

ORIGINAL ARTICLE

Experimental Allergy and Immunology

Identification of specifically reduced Th2 cell subsets in allergic rhinitis patients after sublingual immunotherapy

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Abstract

Background: Although Th2 cells are well known to play important roles in allergic diseases including allergic rhinitis (AR), the factors that induce and sustain the pathogenesis of AR remain unclear. The recent development of sublingual immunotherapy (SLIT) is expected to allow changes to the underlying pathogenesis of AR. However, which Th2 cell subsets are important in house dust mite-induced AR (HDM-AR), the influence of SLIT on the pathogenic Th2 cells, and the association of Th2 cell subsets with SLIT efficacy have not been clarified.

Methods: The cytokine production and frequency of HDM-reactive T-cell subsets in peripheral blood mononuclear cells (PBMCs) were evaluated using flow cytometry in 89 HDM-AR patients (placebo [n = 43] and HDM 300 IR [n = 46]) who participated in a placebo-controlled study of SLIT with HDM tablets. All patients provided samples both before treatment as a baseline and at the end of the 52-week study. The PBMCs were stained with CellTrace™ Violet (CTV) before culture with HDM extract, and HDM-reactive T cells were detected as the proliferated cells with diminished CTV.

Results: HDM-reactive IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺ cells and ST2⁺CD45RO⁺CD4⁺ cells were observed in the peripheral blood from each patient with HDM-AR; these cells significantly decreased after SLIT in the group treated with active tablets. HDM-reactive ST2⁺CD45RO⁺CD4⁺ cells were significantly lower in active-responders.

Conclusion: Allergen-reactive ST2⁺CD45RO⁺CD4⁺ cells or those combined with IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺ cells may be useful as markers indicating the successful treatment of SLIT. These cells may play a crucial role in the pathogenesis of AR as pathogenic memory Th2 cells.

KEYWORDS

allergic rhinitis, house dust mite, ST-2, sublingual immunotherapy, T helper 2 cells

1 | INTRODUCTION

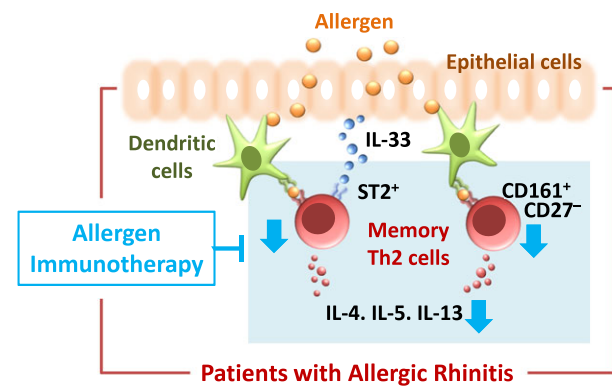
CD4⁺ T cells are crucial for immune cell homeostasis and host defense. Naïve CD4⁺ T cells differentiate into effector T cells and synthesize specific cytokine subsets when T-cell antigen receptors

Fumie Ihara and Daiju Sakurai contributed equally to this study.

are activated in particular cytokine environments. Lineages such as Th1, Th2, Th17, and regulatory T (Treg) cells are defined by their pattern of cytokine production and their transcription factor expression.^{1,2} Although Th2 cells are well known to play important roles in allergic diseases and eosinophilic inflammatory diseases, the factors that induce and sustain chronic eosinophilic inflammation through memory CD4⁺ T cells remain unclear. Recent animal studies have reported the identification of several distinct memory type Th2 cell subsets, which produce a large amount of IL-5 or IL-17 in addition to IL-4 and IL-13.³⁻⁵ These distinct memory Th2 cell populations appear to be important drivers of the pathology of chronic allergic diseases.^{2,6,7} A pathogenic IL-5-producing memory Th2 subpopulation, specifically the CD62L^{low}CXCR3^{low} Th2 cells, was reported to induce eosinophilic airway inflammation in a mouse model.⁴ The memory CD4⁺ T cells were divided by the expression of CD27. The memory T cells lacking CD27 had a strong antigen-recall response.⁸ CD161 is also expressed by memory CD4⁺ T cells. An increased percentage of CD27⁻ Th2 cells co-expressing CD161 and CRTH2 cells were identified in allergic individuals compared with nonallergic individuals, and these cells decreased after oral immunotherapy or subcutaneous immunotherapy.^{9,10}

Mucosal epithelial cells are not simply present as a physical barrier; they also express receptors for various stimulatory signals, and it has been shown that epithelial cells themselves are actively involved in immune reactions through the production of cytokines.¹¹⁻¹³ IL-33 is the representative innate allergic cytokine produced by mucosal epithelium, and large-scale genome-wide association studies have identified this cytokine as being involved in allergic diseases.¹⁴ Higher levels of IL-33 were reported in the respiratory tracts of patients with allergic rhinitis (AR) and asthma than in healthy controls.^{15,16} IL-33 is induced in response to various antigens, pathogens, and tissue damage by airway epithelial cells and is expected to cause eosinophilic inflammation in an antigen-independent manner.^{17,18} Furthermore, we recently identified the pathogenic potential of the IL-33-ST2 pathway in memory Th2 cells in an airway eosinophilic inflammation mouse model as well as in human eosinophilic inflammation, such as chronic rhinosinusitis with nasal polyps.¹⁹ Additionally, we recently demonstrated an enhanced IL-5-producing potential of ST2-expressing memory Th2 cells in AR patients compared with asymptomatic sensitized patients.²⁰ It is possible that the IL-33 receptor may be characteristic of pathogenic Th2 cells that induce and sustain eosinophilic inflammation, including AR.

Although many countries have experienced an increase in the prevalence of AR, natural resolution is not commonly observed. Antigen-specific immunotherapy, such as sublingual immunotherapy (SLIT), is effective for changing the natural course of allergic diseases²¹ and is expected to have long-term effects.^{22,23} To clarify the efficacy of SLIT for AR, we previously conducted several double-blinded, randomized, placebo-controlled trials for Japanese cedar pollen-induced AR or house dust mite (HDM)-induced AR (HDM-AR) and showed that SLIT was effective and safe.^{24,25} However, there remain some problems preventing the wide use of SLIT, including the current lack of objective biomarkers corresponding to the therapeutic effects.



- House dust mite (HDM)-reactive IL-5⁺IL-13⁺CD27⁻CD161⁺ CD4⁺ cells and ST2⁺CD45RO⁺CD4⁺ cells were observed in the peripheral blood from patient with HDM-induced allergic rhinitis; these cells significantly decreased after sublingual immunotherapy.
- HDM-reactive ST2⁺CD45RO⁺CD4⁺ cells were significantly lower after 1 year of sublingual immunotherapy in the responders in active group than those for the nonresponders.
- Allergen-reactive ST2⁺CD45RO⁺CD4⁺ cells or those combined with IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺ cells may be useful as markers indicating the successful treatment of sublingual immunotherapy.

Here, we analyzed HDM-reactive memory Th2 cell subsets in the peripheral blood of patients with HDM-AR who participated in a randomized placebo-controlled study of SLIT with HDM extract. We aimed to clarify the influence of SLIT on these T-cell subsets as well as the association of T-cell subsets with SLIT efficacy and the pathogenic potential of Th2 cell subsets in AR.

2 | MATERIALS AND METHODS

2.1 | Participants

A large, randomized, double-blinded, placebo-controlled with a parallel group, multicenter study (Japan Pharmaceutical Information Center Clinical Trial Information number: JapicCTI-121917), examining the efficacy of SLIT using HDM extract, was conducted in Japan from 2012 to 2013. The study participants were randomized into the 300 index reactivity (IR), 500 IR, and placebo treatment groups. The active tablets contained a 1:1 mixture of standardization extracts of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. All active and placebo tablets were supplied by Stallergenes S.A. (Antony, France). The subjects took the tablets sublingually every day for 52 weeks. The primary endpoint was the change in Average Adjusted Symptom Score (AASS) in the last 8 weeks of the 52-week treatment. Adjusted Symptom Score (ASS) was calculated by adjusting the rhinitis symptom score for rescue medication usage. If a patient took rescue medication, the ASS was equal to Rhinitis Total Symptom Score (RTSS) or the ASS of the previous day, whichever was higher, and the ASS on the next day was equal to the RTSS on the next day or the ASS from the prior day, whichever was higher. An improvement ratio

TABLE 1 Participant demographics and baseline characteristics

		Active (300 IR) (n = 46)	Placebo (n = 43)	P-value
Age	Mean ± SD	33.0 ± 11.5	32.0 ± 9.8	n.s.*
Gender	Male	25	24	n.s.**
	Female	21	19	
Serum level of IgE specific to <i>Dermatophagoides pteronyssinus</i> (Ua/mL)	Mean ± SD	39.3 ± 81.7	83.3 ± 302.1	n.s.*
	<0.35	0	0	
	≥0.35 to <0.70	0	0	
	≥0.70 to <3.5	6	4	
	≥3.5 to <50	32	30	
	≥50 to <100	6	6	
	≥100	2	3	
Serum level of IgE specific to <i>D. farinae</i> (Ua/mL)	Mean ± SD	35.6 ± 68.1	71.2 ± 208.1	n.s.*
	<0.35	0	0	
	≥0.35 to <0.70	2	0	
	≥0.70 to <3.5	4	3	
	≥3.5 to <50	34	30	
	≥50 to <100	4	7	
	≥100	2	3	
AASS at baseline	Mean ± SD	8.60 ± 1.79	8.90 ± 1.76	n.s.*
AASS at week 52	Mean ± SD	4.33 ± 2.00	6.56 ± 2.57	P < .0001*
Change in AASS (%)	Mean ± SD	−48.4 ± 22.6	−26.0 ± 27.0	P < .0001*

IR, index reactivity; AASS, average adjusted symptom score; n.s., not significant.

Change in AASS (%); {(final AASS – baseline AASS)/baseline AASS} × 100.

*P-values were analyzed using unpaired t test.

**P-values were analyzed using Fisher's exact test.

of the final AASS to the baseline AASS from 1 week before the initiation of SLIT was calculated for each subject. The details of the SLIT study were reported previously.²⁵ This study was conducted using peripheral blood samples from 89 of the participants who had been enrolled from across 37 medical institutions and had completed the clinical study in the placebo (n = 43) or 300 IR (n = 46) treatment groups (Table 1). Their symptoms were recorded, and blood samples were collected in the same season for all patients. The key for the original double-blinded clinical study was not revealed until after the immunological analyses were completed. The protocol was approved by the Ethics Committee of Chiba University and Shionogi & Co., Ltd., and written informed consent was obtained from each patient prior to participation in the study. The study was approved by the Institutional Review Board of Chiba University and Shionogi & Co., Ltd.

2.2 | Blood samples

Peripheral blood was obtained from each patient just before and at the end of the SLIT study, which lasted for 52 weeks. Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque

PLUS (GE Healthcare BioSciences, Uppsala, Sweden) and stored in a freezer at −80°C with CELLBANKER-1® cell freezing medium (Takara Bio Inc., Shiga, Japan) until use.

2.3 | Cell culture

The cytokine production and frequency of HDM-reactive T cells in PBMCs obtained from patients were evaluated using flow cytometry before treatment as a baseline and at week 52 of SLIT. Thawed PBMCs were labeled using a CellTrace™ Violet Cell Proliferation Kit (CTV) (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. CTV-labeled PBMCs were cultured with 6.5 µg/mL HDM extract with low endotoxin levels (LoTox™ *D. pteronyssinus* antigen, INDOOR Biotechnologies, USA) in the presence of IL-2 (20 IU/mL) for 7 days, followed by culturing with 10 ng/mL phorbol 12-myristate 13-acetate, 1 µmol L^{−1} ionomycin, and 1 µL/1.5 mL Goldistop solution for 4 hours. To measure ST2 expression, CTV-labeled PBMCs were cultured with 6.5 µg/mL HDM extract in the presence of IL-2 (20 IU/mL) and IL-33 (100 ng/mL) for 7 days. The harvested cells were stained with various specific antibodies and analyzed by flow cytometry.

2.4 | Antibodies and flow cytometry

PBMCs were stained with the following anti-human antibodies: anti-CD3-FITC (BD Bioscience, San Diego, CA, USA), anti-CD4-PerCPy5.5 (BD Bioscience), anti-CD8-PE-Cy7 (BioLegend, San Diego, CA, USA), anti-CD27-Brilliant violet510 (BioLegend), anti-CD45RO-APC (BioLegend), anti-CD161-PE (BioLegend), and anti-ST2-PE (LifeSpan BioSciences, Seattle, WA, USA) for surface staining and anti-IL-4-Alexa 488 (eBioscience, Santa Clara, CA, USA), anti-IL-5-APC (BD Bioscience), anti-IL-13-PE (BioLegend), anti-IL-17-FITC (eBioscience), anti-IL-10-PE (BioLegend), and anti-Foxp3-Alexa Fluor 647 (BioLegend) for intracellular staining. Dead cells were identified by staining with Fixable Viability Dye-eFlour 780 (eBioscience). Cell surface and intracellular staining were performed according to the manufacturer's protocols. The phenotypes of the cells were evaluated using the FACSCanto II system (BD Biosciences). The data were analyzed using the FlowJo software program (Tree Star Inc., Ashland, OR, USA).

2.5 | Statistical analyses

The statistical analyses of the means were evaluated using the unpaired *t* test or the paired *t* test, and the correlations were assessed using the Pearson's correlation coefficients. All *P*-

values were two-sided, and *P*-values of $<.05$ were statistically significant.

3 | RESULTS

3.1 | Patient profiles and nasal symptoms after SLIT for HDM

The profiles of all 89 enrolled patients are shown in Table 1. There were no significant differences in the age, serum HDM-specific IgE level, or baseline AASS before treatment between the active and placebo groups. The change ratio of AASS in the active group was significantly lower than that in the placebo group ($P < .0001$) (Table 1).

3.2 | Cytokine and surface molecule expression of HDM-reactive CD4⁺ T cells

The HDM-reactive cells were identified as those with diminished CTV levels (CTV^{dim} cells) after HDM-antigen stimulation. Representative flow cytometry profiles of IL-4, IL-5, IL-13, IL-17, and Foxp3 expression in the CTV^{dim}CD4⁺ cells from a patient at baseline are shown in Figure 1A. The IL-5 expression and IL-13 expression in CD27⁺CD161⁺ cells among CTV^{dim}CD4⁺ cells (Figure 1B) and the ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells (Figure 1C) in another patient at baseline are also shown. There were no significant

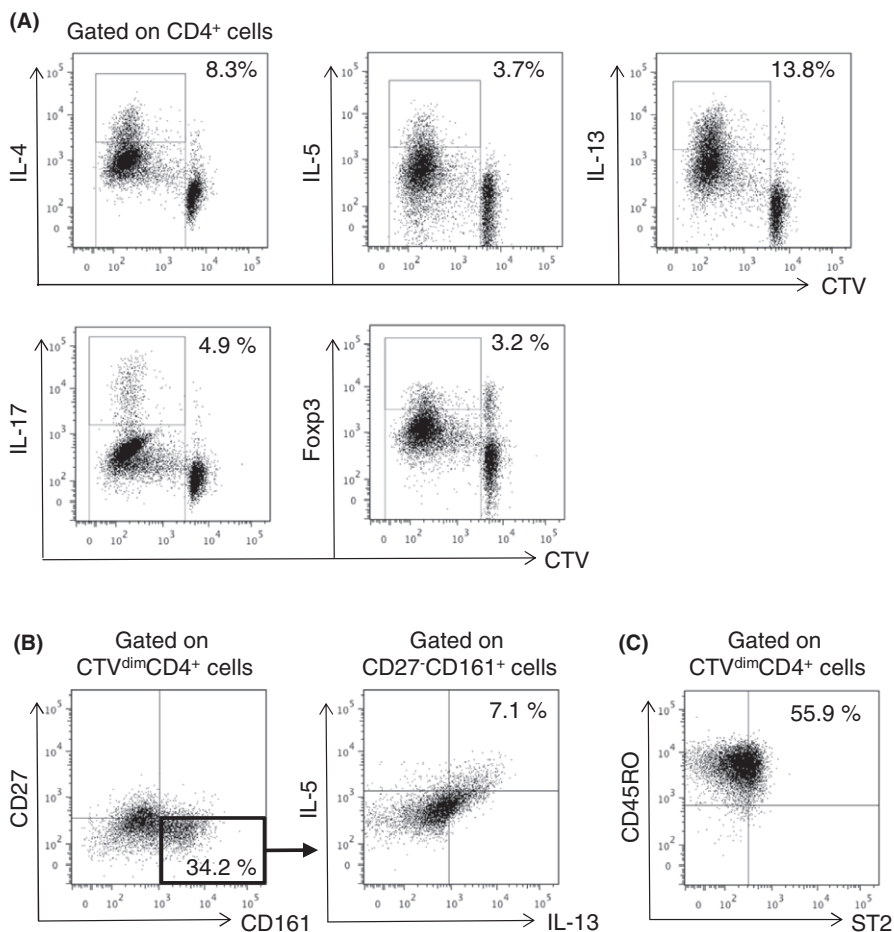


FIGURE 1 Identification of HDM-reactive T-cell subsets. (A-C) Representative flow cytometry plots showing the phenotype of HDM-reactive T cells at baseline. The percentages of various cytokine-producing cells or Foxp3⁺ cells in CTV^{dim} cells among CD4⁺ cells (A). The percentages of IL-5⁺IL-13⁺ cells in the subset of CD27⁺CD161⁺ cells among CTV^{dim}CD4⁺ cells (B), and the percentages of ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells (C)

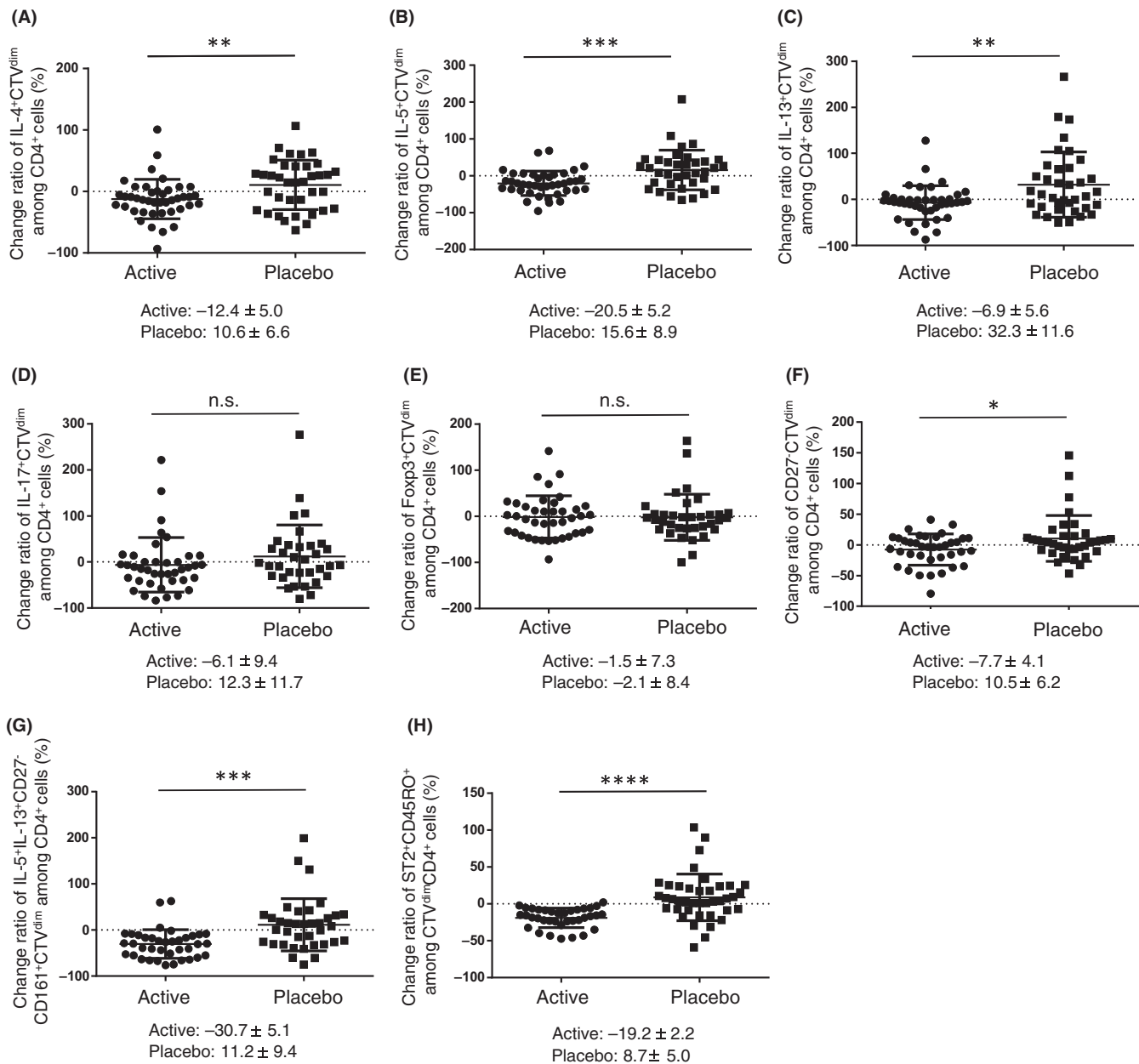


FIGURE 2 Changes in the frequency of HDM-reactive CD4⁺ cell subsets before and after treatment. (A-D) The change ratios of IL-4- (A), IL-5- (B), IL-13- (C), and IL-17- (D) producing CTV^{dim} cells among CD4⁺ cells before and after treatment between the SLIT group (active) and placebo group (placebo). The change ratios of Foxp3⁺ cells (E), CD27⁺ cells (F), IL-5⁺IL-13⁺CD27⁺CD161⁺ cells (G) in CTV^{dim} cells among CD4⁺ cells, and ST2⁺CD45RO⁺ cells (H) among CTV^{dim}CD4⁺ cells before and after treatment between the active and placebo groups. Two-group comparisons were performed using an unpaired Student's *t* test. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001

differences in the percentages of these expressed cytokines or surface molecules on CD4⁺ cells between the active and the placebo groups before treatment (data not shown).

3.3 | SLIT-induced changes to HDM-reactive Th2 cytokine-producing cells and CD27⁺CD161⁺ and ST2⁺CD45RO⁺ subsets among CD4⁺ cells

The percentages of IL-4⁺CTV^{dim}, IL-5⁺CTV^{dim}, IL-13⁺CTV^{dim}, CD27⁺CTV^{dim}, and IL-5⁺IL-13⁺CD27⁺CD161⁺CTV^{dim} cells among

the CD4⁺ cells, and ST2⁺CD45RO⁺ cells among the CTV^{dim}CD4⁺ cells were each significantly decreased after SLIT in the active group, but not in the placebo group (Figure S1). These change ratios were each significantly lower in the active group compared with those in the placebo group (Figure 2A-C and F-H). No significant changes were observed in the percentages of IL-17⁺CTV^{dim} and Foxp3⁺CTV^{dim} cells after treatment (Figure S1), and there was no significant difference in these changes between the active and placebo groups (Figure 2D and E). The change ratio of ST2⁺CD45RO⁺ cells and CTV^{dim} T-cell subsets other than IL-

17⁺CTV^{dim} and Foxp3⁺CTV^{dim} cells among total lymphocytes was significantly lower in the active group compared with the placebo group (Figure S2).

3.4 | Correlation of the HDM-reactive CD4⁺ T-cell subsets with the AASS change after SLIT

Significant correlations were observed between the percentage change in AASS and the change differences in IL-5⁺IL-13⁺CD27⁻CD161⁺CTV^{dim} cells among CD4⁺ cells and of ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells ($P < .05$ and $P < .01$, respectively) in all patients of both groups (Figure 3A and B). When these CD4⁺ T-cell subsets were compared in the active or placebo groups separately, these correlations were not significant. No significant correlations were observed between the percentage improvement of AASS and the change differences in IL-4⁺CTV^{dim}, IL-5⁺CTV^{dim}, IL-13⁺CTV^{dim}, or CD27⁻CTV^{dim} cells (Figure 3C-F). The baseline percentages of HDM-reactive CD4⁺CTV^{dim} cells or the ratio of specific IgE to total IgE in the active or placebo groups showed no significant correlation with the AASS change.

3.5 | Nonresponders in the active group showed no reduction in either the HDM-reactive ST2⁺CD45RO⁺ cells or IL-5⁺IL13⁺CD27⁻CD161⁺ cells

The patients were divided into additional 2 subgroups based on their individual AASS reduction; responders were defined as patients with an AASS reduction of more than 30% from their baseline AASS, and nonresponders were defined as those with an AASS reduction of less than 30%. Across the 4 subgroups, the responders in the active group (active-responders; $n = 31$), the nonresponders in the active group (active-non-responders; $n = 6$), the responders in the placebo group (placebo-responders; $n = 17$), and the nonresponders in the placebo group (placebo-non-responders; $n = 23$), the reduction of the percentage of ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells for the active-responders was significantly larger than those for the active-non-responders or the placebo-responders ($P < .05$ and $P < .01$, respectively) (Figure 4A). The reduction of the percentage of IL-5⁺IL-13⁺CD27⁻CD161⁺CTV^{dim} cells for the active-responders was significantly larger than that for the placebo-responders ($P < .01$) (Figure 4B). No significant difference was observed in those of the IL-4⁺CTV^{dim}, IL-5⁺CTV^{dim}, IL-13⁺CTV^{dim}, or CD27⁻CTV^{dim} cells between the responders and the nonresponders in either the active group or the placebo group (data not shown).

A significant correlation was observed between the change difference of the percentage of IL-5⁺IL-13⁺CD27⁻CD161⁺CTV^{dim} cells and the percentage of ST2⁺CD45RO⁺ cells in all patients ($P < .05$), but not in the active or the placebo groups separately. The distribution of these cells in the active group was substantially different from that in the placebo group, and no major reductions in these subpopulations were observed in active-non-responders (Figure 4C).

When the cutoff values of IL-5⁺IL-13⁺CD27⁻CD161⁺CTV^{dim} cells and ST2⁺CD45RO⁺ cells were defined as -0.74 and -2.5 , respectively, those were the minimum values of their change differences in active-non-responders (Figure 4C; dotted lines), and these 2 parameters were combined and used to discriminate all the active-non-responders from active-responders, the sensitivity and specificity were 82.1% and 100%, respectively, and the diagnosis accuracy was 85.3%. The 68% and 32% of patients in the active group were assessed as active-responders and active-non-responders, respectively. Meanwhile, the diagnosis accuracy using IL-5⁺IL-13⁺CD27⁻CD161⁺ cells or ST2⁺CD45RO⁺ cells alone as a single marker was 50% or 78.3%, respectively.

4 | DISCUSSION

Although the total number of Th2 cells is much lower than that of Th1 cells, the amount of allergen-specific Th2 cells is significantly higher than the amount of allergen-specific Th1 cells among the PBMCs of patients with AR.^{26,27} In this study, after a 52-week treatment of SLIT with HDM extract, the percentages of IL-4⁺CTV^{dim}, IL-5⁺CTV^{dim}, IL-13⁺CTV^{dim} cells were significantly decreased in the active group, but the differences between active-responders and active-non-responders were not significant. Meanwhile, no significant change was observed in the percentages of IL-17-producing CD4⁺CTV^{dim} cells or Foxp3⁺CD4⁺CTV^{dim} cells, either in the active or placebo groups.

In previous studies, we found that the number of allergen-reactive Th2 cytokine-producing cells increased during cedar pollen dispersal season.²⁷ Furthermore, in the placebo-controlled clinical studies of SLIT for AR induced by cedar pollen using cedar pollen extract, the seasonal increase in cedar pollen-reactive Th2 cytokine-producing cells was suppressed by SLIT, while the number of IL-10-producing Treg cells increased after SLIT.^{28,29} These changes were associated with the clinical efficacy of SLIT.³⁰ The difference between the results of the present study and those of previous studies might be due to the difference in allergen exposure or in the dispersal periods. The analysis methods were also different between previous work and the current study. Earlier work examined the antigen-reactive CD4⁺ T cells using an ELISPOT assay; however, in this study, we used CTV staining, which allowed proliferated cells that were responsive against a specific antigen to be detected via their diminished CTV intensity. Although the method using a specific peptide-MHC tetramer is efficient for identifying and quantifying antigen-specific T cells, this method requires the generation of different peptide-MHC tetramers for each different antigen, and the subsequent detection then depends on the type of MHC allele. In contrast, the CTV method used in this study can be applied for all patients with AR as well as for various allergens.

Reportedly, Treg cells are divided into 2 distinct functional subsets: effector Tregs and naïve Tregs.³¹ The change ratio of Foxp3⁺CD4⁺CTV^{dim} cells might not reflect the change in HDM-specific

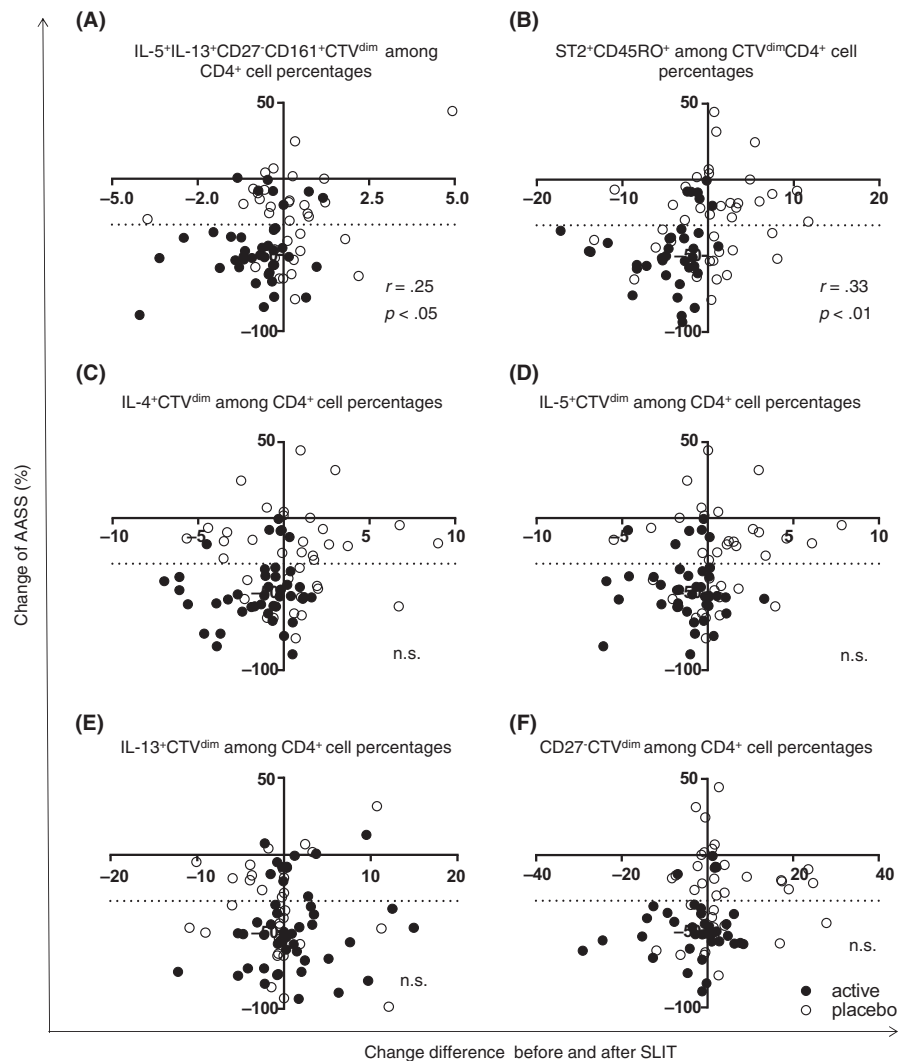


FIGURE 3 Correlation between the percentage change in AASS and the change in T-cell subsets before and after SLIT treatment. The correlation between the post-SLIT percentage change in AASS and the change differences in the percentages of ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells (A), IL-5- and IL-13-producing CD27⁻CD161⁺ cells (B), IL-4- (C), IL-13- (D), and IL-5- (E) producing cells, and CD27⁻ cells (F) in CTV^{dim} cells among the CD4⁺ cells before and after SLIT. The dotted line shows a -30% change in AASS. n.s., not significant; AASS, average adjusted symptom score

functional Treg cells. Further studies are needed to assess Treg cells in patients with AR.

ST2 is the receptor for IL-33, a clone of an IL-1 family member and the representative innate allergic cytokine produced by mucosal epithelium and known to be involved in allergic disease.^{32,33} Recently, we identified ST2-expressing CD4⁺ T cells as a new subset of Th2 cells.^{2,19} Upon activation, these Th2 cells can produce abundant levels of Th2 cytokines, such as IL-5 and IL-13, and are involved in the pathogenesis of diseases such as asthma and chronic airway inflammation. The ST2-expressing, IL-5-producing CD4⁺ cells are called pathogenic memory Th2 cells.²

The expression levels of CD27 and CD161 were reported to be associated with the function of memory CD4⁺ T cells.^{8,34} Recently, Wambre et al. reported that allergen-specific CD27⁻CD161⁺ CRTH2⁺CD4⁺ T cells were significantly increased in allergic individuals, and peanut-specific CD27⁻CD161⁺CRTH2⁺CD4⁺ T cells were significantly decreased in patients with peanut food allergy after oral immunotherapy.⁹ Here, HDM-reactive CD27⁻CD4⁺ cells, IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺ cells, and ST2⁺CD45RO⁺CD4⁺ cells were detected in the peripheral blood in all patients with HDM-AR, and

the amounts of each of these cell types were significantly decreased after SLIT. Furthermore, significant correlations were observed between the change in AASS and the change differences in HDM-reactive IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺ cells and of ST2⁺CD45RO⁺CD4⁺ cells in all patients. However, these correlations were not significant when they were compared in the active groups separately. When the placebo group was included, less symptomatic improvement with less change in the memory Th2 cell population may have increased the likelihood of showing a significant correlation. Meanwhile, the percentage of HDM-reactive ST2⁺CD45RO⁺CD4⁺ cells for the active-responders showed a significant reduction compared with the active-non-responders, suggesting the association with the SLIT efficacy. Recently, we have demonstrated that the proliferation and IL-5 production of ST2⁺CD45RO⁺CD4⁺ cells in symptomatic AR patients were significantly increased compared with nonsensitized or asymptomatic sensitized patients following the stimulation by allergen in the presence of IL-33.²⁰ ST2⁺CD45RO⁺CD4⁺ cells were suggested to play a crucial role in the progression of AR. SLIT could improve the underlying conditions of AR by suppressing allergen-specific ST2⁺CD45RO⁺CD4⁺ cells and IL-5⁺IL-

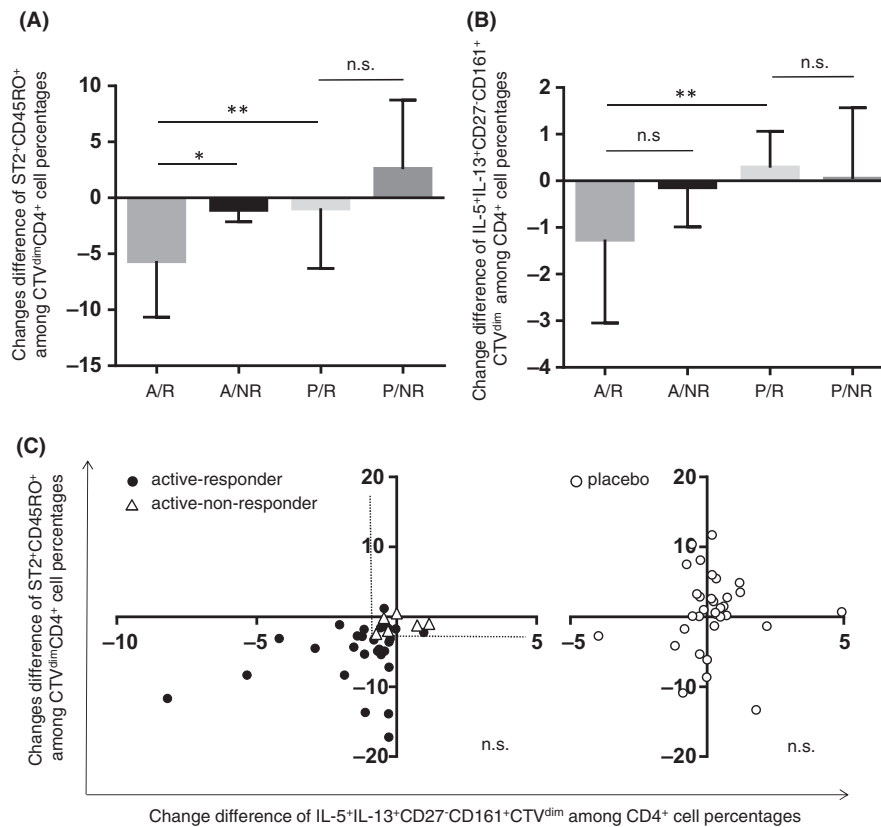


FIGURE 4 Association of SLIT clinical efficacy with the change in HDM-reactive T-cell subsets. (A–B) The change difference in ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells (A) and IL-5⁺IL-13⁺CD27⁻CD161⁺ CTV^{dim} cells among CD4⁺ cells (B) in an active-responder (A/R), active-non-responder (A/NR), placebo-responder (P/R), and placebo-non-responder (P/NR) before and after SLIT. Two-group comparisons were performed using an unpaired Student's *t* test. **P* < .05, ***P* < .01, n.s. not significant. (C) The correlation of the change difference in ST2⁺CD45RO⁺ cell and IL-5⁺IL-13⁺CD27⁻CD161⁺ cell percentages in active-responder, active-non-responder, and placebo groups before and after SLIT. The dotted lines indicate the cutoff points of IL-5⁺IL-13⁺CD27⁻CD161⁺ CTV^{dim} cells among CD4⁺ cells and ST2⁺CD45RO⁺ cells among CTV^{dim}CD4⁺ cells, which are -0.74 and -2.5, respectively, to detect a nonresponder in the active group

13⁺CD27⁻CD161⁺CD4⁺ cells. The suppression of these IL-5-producing memory Th2 cells might lead to suppression of allergic inflammation, including the activation of eosinophils. Although the mechanism of specific suppression of ST2⁺Th2 cells during SLIT is unclarified, the suppressor cells such as tolerogenic dendritic cells, Treg cells and regulatory B cells induced by SLIT might be involved.^{35,36}

When the patients were divided into 2 subgroups based on the AASS reduction after 1 year of SLIT, with the responders defined as patients with an AASS reduction of more than 30% from the baseline, 86% of patients in the active group were assessed as responders. Additionally, 55.8% of patients in the placebo group were also identified as responders. However, this is not surprising given that placebo effects are well-documented to occur in SLIT.^{25,26} The nasal symptoms were improved in approximately 30% of AR patients even in the placebo group. The placebo effects were suggested to be involved in the active group as well. As such, previous reports have highlighted the difficulty of precisely evaluating the therapeutic efficacy and the necessity of finding an objective biomarker for SLIT. Although various biomarkers have been suggested, for them to be useful, potential markers must exclude the placebo effects as much as possible and

show a significant correlation with the clinical efficacy. To date, no reliable biomarkers of SLIT efficacy have been established.

A decrease in the amount of allergen-specific CD27⁻CD4⁺ cells after SLIT has been mentioned in previous reports.^{10,37,38} In this study, the percentage of CD27⁻CTV^{dim} cells among CD4⁺ cells was significantly decreased in the active group after SLIT, and the reduction ratio in the active group was significantly greater than that in the placebo group. However, by itself, the change in the percentage of CD27⁻CTV^{dim} cells in the CD4⁺ cells did not have a significant correlation with AASS improvement.

The percentages of ST2⁺CD45RO⁺ cells in CD4⁺CTV^{dim} cells in the active-responders were significantly lower than those in the active-non-responders, and the percentages of IL-5⁺IL-13⁺CD27⁻CD161⁺ CTV^{dim} cells in the active-responders were significantly lower than those in the placebo-responders. Mitson-Salazar et al. reported that CD161⁺ Th2 cells express IL-33 receptors on their cell surfaces.²³ In our previous study, the ST2⁺CD45RO⁺CD4⁺ cells produced large amounts of IL-5 in response to allergen in the presence of IL-33 in AR patients.²⁰ Therefore, the ST2⁺CD45RO⁺CD4⁺CTV^{dim} cells and IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺CTV^{dim} cells are suggested to

be an overlapping population; however, their association was not clear in this study.

When the cutoff value for the change in the percentages of HDM-reactive ST2⁺CD45RO⁺ cells and IL-5⁺IL-13⁺CD27⁺CD161⁺ cells was defined as −2.5% and −0.74%, respectively, the sensitivity and specificity were 82.1% and 100%, respectively. The responders in the active group could be distinguished from the nonresponders with high sensitivity and specificity. Together, our results suggest that allergen-reactive ST2⁺CD45RO⁺ cells and their combination with IL-5⁺IL-13⁺CD27⁺CD161⁺ cells could be useful biomarkers to reflect the SLIT efficacy.

5 | CONCLUSION

HDM-reactive ST2⁺CD45RO⁺CD4⁺ cells and IL-5⁺IL-13⁺CD27⁺CD161⁺CD4⁺ cells were both observed in the peripheral blood of patients with HDM-AR. The amounts of each of these cell types significantly decreased after 1 year of SLIT, and HDM-reactive ST2⁺CD45RO⁺CD4⁺ cells were correlated with the SLIT clinical efficacy. These results indicate that 2 cell populations could be useful biomarkers to reflect the clinical efficacy of SLIT and that these cells may play a crucial role in the pathogenesis of AR by acting as pathogenic Th2 cells. A further study of longer duration is required to determine the parameter changes during SLIT, which should be undertaken for a longer duration (2–3 years), and the study of T-cell subsets in the local nasal mucosa will be required to confirm the mechanisms responsible for the pathogenesis in AR.

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CONFLICT OF INTEREST

YO has received payments for lectures from Shionogi & Co., Ltd., MSD, and Torii Pharmaceuticals. The remaining authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

FI, DS, SY, RY, KK, TN, and YO designed and developed the protocol for the study. DS, SY, TI, TS, YM, TA, and SS conducted the clinical study and the acquisition of data. TI, AM conducted the analysis of data. FI and DS performed the analysis of results and wrote the manuscript. TN and YO supervised the study and reviewed the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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