The pathogenic Th2 (Tpath2) cells constitute a population of Th2 cells with additional potentially pathogenic

characteristics. We examined the relationship between T_{path2} cells and the stages of allergic rhinitis by focusing on ST2, which is an IL-33 receptor.

Methods: Patients with Japanese cedar pollen-induced AR (JCP-AR) and healthy volunteers were divided into “non-sensitized,” “asymptomatic sensitized (AS),” and “JCP-AR” groups. We analyzed the ST2 expression and the Th2 function of cultured CD4⁺ T cells. Next, we observed the progress of patients in the AS stage around the time of seasonal pollen dispersal, with the characteristics of Th2 cells.

Results: The ST2 expression of T cells was only upregulated in the AR group. The production of IL-4 and IL-13 was found in CD4⁺ T cells obtained from AS by stimulation with JCP, but reactivity to IL-33 was not observed. Although IL-33 did not induce the elevation of IL-4 production in the JCP-AR group, IL-33 substantially increased the production of IL-5 and IL-13 in comparison to antigen stimulation alone. In newly afflicted patients, the increased expression of ST2 and elevated reactivity to IL-33 was observed, even before the pollen dispersal season.

Conclusions: Our study demonstrated that the pathogenicity of memory Th2 cells is linked to sensitization and the stage of allergic rhinitis. Therefore, T_{path2} cells may provide useful insights into the mechanism of the onset and progression of allergic rhinitis.

Key words

Allergic rhinitis, Th2, Japanese cedar, IL-33, ST2

INTRODUCTION

Allergic rhinitis (AR) is a common allergic disease in both Japan and the West (1). In Japan, perennial AR is often caused by mites, whereas seasonal AR is typically caused by Japanese cedar pollen. Japanese cedar pollen-induced AR (JCP-AR) prompts intense nasal symptoms from February to April, and affects up to one-third of the whole Japanese population (2).

With the number of afflicted people increasing each year, it is posing immense social problems. Thus, in addition to studies on normal therapeutic treatment, research is being conducted to establish methods to prevent sensitization to JCP and the occurrence of the disease. However, given the abundance of Japanese cedar pollen, allergen avoidance as a primary prophylactic measure against JCP-AR is not a realistic option (3). Although experiments involving the oral administration of probiotics are also being conducted, they have yet to yield satisfactory results (4, 5).

Not everyone who is sensitized to the allergen develops AR: a stage called asymptomatic sensitization (AS) occurs before the onset. In other words, Type I allergic diseases consist of three stages of development that depend on the presence/absence of antigen-specific IgE and

symptoms: (1) the non-antigen-sensitized stage, (2) the sensitized but barely symptomatic stage, and (3) the symptomatic stage. Although researchers have been aware of the presence of AS, despite research on how to distinguish AS-stage patients and studies implying the significance of IL-5 in this phenomenon, it has remained unclear why this condition exists (6, 7). The clarification of the difference between the AS stage and the symptomatic stages may help to elucidate the pathogenic mechanism and be of use in developing secondary prophylactic measures.

In our previous studies, we demonstrated the presence of pathogenic Th2 (T_{path2}) cells that cause pathological conditions in patients with allergic diseases (8-10). A certain population of non-sensitized T cells develops into memory T cells after sensitization to antigens. They form a subpopulation of cells with peculiar functions through further iterations of differentiation (11). T_{path2} cells constitute a population of Th2 cells with additional potentially pathogenic characteristics. Using a mouse model of bronchial asthma, we classified Th2 cells by identifying the presence of CXCR3 and CD62L, thereby substantiating their pathogenicity.

We also demonstrated that these cells display high expression levels of ST2, an IL-33 receptor (12). T cells that express ST2 at high levels are also present in patients with eosinophilic chronic rhinosinusitis, which suggests their possible contribution to its pathogenicity (13, 14). A report by Islam et al. (15), revealed that Th2 cells that co-express IL-17RB and CCR8 played pivotal roles in a mouse model of atopic dermatitis. IL-25 and

IL-33 are representative epithelium-derived cytokines that cause Th2-skewed inflammation (16, 17). These cytokines are generated by airway epithelial cells in response to various antigens and pathogens via Toll-like receptors or epithelial injury (18, 19). Thus, T_{path2} cells can be activated by both adaptive immunity and innate immunity. Meanwhile, Wambre et al. (20) reported that the implementation of antigen-specific immunotherapy transformed functional Th2 cells that were distinguished by CD27. As demonstrated by these examples, extensive research on T_{path2} cells is currently underway, and is gradually revealing their involvement in human diseases (21).

AR is an archetypal type I allergic disease wherein T and B cells play important roles in the stages of sensitization and pathogenicity (3). In the present study, we focused on patients with JCP-AR and examined the relationship between the stages of AR and T-cell-derived pathogenicity, along with the presence of T_{path2} cells in which the expression of ST2 was increased.

METHODS

Patients

Three groups of subjects were investigated. First, for a histological analysis, inferior turbinates were obtained during turbinectomy from patients with house dust mite-induced allergic rhinitis (HDM-AR), because we could not obtain a sufficient number of inferior

turbinate from patients with JCP-AR during the pollen dispersal season. Patients with HDM-AR, patients with asymptomatic-mite-sensitized hypertrophic rhinitis and patients with non-atopic hypertrophic rhinitis were recruited from Chiba University Hospital and Kimitsu Chuo Hospital. Second, volunteers (n = 29) were recruited and divided into three groups: the Japanese cedar non-sensitized (NS) group, the AS group, and the JCP-AR group. Finally, asymptomatic Japanese cedar-sensitized subjects (n = 19) were enrolled and followed from January 2013 to May 2014 to observe the onset of JCP-AR. The characteristics of the subjects are summarized in Supplementary Tables 1 and 2 and Table 1. None of the patients received systemic or topical steroids or anti-histamines for at least 4 weeks before surgery or examination. All subjects gave their written informed consent and the studies were approved by the Ethics Committee of the Chiba University Graduate School of Medicine and Kimitsu Chuo Hospital (UMIN000006180, UMIN000007178).

The definitions of the phenotypes

In the histological analysis, patients with no nasal clinical history and no specific IgE (sIgE) for house dust mites, Japanese cedar pollen, orchard grass pollen, ragweed pollen, *Candida*, *Aspergillus*, *Alternaria*, *Penicillium*, and *Cladosporium* were included as non-atopic controls (immune CAP method; Phadia, Inc., Uppsala, Sweden) (sIgE < 0.34 UA/ml). Subjects with

no clinical history and who were sensitized to house dust mites were included as AS in the histological analysis. The HDM-AR subjects had a clinical history and were sensitized to house dust mites.

For the other analyses, we focused on JCP-AR because it is relatively easy to distinguish the AS stage in patients with JCP-AR, and because JCP-AR patients only show very severe nasal symptoms between February and April. Subjects were assigned to one of three groups based on the presence of Japanese cedar-specific IgE, the presence of seasonal symptoms (for at least two years) and the results of a nasal provocation test (NPT). The NS group was composed of subjects without sIgE or seasonal symptoms and with a negative result on an NPT. The AS group was composed of subjects with Japanese cedar sIgE, without seasonal symptoms and with a negative result on an NPT. The JCP-AR group was composed of subjects with sIgE, seasonal symptoms and a positive result on an NPT.

The total nasal symptom score (TNSS) was used to define the seasonal symptoms that occurred with the onset of JCP-AR. Nasal obstruction, itching, sneezing and secretion were scored separately on a severity scale of between 0 and 3, and were combined. If the score increased by more than 2 points in the pollen season in comparison to the preseason, the subject was defined as having seasonal symptoms.

The nasal provocation test

The NPTs were performed with discs of filter paper according to Japanese guideline of Allergic rhinitis (22). Discs containing control solution or Japanese cedar pollen allergen extract (Torii Co., Tokyo, Japan) were placed on the surface of the inferior turbinate for 15 min. After checking the patient's nasal symptoms with a control disc, the results of exposure to the allergen were evaluated by otolaryngologists based on the presence of sneezing, the swelling of inferior turbinate or an increase of rhinorrhea. The NPT result was considered to be positive if a subject was positive for one of the three symptoms.

Immunofluorescence

Frozen sections of inferior turbinates obtained from non-atopic, AS and HDM-AR subjects were prepared and stained as previously described (13). The sections were incubated overnight with anti-IL-33, anti-IL1RL1 (both 1:200; Sigma-Aldrich, St Louis, MO, USA) and anti-CD3 (1:100; Abcam, Cambridge, MA, USA). They were subsequently incubated with Alexa Fluor[®] 488- or 555-labeled secondary antibodies. Otherwise, we used Alexa Fluor 488-anti-CD4 (1:20; BioLegend, San Diego, CA), and APC-anti-CD8 (1:20; BD Pharmingen, San Diego, CA, USA). The same concentrations of isotype controls were used. Nuclei were counterstained with TO-PRO3 iodide (Invitrogen, Carlsbad, CA, USA) or DAPI

(Sigma-Aldrich). The sections were examined using a confocal laser microscope (LSM710; Carl Zeiss Inc., Oberkochen, Germany) and were analyzed using the ZEN lite 2012 system (Carl Zeiss Inc.). Counting of ST2-expressing T cells was carried out manually by a blinded observer. At least fifty CD3 positive cells were analyzed.

ELISA

Tissue specimens of inferior turbinates were weighed and minced as previously described (13). Cytokine levels in tissue homogenates were measured using ELISA. The IL-33 levels were measured using an ELISA development kit (PeproTech, Rocky Hill, NJ, USA).

Cell culture

Peripheral blood mononuclear cells (PBMCs) were purified from whole blood using the Ficoll-Hypaque technique and cultured as previously described (13). RPMI 1640 (Sigma-Aldrich) supplemented with fetal calf serum (10%), L-glutamine (2 μ M), penicillin (100 U/ml), streptomycin (100 μ g/ml), HEPES (10 mM), 2-mercaptoethanol (55 μ M), nonessential amino acids (1%), and sodium pyruvate (1 mM) (all from Gibco BRL, Grand Island, NY) was used as the culture medium. PBMCs obtained from patients in the NS, AS and JCP-AR groups were cultured (1×10^6 cells/ml) at 37°C for 2 days for real-time PCR or 8

days for flow cytometry. Cells were stimulated with or without *Sugi* (Japanese cedar in Japanese) basic protein (SBP; 2 µg/ml; HAYASHIBARA, Okayama, Japan) in the presence or absence of IL-33 (100 ng/ml; R&D Systems, Minneapolis, MN). SBP is composed of major Japanese cedar pollen allergen Cry j1 and Cry j2. For intracellular staining, cells were re-stimulated with 25 ng/ml of phorbol-12-myristate-13-acetate and 1 µg/ml of ionomycin (Sigma-Aldrich) in the presence of 2 µM monensin (BioLegend) for the final 4 h.

Flow cytometry

To prevent non-specific binding, samples were pre-incubated on ice with human FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) and stained with a combination of antibodies. The re-stimulated cells were fixed and permeabilized (Intracellular Fixation and Permeabilization Buffer Set; eBioscience, San Diego, CA, USA), blocked with 3% bovine serum albumin (Sigma-Aldrich), and then stained for intracellular cytokines. We used the following reagents: FITC-anti-CD3 (BD, Franklin Lakes, NJ), PE-anti-ST2/IL-1R4 (R&D Systems), PerCP-Cy5.5-anti-CD4 (BioLegend), APC-anti-CD45RO (BioLegend), APC-anti-IL-5 and anti-IL-13 (BioLegend) and Alexa Fluor[®] 647-anti-IL-4 (BioLegend). The same concentrations of isotype controls were used. Lymphocytes were first identified by forward and side scatter characteristics. Gates were

restricted to the CD3⁺ CD4⁺ subset for T cell analysis, and at least 2×10^5 cells were acquired.

Real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A semi-quantitative real-time PCR was performed using the SYBR® Green method with an Applied Biosystems Step One Plus Real-time PCR System. Primer and probe sets were purchased from Eurofins Operon MWG (Ebersberg, Germany). The relative gene expression was calculated by the comparative CT method. β -actin was used as a housekeeping gene for normalization. The primer sequences were as follows: IL-4 sense GCACCGAGTTGACCGTAACA, antisense TGTCGAGCCGTTTCAGGAAT; IL-5 sense AGCTGCCTACGTGTATGCCA, antisense GCAGTGCCAAGGTCTCTTTCA; IL-13 sense CAGAGGATGCTGAGCGGATT, antisense TTGAACTGTCCCTCGCGAAA; GATA-3 sense GCGGGCTCTATCACAAAATGA, antisense GCTCTCCTGGCTGCAGACAGC; β -actin sense CCAGCCTTCCTTCTTGGGTAT, antisense TGGCATAGAGGTCTTTACGGATGT.

Statistics

The values are shown as the mean $\pm$ standard deviation. The Mann–Whitney U test and Fisher’s exact test were used for the intergroup comparisons, while the Wilcoxon signed-rank test was used for the intragroup comparisons. Correlation analysis was performed using the Spearman rank correlation coefficient. P values of <0.05 were considered to indicate statistical significance.

RESULTS

The ST2 expression of T cells in the three stage groups of allergic rhinitis

The ST2 expression of CD4⁺ cells and CD3⁺ cells in inferior turbinates was analyzed by immunofluorescence (Fig. 1A and 1B). While ST2 was expressed in a small population of CD3⁺ cells in non-atopic or AS subjects, significantly more ST2⁺ CD3⁺ cells were detected in the inferior turbinates obtained from patients with HDM-AR (Fig. 1C). The IL-33 expression was no different in the three stages of disease (Supplementary Figure 1)

Next, the CD4⁺ T cells in PBMCs were examined in non-sensitized subjects, and subjects with AS and JCP-AR by flow cytometry (Fig. 2). The stimulation of Cry j or Cry j together with IL-33 caused CD4⁺ T cells in PBMCs from JCP-AR to express ST2, and most of the ST2⁺ CD4⁺ T cells expressed CD45RO (Fig. 2A). In CD4⁺ T cells from patients with

JCP-AR, the ST2-positive proportion and absolute number were significantly upregulated in comparison with AS patients after stimulation with Cry j (both $P < 0.05$) and further upregulation was observed by costimulation with IL-33 (both $P < 0.01$) (Fig. 2B). However, the same enhancement was not observed in CD4⁺ T cells from subjects in the NS and AS groups.

We also investigated the ST2 expression on CD4⁺ T cells. We found that there were only a small number of ST2⁺ CD4⁺ T cells in the inferior turbinate and among PBMCs (Supplementary Figure 2–C).

The pattern of Th2 cytokine production differs in the three stage groups

The mRNA level of IL-4 in PBMCs was significantly elevated by Cry j stimulation in the AS and JCP-AR groups (both $P < 0.05$) in comparison to the NS group, however there was no significant difference between AS and JCP-AR (Fig. 3A). Although stimulation with Cry j increased the level of *IL-4* in subjects in the AS and JCP-AR groups, IL-33 did not provide additive upregulation in either group. In contrast, Cry j stimulation did not alter the mRNA level of IL-5 in the NS or AS groups; however, the *IL-5* level was significantly elevated by Cry j stimulation in the JCP-AR group in comparison to the subjects in the NS and AS group (both $P < 0.05$) and further upregulation was observed in response to IL-33 stimulation in the

JCP-AR group ($P < 0.01$) (Fig. 3B). Different from *IL-4* and *IL-5*, the level of *IL-13* was significantly elevated by Cry j stimulation in the AS and JCP-AR groups ($P < 0.05$ and < 0.01 , respectively) (Fig. 3C). Further upregulation by IL-33 was only observed in the JCP-AR group ($P < 0.01$) and, the *IL-13* level of JCP-AR group was higher than that of AS group by stimulation with IL-33 ($P < 0.05$). The mRNA level of GATA3 activation in response to Cry j stimulation was the highest in the JCP-AR group, followed by the AS group, in comparison to the NS group ($P < 0.01$ and < 0.05) (Fig. 3D).

IL-4⁺ ST2⁺ CD4⁺ T cells were observed in the JCP-AR group following stimulation with Cry j and IL-33, and the proportion of IL-4⁺ cells was increased compared with stimulation with Cry j alone (Fig. 4A). Moreover, the proportion of IL-4-producing ST2⁺ CD4⁺ T cells in the JCP-AR group was the same as that in the AS group (Fig. 4B). Notably, IL-5⁺ ST2⁺ and IL-13⁺ ST2⁺ CD4⁺ T cells were increased following stimulation with Cry j and IL-33, but only in the JCP-AR group (Fig. 4C, 4D, 4E and 4F). The proportion of IL-13-producing CD4⁺ T cells observed in the AS group was lower than that observed in the JCP-AR group, although the IL-13⁺ ST2⁺ CD4⁺ T cells were the same. A positive correlation between the rate of ST2 expression and the rate of Th2 cytokine expression by CD4⁺ T cells is shown in Supplementary Figure 3.

The expression of ST2 was high before the onset of JCP-AR

In January 2014, 19 asymptomatic Japanese cedar-sensitized subjects were enrolled. In May 2014, JCP-AR developed in five subjects, and three subjects were excluded because the TNSS and NPT results did not match (Fig. 5A). Japanese cedar-specific IgE in the preseason was higher in newly afflicted patients (AS-AR) than it was in patients with ongoing AS (AS-AS) (Table 1). Stimulation with Cry j and IL-33, resulted in the significant elevation of the ST2 expression in CD4⁺ T cells in the subjects with AS-AR in both the preseason and post-season in comparison to the AS-AS subjects ($P < 0.01$ and < 0.001) (Fig. 5B and 5C). In both *IL-5* and *IL-13*, reactivity to IL-33 was significantly upregulated in the AS-AR subjects in the preseason ($P < 0.05$ and < 0.05) (Fig. 5D). Furthermore, serum anti-Japanese cedar sIgE levels were significantly positively correlated with ST2 expression ($p = 0.02$, $r = 0.51$) (Fig. 5E).

DISCUSSION

In the present study, we revealed that only patients who were in the symptomatic stage of JCP-AR had memory CD4⁺ T cells, which indicated the expression of ST2. We then showed that patients in the three stages (NS, AS, and JCP-AR) exhibited different patterns of cytokine production by Th2-cells in response to antigen stimulation and that they reacted differently to IL-33. First, in the NS stage, the T cells in the peripheral blood did not react to

either JCP or IL-33 stimulation. In the AS stage, stimulation with JCP induced the production of IL-4 and small amounts of IL-13; however, no reaction to IL-33 was observed. In contrast, in the JCP-AR stage, stimulation with JCP triggered the production of large amounts of IL-5 and IL-13. Although the production of IL-4 only differed slightly from that in the AS stage, the cells in the JCP-AR stage reacted to IL-33 and produced abundant amounts of IL-5 and IL-13. Based on the hypothesis that reactivity to IL-33 is responsible for the pathogenicity, we observed the progress of patients in the AS stage around the time of seasonal pollen dispersal. With respect to the results, not only did the newly afflicted patients exhibit high levels of ST2 expression after the start of pollen dispersal but they also showed increased ST2 expression and elevated reactivity to IL-33, even prior to the pollen dispersal season, i.e., before developing symptoms. The positive correlation between preseason serum anti-Japanese cedar sIgE and ST2 expression of CD4⁺ T cells supported these results.

In the AS group, T cells that reacted to the Japanese cedar pollen allergen primarily produced IL-4 and -13, with the production of IL-4 particularly being almost comparable to that in the JCP-AR group. IL-4 is a cytokine that induces class switch from IgG to IgE antibodies in conformity with the presence of specific IgE antibodies at the AS stage. The most noteworthy difference from the JCP-AR group is that antigen stimulation alone did not induce the production of IL-5. This may be attributable to the difference in the expression of *GATA3*. In

the present study, the activation of *GATA3* in response to antigen stimulation was the highest in the JCP-AR group, followed by the AS group, and then the NS group (Fig. 3D). According to other studies, while *IL-4*, *IL-5*, and *IL-13* are all equally dependent on *GATA3* for the chromatin remodeling of the gene loci, *IL-5* is the most reliant on *GATA3* for transcriptional activation (23-25). Consequently, the difference in the expression of *GATA3* may have been reflected in the difference in the production of *IL-5*. The present study did not reveal why *GATA3* was poorly expressed in the AS group, and it was unclear whether this difference in the expression of *GATA3* was the cause or the result of the development of disease in the subjects of the present study. Furthermore, in the present study, we analyzed mainly $CD4^{+}$ T cells, however, stimulation by allergen, infection or injury may induce activation of $CD8^{+}$ T cells and the production of *IL-4*, *IL-5* and *IL-13* (26, 27). Thus, the influence of $CD8^{+}$ T cells also requires further examination.

Although Japanese cedar-specific Th2 cells triggered the production of IgE in both the AS and JCP-AR groups, it is assumed that the AS group may still lack some of the factors that are associated with the onset of symptoms. With regard to these factors, the present study demonstrated two factors; the intensity of ST2 expression in Th2 cells in reaction to exposure to the specific antigen, as well as the reactivity to *IL-33*. In the JCP-AR group, the expression of ST2 was observed to be increased in the peripheral blood T cells, while *IL-33* substantially

increased the production of IL-5 and IL-13 in comparison to antigen stimulation alone. It is possible that IL-5 and IL-13 are associated with the activation of eosinophils and production of mucin in epithelial cells, thereby leading to the manifestation of symptoms (4). The results of genome-wide association studies have also indicated the presence of numerous single-nucleotide polymorphisms in IL-33 and ST2 in patients with allergies (28, 29). It is therefore possible that individuals who are prone to high expression of ST2 in Th2 cells after repeat exposure to the antigen are predisposed to allergic disease, with ST2-positive T cells being responsible for the pathogenesis. We hypothesize that T_{path2} cells increase in individuals with ST2 polymorphism as a result of repeated antigen exposure, which then provokes onset. However, the stimulation of IL-33 did not increase the production of IL-4, even in Th2 cells that expressed ST2. The difference in the downstream signaling may be responsible for this discrepancy. While the stimulation of T-cell receptors is known to activate both MAPK and NFAT and result in the transcription of cytokines, including IL-4, the stimulation of IL-33 is considered to activate MyD88/MAPK, and to result in the production of IL-5 and IL-13 (17, 30). Thus, the T-cell-mediated activity of IL-33 may only have a limited impact on the decrease in the sensitization process that occurs in response to an increase in IL-4, despite its involvement in other pathogenic behaviors, such as the infiltration of eosinophils and the activation of goblet cells (31).

We examined the relationship between the pathogenicity of T cells and the intensity of ST2 expression in newly afflicted patients. The results demonstrated that these new patients already expressed high levels of ST2, even prior to the dispersal of pollen and that they displayed similar IL-33 reactivity to symptomatic patients. These results indicated the possibility that the emergence of T_{path2} cells may precipitate the manifestation of allergic rhinitis. IL-33, which is always present within the nuclei of the nasal epithelial cells, is released following the stimulation of the epidermis (17, 32). Because this phenomenon also occurs in patients with non-allergic rhinitis, the differentiation of T cells into T_{path2} cells as IL-33 receptors may induce symptoms.

We are presently attempting to determine how to prevent progression from the AS stage to the AR stage. Other research groups have reported that certain antigen-specific immunotherapies lead a reduction in the number of T_{path2} cells as a part of their mechanisms of action (33). Accordingly, we hypothesize that it may be possible to prevent the pathogenicity of allergic rhinitis by restricting the increase in the number of T_{path2} cells in AS patients who receive immunotherapy. We are currently working on predictive sublingual immunotherapy and proceeding with its evaluation with the aim of establishing effective prophylactic measures (UMIN000015603). The present study implies the possibility that the increased expression of ST2 in T cells may be used as a predictive marker of pathogenicity.

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IL-17RB and CCR8 have also been used in addition to ST2 as T_{path2} cell markers (15).

Other groups have reported the use of CD27 (33), however, a specific marker is currently unknown.

The age of disease onset of HDM-induced AR is basically from childhood and it is difficult to be detected because the symptoms in children are ambiguous. In contrast, the symptomatic period of JCP-induced AR is only three months, and the number of patients who undergo surgical treatment is small. Thus, in the present study, we could not use paired mucosa and PBMCs. Therefore, we used mucosa of HDM-induced AR patients for localization experiments and PBMCs of JCP-induced AR patients for all the other experiments. Hence the relationship between findings in the mucosa and the periphery still needs to be investigated in future studies.

In conclusion, we have shown the possibility that the functions of Th2 cells are linked to the pathological progress of allergic rhinitis. It is therefore believed that understanding the functionality of T_{path2} cells as an endotype may provide useful insights into the mechanism of action of ST2 as a marker and the development of novel therapeutic techniques (34, 35).

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Figure legends

Figure 1. The immunofluorescence analysis of the T cells from tissue specimens of patients in the three stages. (A) The expression of ST2 and CD4 in the inferior turbinates obtained from patients with house dust mite-induced allergic rhinitis (HDM-AR) (Original magnification, ×20). (B) The expression of ST2 in T cells in the inferior turbinates obtained from non-atopic patients, patients with asymptotically-mite-sensitized hypertrophic rhinitis and patients with HDM-AR was observed (Original magnification, ×80). The data are representative of four to five independent experiments. (C) The proportion of ST2⁺ CD3⁺ T cells in inferior turbinates. from non-atopic patients (n = 5), patients with

asymptomatic-mite-sensitized hypertrophic rhinitis (n = 4) and patients with house dust mite-induced allergic rhinitis (HDM-AR) (n = 4)

Figure 2. The ST2 expression of CD4⁺ T cells in the blood. The expression levels of CD45RO and ST2 on CD3⁺ CD4⁺ T cells in PBMCs cultured with Japanese cedar pollen antigen named Cry j and IL-33 were measured by flow cytometry. (A) The figure shows the percentage of ST2⁺CD45RO⁺ cells gated on CD3⁺ CD4⁺ cells. (B) The statistical analysis of ST2⁺ CD45RO⁺ cells obtained from non-sensitized subjects (n = 9), asymptomatic sensitized subjects (n = 11) and subjects with Japanese cedar pollen induced-allergic rhinitis (JCP-AR) (n = 9). The left figure represents proportion of positive cells and the right represents absolute number. **P* < 0.05, ***P* < 0.01, N.S, not significant.

Figure 3. (A-C) The expression of Th2 cytokines in PBMCs cultured with JCP antigen named Cry j and IL-33. The real-time PCRs of IL-4 (A), IL-5 (B) and IL-13(C) were performed in non-sensitized subjects (n = 9), subjects with asymptomatic sensitization (n = 11) and subjects with Japanese cedar pollen (JCP) induced-allergic rhinitis (n = 9). (D) The expression of GATA3 in PBMCs cultured with the JCP antigen and JCP together with IL-33. **P* < 0.05, ***P* < 0.01, N.S, not significant.

Figure 4. Flow cytometry of Th2 cytokine-producing cells in non-sensitized subjects (n = 4), subjects with asymptomatic sensitization (n = 8) and subjects with Japanese cedar pollen (JCP) induced-allergic rhinitis (n = 6). Intracellular staining of IL-4 (A and B), IL-5 (C and D) and IL-13 (E and F) and the expression of ST2 were observed in CD4⁺ T cells stimulated with or without the Japanese cedar pollen antigen and IL-33. The percentage of positive cells is shown (A, C, and E). The statistical analysis of ST2^{+/+} CD4⁺ T cells is shown (B, D, and F). NS; non-sensitized subjects, AS; Asymptomatic sensitization, JCP-AR; Japanese cedar pollen induced-allergic rhinitis

Figure 5. The clinical progress and the change in the T cell function of patients in the asymptomatic sensitization stage around the time of seasonal pollen dispersal. (A) An outline of the follow-up and screening procedures. (B–C) The expression of ST2 in CD4⁺ T cells stimulated with the Japanese cedar pollen (JCP) antigen and IL-33 was observed in newly afflicted patients (AS-AR, asymptomatic sensitization to allergic rhinitis) and patients with ongoing AS (AS-AS), in the pollen preseason in comparison to the postseason. The percentage of positive cells is shown. (D) The expression of Th2 cytokines in PBMCs cultured with the JCP antigen and IL-33 were compared in newly afflicted patients and patients with ongoing AS. TNSS, total nasal symptom score; NPT, nasal provocation test.

Supplementary Figure 1. The expression of IL-33 in the inferior turbinates obtained from non-atopic patients, patients with asymptomatic-mite-sensitized hypertrophic rhinitis and patients with house dust mite-induced allergic rhinitis (HDM-AR) was observed by immunofluorescence (A). Measurement of IL-33 in inferior turbinate homogenates by ELISA obtained from non-atopic patients (n = 9), patients with asymptotically-mite-sensitized hypertrophic rhinitis (n = 6) and patients with house dust mite-induced allergic rhinitis (HDM-AR) (n = 10) (B)

Supplementary Figure 2. The ST2 and Th2 cytokine expression of CD4⁺ T cells. (A) Immunofluorescence analysis of CD4 or CD8 positive cells for ST2 expression. The inferior turbinates obtained from house dust mite-induced allergic rhinitis (HDM-AR) were observed. (B-D) The flowcytometric analysis of ST2 and Th2 cytokine expression in CD4⁺ T cells stimulated with the Japanese cedar pollen antigen and IL-33. PBMCs obtained from non-sensitized subjects (n = 4), subjects with asymptomatic sensitization (n = 8) and subjects with Japanese cedar pollen (JCP)-induced allergic rhinitis (n = 6) were analyzed. The percentage of positive cells is shown (B). The left figure represents proportion of ST-2 positive cells and the right represents absolute number (C).

Supplementary Figure 3. The correlation between the rate of ST2 expression and the rate of Th2 cytokine expression by CD4⁺ T cells. Intracellular staining of IL-4, IL-5 and IL-13 and the expression of ST2 were observed in CD4⁺ T cells stimulated with the Japanese cedar pollen antigen and IL-33 by flow cytometry. Correlations were analyzed in a mix of non-sensitized subjects, subjects with asymptomatic sensitization and subjects with Japanese cedar pollen (JCP) induced-allergic rhinitis (n = 18).

Table 1. The characteristics of the asymptomatic Japanese cedar-sensitized patients

	AS-AS	AS-AR	P value
Patients, n	11	5	
Age, median	32	28	
Sex male/female, n	5/6	3/2	
Preseason blood total IgE level, IU/ml	411	152	0.58
Preseason blood cedar IgE level, UA/ml	5.6	23	0.028
HDM-AR, n	6	2	0.64
Preseason TNSS	0.73	0.40	0.68
Seasonal TNSS	0.73	3.8	0.0011
Postseason TNSS	0.73	0.50	0.70

AS, asymptomatic sensitization; AR, allergic rhinitis; TNSS, Total nasal symptom score.

HDM-AR, House dust mite-induced allergic rhinitis

Statistical analysis was performed by Mann-Whitney-U test and Fisher exact test.

Figure 1

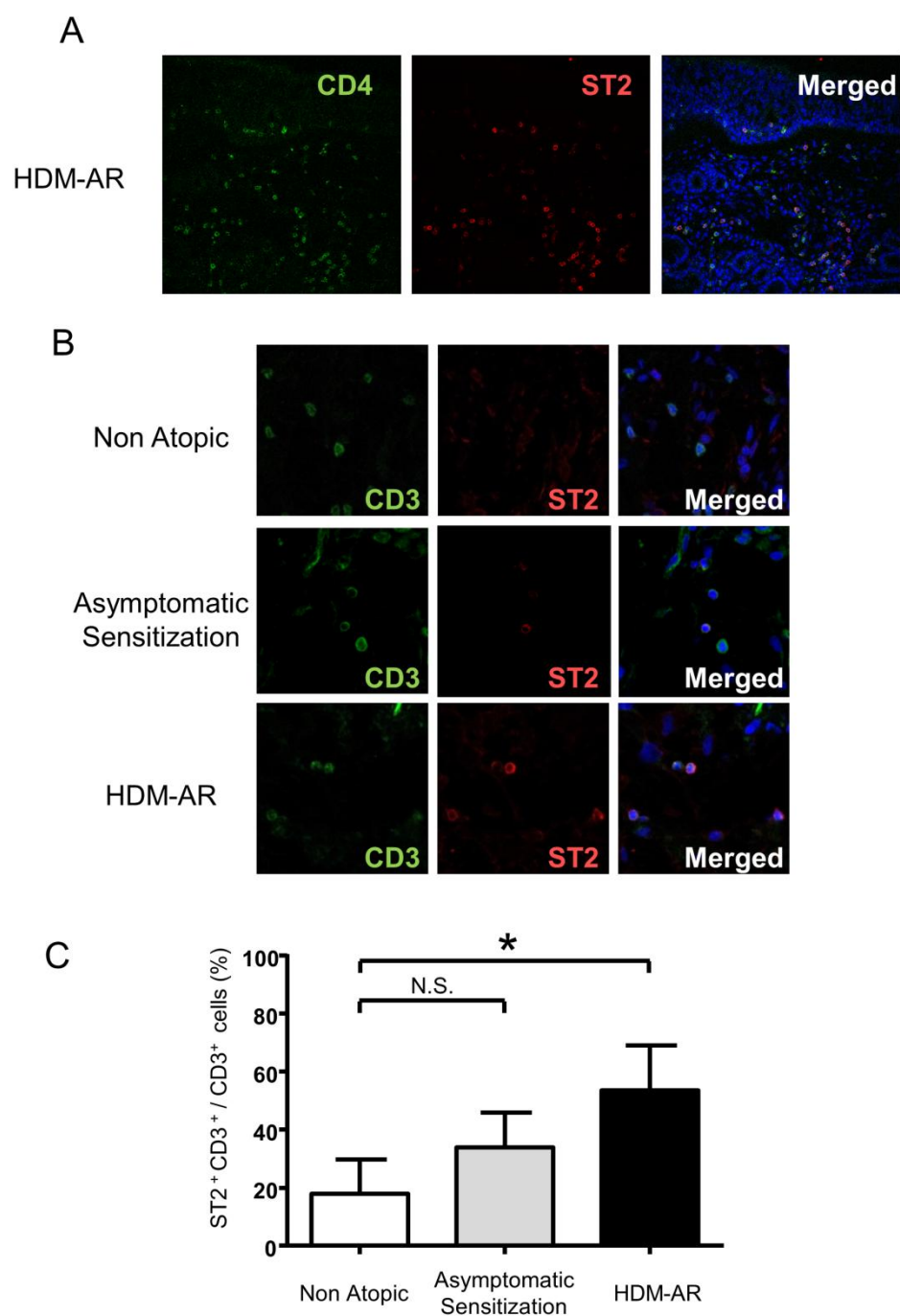


Figure 2

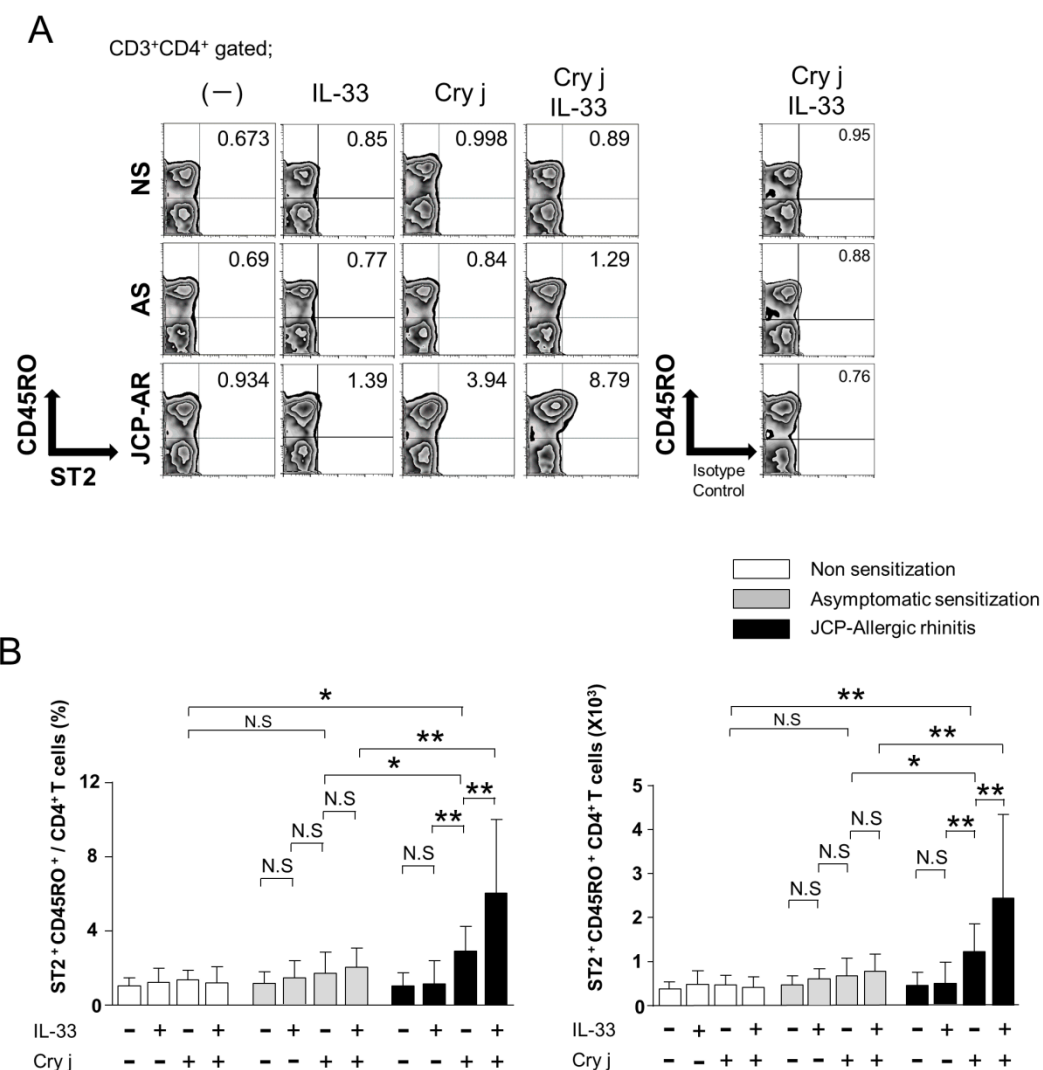


Figure 3

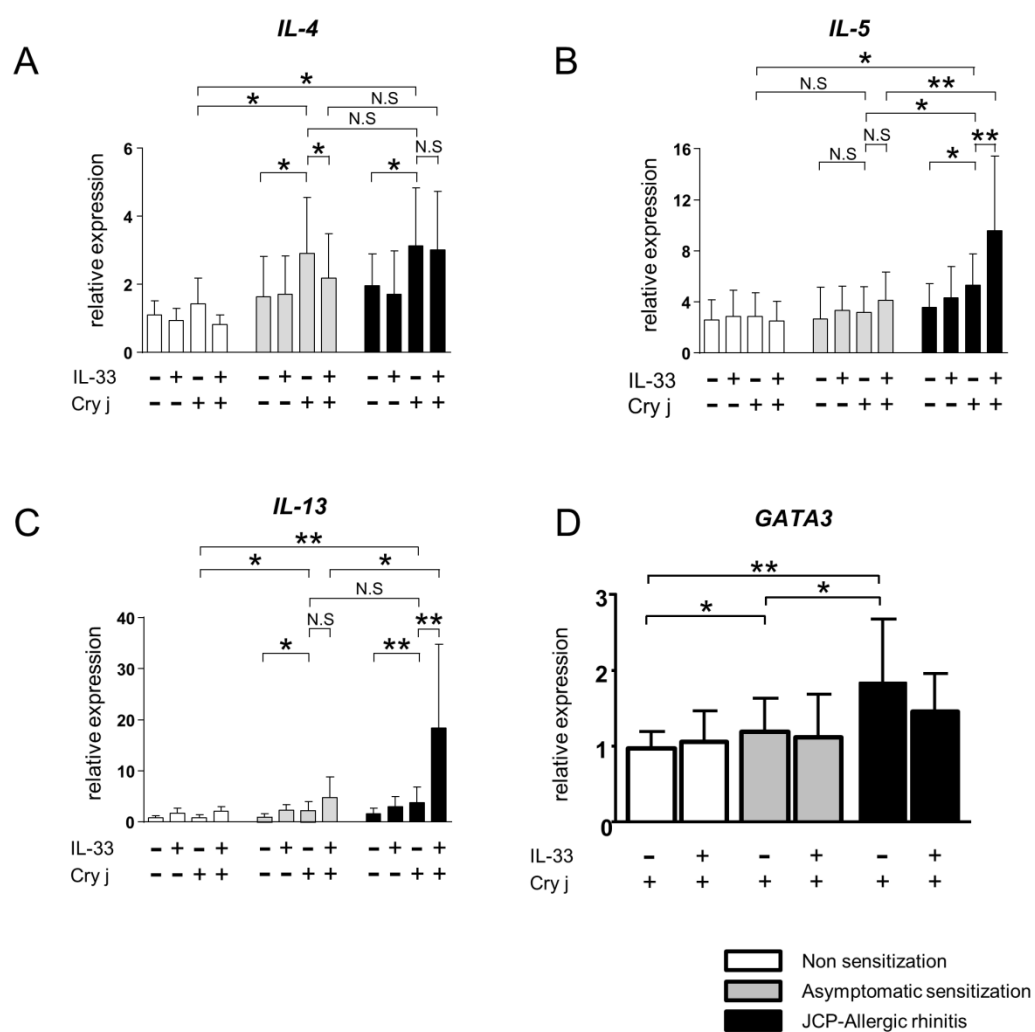


Figure 4

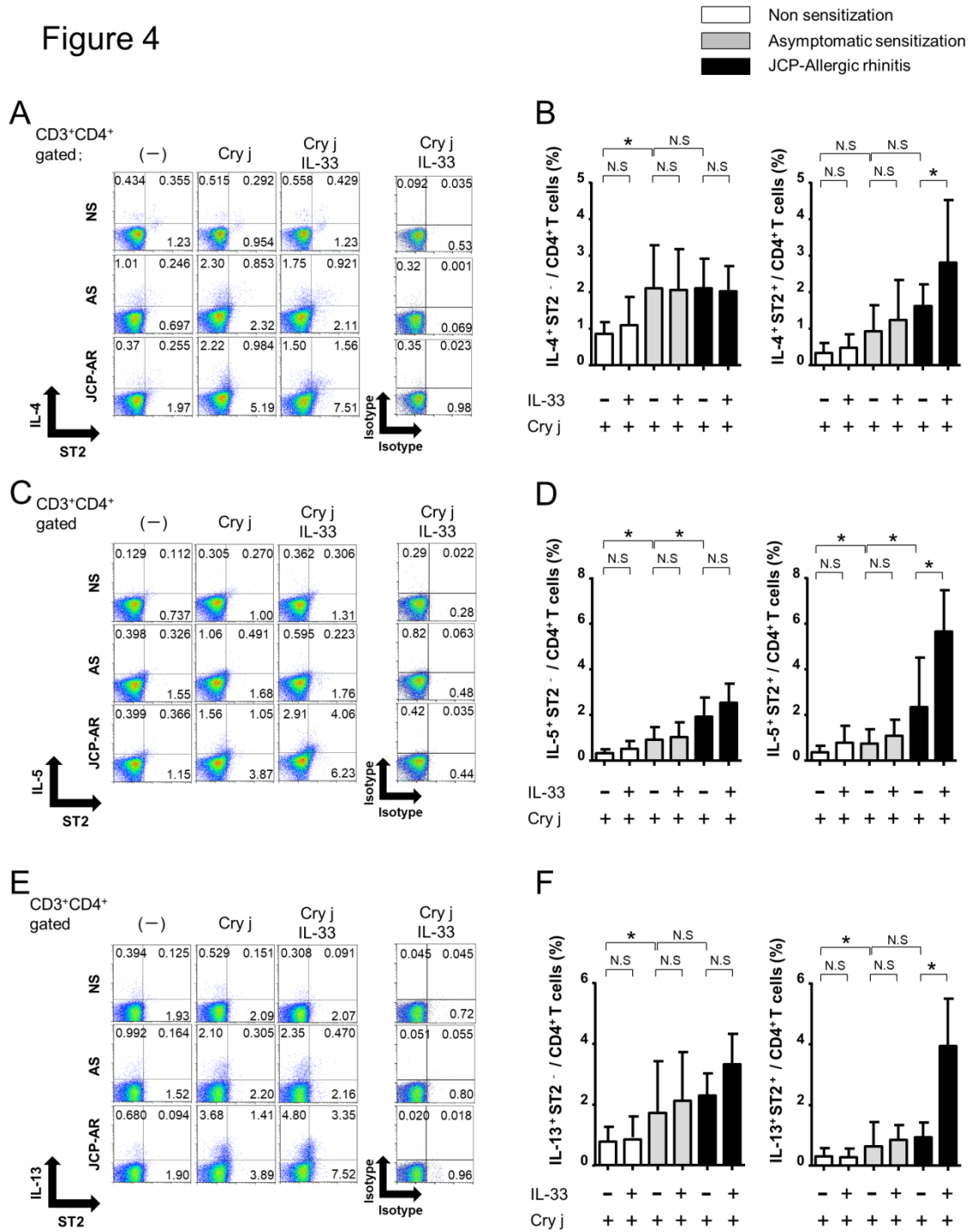
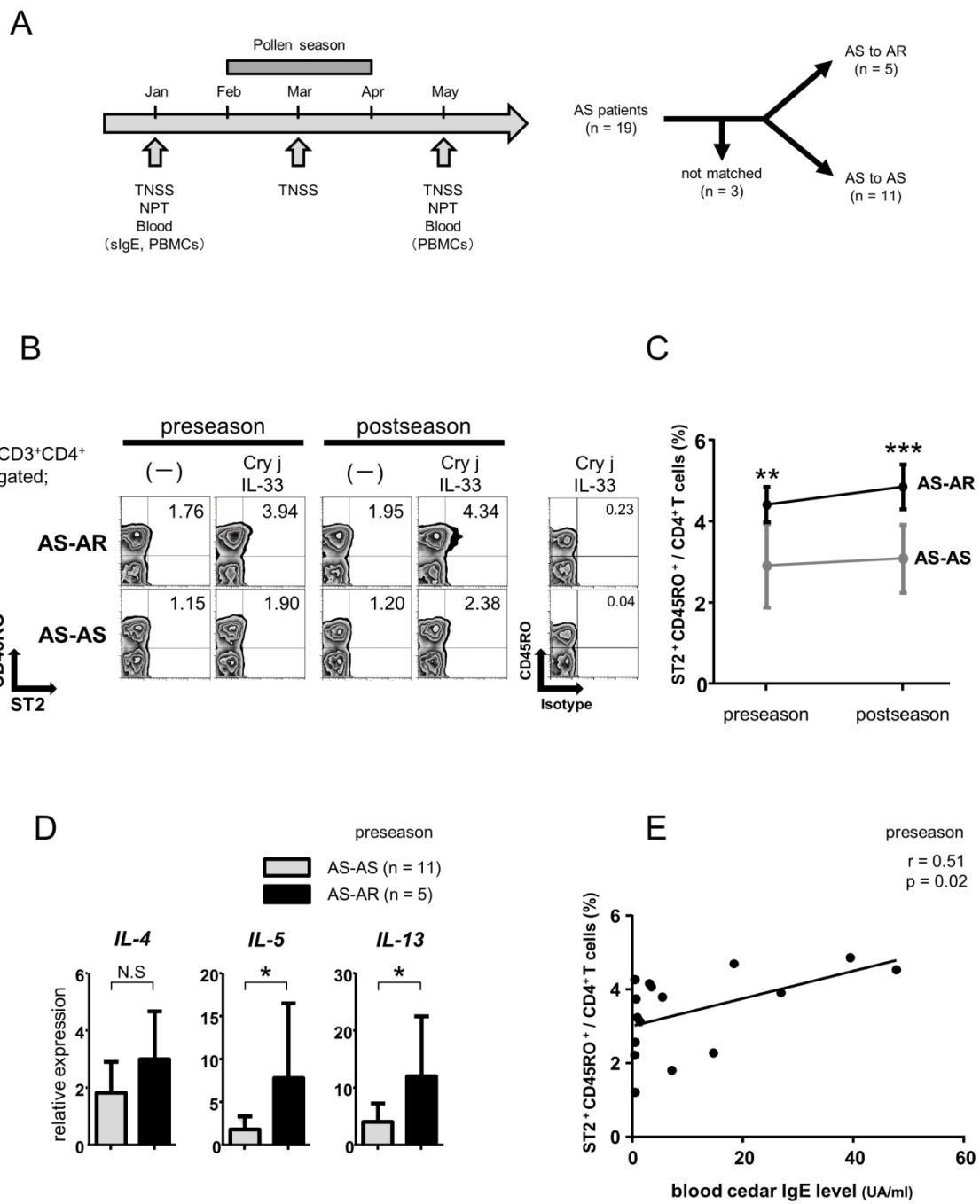
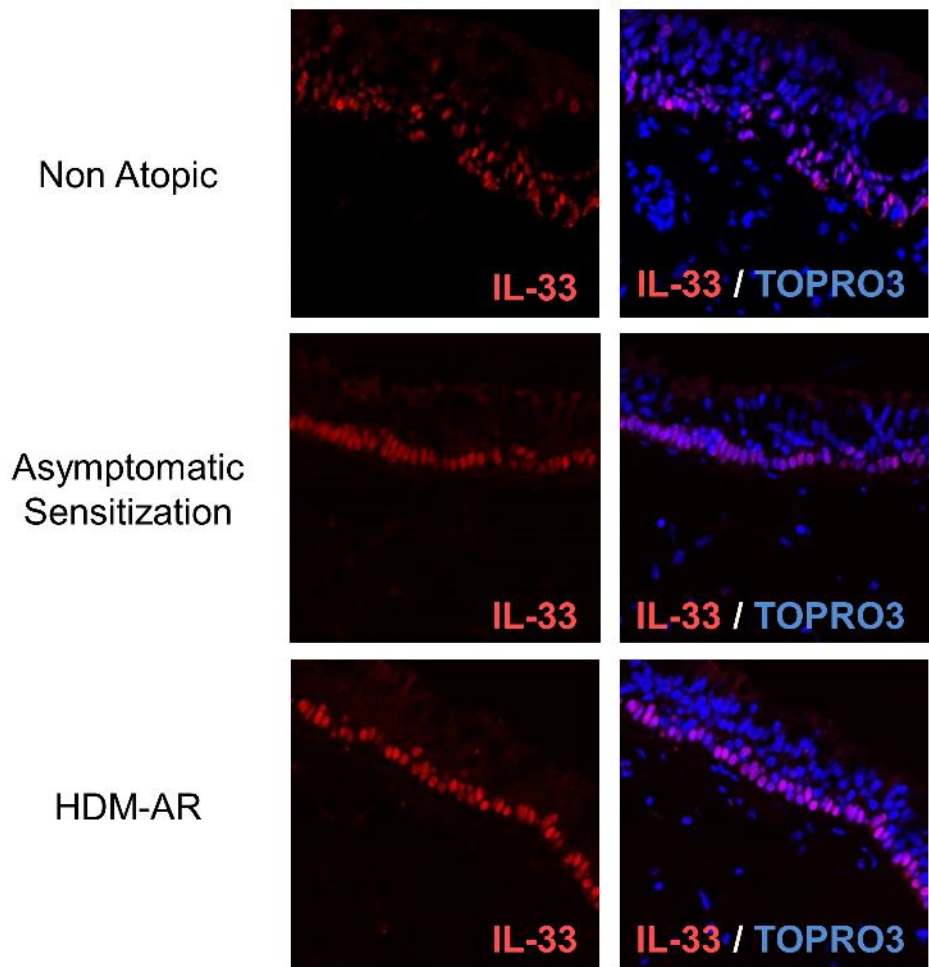


Figure 5

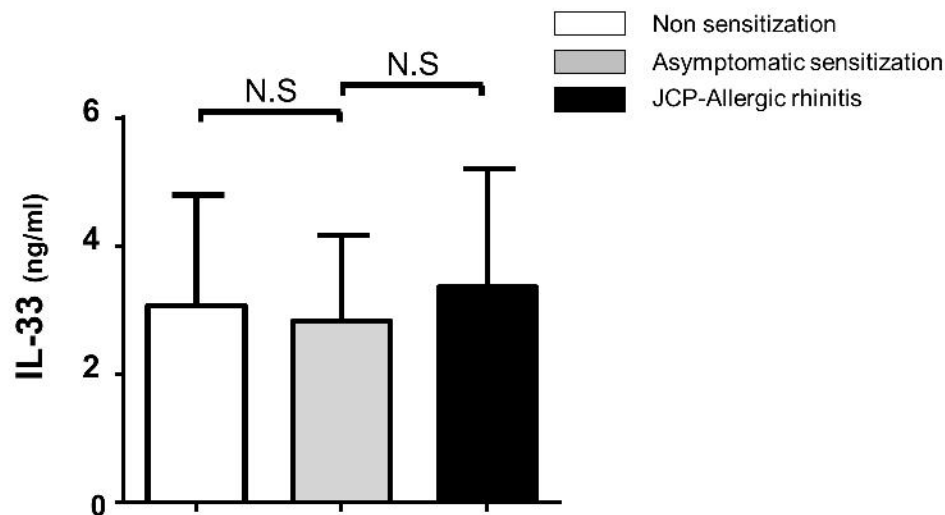


Supplementary Figure 1

A

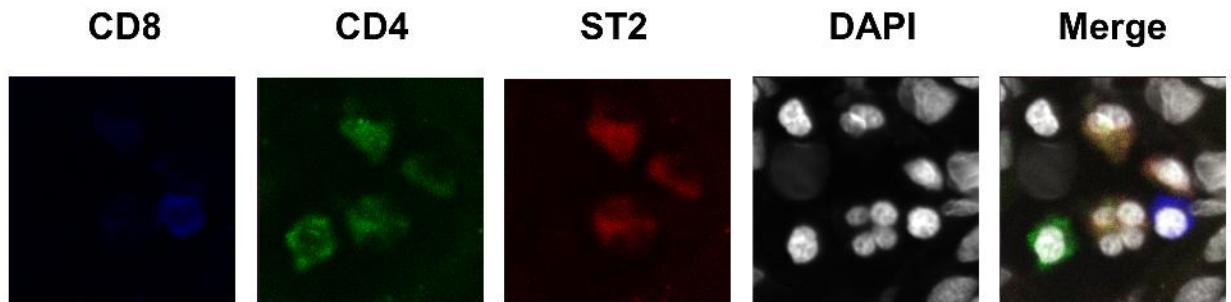


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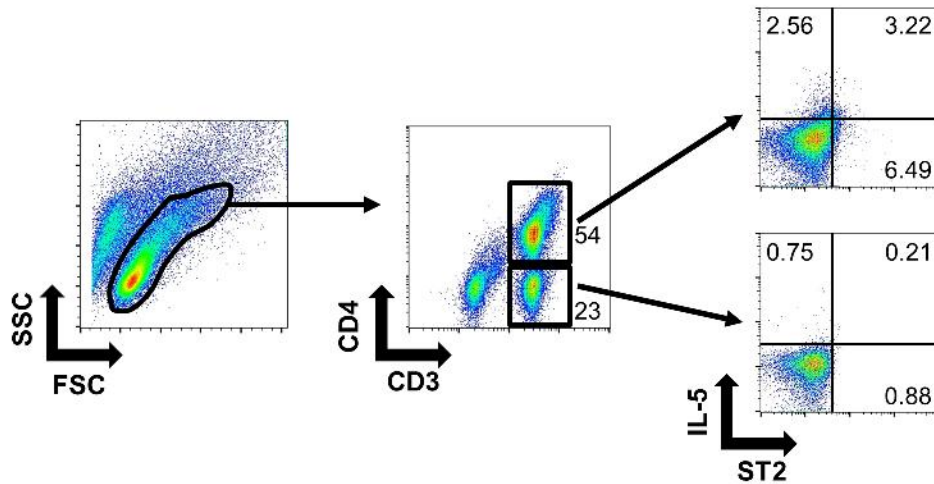


Supplementary Figure 2

A



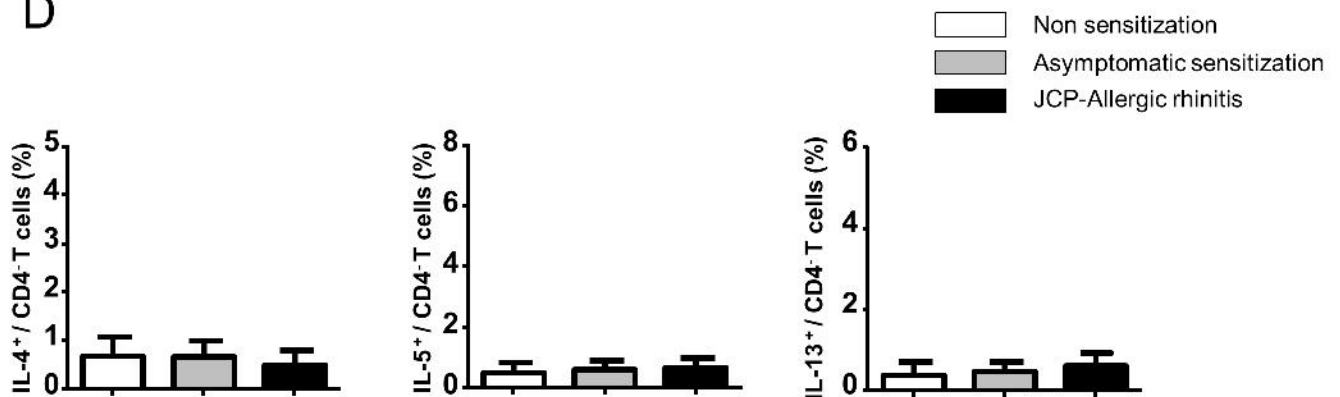
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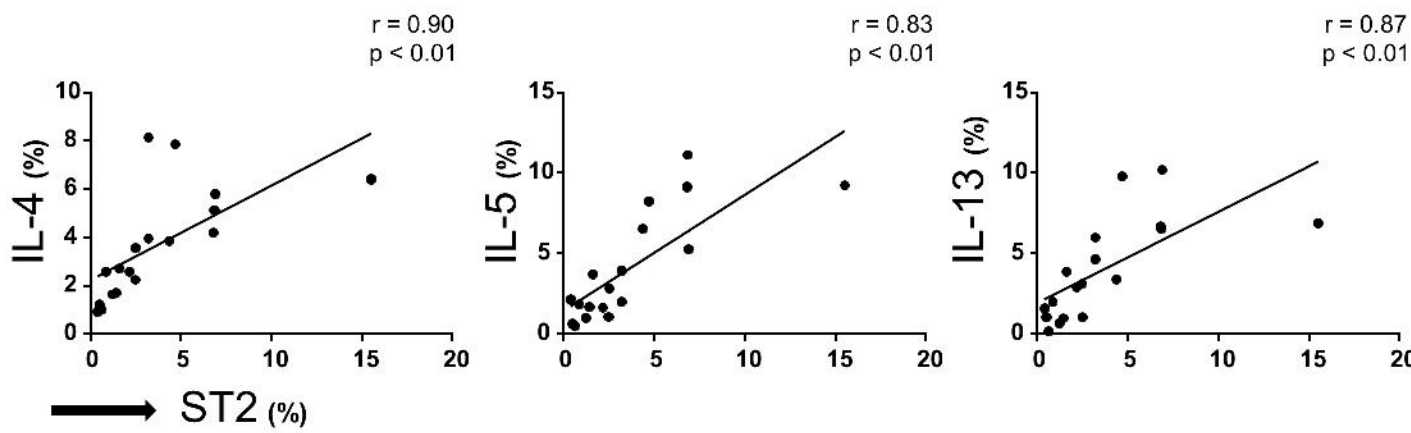
C



D



Supplementary Figure 3



Supplementary table 1

The characteristics of the of the subjects of the tissue analysis

	Non-sensitized	Asymptomatic sensitization	HDM-AR
patients, n	5	4	4
age, median	57.6	45.3	38.2
sex male/female, n	4/1	4/0	4/0
blood HDM IgE UA median	0	8.4	28.2

HDM-AR, House dust mite-induced allergic rhinitis

Supplementary table 2

The characteristics of the subjects and the results of the peripheral blood analysis

	Non-sensitized	Asymptomatic sensitization	JCP-AR
Subjects, n	9	11	9
Age, median	37	36	39
Sex male/female, n	7/2	8/3	5/4
Blood total IgE level, IU/ml	43.7	113.8	495.1
Blood cedar IgE level, UA/ml	0.0	8.3	100.1
HDM-AR, n	2	6	6

JCP-AR, Japanese cedar pollen-induced allergic rhinitis

HDM-AR, House dust mite-induced allergic rhinitis