



Sublingual immunotherapy for 4 years increased the number of Foxp3⁺ Treg cells, which correlated with clinical effects

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Abstract

Objective At least 3 years of sublingual immunotherapy (SLIT) is required to achieve long-term clinical tolerance for allergens. However, immunological changes with more than 3 years of SLIT have not yet been elucidated in detail. The present study investigated whether the numbers of regulatory T (Treg) cells and regulatory B (Breg) cells increased with 4 years of SLIT and if these increases correlated with clinical effects for pollinosis.

Methods Seven Japanese cedar pollinosis patients received SLIT in 2014 or 2015 and continued treatment until May 2019. In May 2017 and May 2019, peripheral blood mononuclear cells (PBMCs) were collected from the patients, and analyzed by flow cytometer.

Results (1) The visual analogue scale (VAS) was significantly higher in 2019 than in 2017. (2) The percentages of Foxp3⁺ Treg cells, type 1 regulatory T (Tr1) cells, and Breg cells in PBMCs were significantly higher in 2019 than in 2017. (3) The percentage of Foxp3⁺ Treg cells in PBMCs positively correlated with VAS, whereas those of Tr1 cells and Breg cells did not.

Conclusions These results suggest that 4 years of SLIT is needed to achieve sustained increases in Foxp3⁺ Treg cells, which are closely associated with the efficacy of SLIT.

Keywords Allergic rhinitis · Immunotherapy · Japanese cedar pollinosis · Regulatory T cells · Regulatory B cells

Introduction

In recent decades, the number of allergic patients has been increasing worldwide [1]. Allergic diseases are commonly treated by pharmacotherapies, such as corticosteroids [2], anti-histamines [3], and anti-leukotrienes [4]. However, pharmacotherapies are not causative treatments for allergic diseases. Allergen immunotherapy is the only causative

treatment that induces long-lasting tolerance to allergens and prevents further allergen sensitization [5]. Subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) are frequently administered forms of allergen immunotherapy [5]. Although SCIT is highly effective for allergic diseases, adverse effects, such as anaphylactic reactions, are often reported [6]. A recent study reported that SLIT is an effective treatment for seasonal allergy and safe alternative to SCIT [7]. Japanese cedar pollinosis is the most common seasonal allergy in Japan, lasting approximately 3 months from February to April [8]. During this season, widely dispersed airborne cedar pollen causes allergic rhinitis and/or conjunctivitis [8]. More than 30% of the Japanese population has cedar pollinosis, and its prevalence has been gradually increasing [8]. A clinical study demonstrated the efficacy and safety of SLIT for Japanese cedar pollen-induced allergic rhinitis [9].

SLIT requires long-term treatment to achieve clinical effects for allergic diseases. Two years of SLIT effectively attenuated nasal symptoms in patients with moderate to

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severe seasonal allergic rhinitis to grass pollen; however, following its discontinuation for 1 year, SLIT was no longer effective [10]. On the other hand, SLIT for 3 years achieved long-lasting efficacy after its discontinuation [11, 12]. Therefore, at least 3 years of SLIT is needed to achieve long-term efficacy [13, 14]. However, the mechanisms underlying the clinical effects of long-term SLIT have not yet been elucidated in detail. Furthermore, immunological evidence to support the clinical effects of SLIT has been insufficient.

Regulatory T (Treg) cells have been identified as one of the immunological parameters that fluctuate in individuals receiving allergen immunotherapy. Treg cells have been shown to play essential roles in the induction of immune tolerance to allergens [15]. Treg cells are mainly classified into two groups: thymus-derived CD25⁺ forkhead box protein 3 (Foxp3)⁺ Treg cells and peripherally-induced Treg cells [16]. Peripherally-induced Treg cells are further subdivided into peripherally-induced Foxp3⁺ Treg cells and type 1 regulatory T (Tr1) cells [17]. Foxp3⁺ Treg cells suppress excessive immune responses by releasing inhibitory cytokines, such as interleukin (IL)-10, IL-35, and transforming growth factor (TGF)- β [17]. Tr1 cells produce large amounts of IL-10 and are negative for Foxp3 [18]. On the other hand, serum allergen-specific IgG4 levels have been shown to increase in SLIT-treated patients [6]. IgG4 has been characterized as a blocking antibody that competes with IgE for antigen binding [6]. Veen et al. [19] found that human Breg cells produced large amounts of IgG4. Breg cells also play critical roles in the induction of immune tolerance to allergens by suppressing allergen-mediated inflammation via IL-10 and IgG4 [20].

The present study was performed to investigate (1) whether the clinical effects of SLIT in the second or third year after its initiation are strengthened by a further 2-year treatment, (2) whether the numbers of Foxp3⁺ Treg cells, Tr1 cells, and Breg cells and allergen-specific IgG4 levels in peripheral blood are also increased by the additional 2-year treatment, and (3) whether increases in regulatory cells and allergen-specific IgG4 correlate with clinical effects. The clarification of these issues will contribute to more accurate decisions regarding the duration of SLIT needed to achieve long-lasting efficacy.

Experimental methods

Patient characteristics

The present study was approved by the Ethics Committees of Osaka Medical College (Osaka, Japan) and Setsunan University (Osaka, Japan), and written consent was obtained from all participants; approval numbers are RIN-37 (1568-01) and 2015-032, respectively. Patient characteristics are

summarized in Table 1. SLIT was initiated for 7 Japanese cedar pollinosis patients in 2014 or 2015 and continued until May 2019. SLIT-treated patients were initially treated sublingually with 0.2 ml of 200 Japanese allergy units (JAU)/ml of Japanese cedar pollen extract (Torii Pharm. Co., Ltd, Tokyo, Japan). This dose was gradually increased over 2 weeks, followed by a maintenance dose of 1.0 ml of 2000 JAU/ml of Japanese cedar pollen extract administered daily. In May 2017 and May 2019, clinical effects for nasal symptoms were assessed by VAS, and PBMCs were collected from patients and analyzed using a flow cytometer.

Airborne cedar pollen sampling

The amounts of Japanese cedar pollen during the pollen season in 2017 and 2019 in Takatsuki city were assessed every day using Durham pollen samplers at Osaka Medical College and then summed. In 2017, the Japanese pollen season started on February 24 and lasted until April 20. As shown in Fig. 1b, the total pollen count in 2017 was 1483/cm². In 2019, the pollen season was from February 19 to April 17. The total pollen count in 2019 of 3735/cm² was larger than that in 2017.

Visual analogue scale (VAS)

At the end of the respective pollen seasons, patients were asked to score their allergic symptoms during each pollen season in comparisons with those experienced during the season before the start of SLIT. VAS ranging from 0 (unchanged symptoms, no improvement) to 10 (total symptom relief, complete recovery) was used.

Measurement of total IgE, allergen-specific IgE, IgG, and IgG4 levels in serum

Total IgE and allergen-specific IgE, IgG, and IgG4 levels in serum were measured using Immulite[®] total IgE III, Immulite AlaSTAT IgE II, Immulite 2000 Allergen-Specific IgG, and Immulite 2000 Allergen-Specific IgG4, respectively (Siemens Healthineers, Tokyo, Japan).

Table 1 Subject characteristics

Factor	SLIT-treated pollinosis patients
Number	7
Sex (male/female)	6/1
Age (years)	60 (35–78)

Age is presented as a median (range)

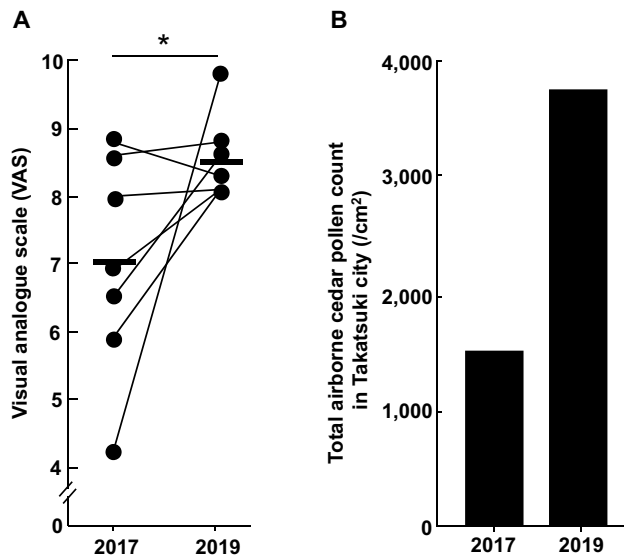


Fig. 1 Airborne cedar pollen counts and the visual analogue scale (VAS) in 2017 and 2019. **a** Clinical effects of sublingual immunotherapy (SLIT) in pollinosis patients ($N=7$) were analyzed by VAS. **b** Airborne cedar pollen counts in 1 day during the Japanese pollen season in 2017 and 2019 in Takatsuki city were summed. Each point represents values of respective patients. Each bar represents the mean. * $p < 0.05$

FACS analyses

PBMCs were isolated from heparinized blood by centrifugation over Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). The PBMCs obtained were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (all from Thermo Fisher Scientific) at a concentration of 10^6 cells/ml. In analyses of IL-10-producing cells, PBMCs were incubated with oligonucleotide (ODN) 2006 (toll-like receptor 9 (TLR9) agonist, 1 µM, InvivoGen, San Diego, CA, USA) or Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) at 37 °C for 24 h in the presence of the protein transport inhibitor, monensin sodium salt (2 µM, Calbiochem, San Diego, CA, USA). To detect CD25⁺ Foxp3⁺ CD4⁺ T cells, PBMCs were not treated by the above-described culture conditions. To exclude dead cells, cells were stained with eFluor® 780-conjugated Fixable Viability Dye (Thermo Fisher Scientific). After washing with 2% FBS-containing PBS, cells were treated with Human BD Fc Block™ (BD Biosciences, San Jose, CA, USA) to reduce potential non-specific antibody binding, followed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (HIT3a), PE-conjugated anti-human CD19 (HIB19), Pacific blue-conjugated anti-human CD4 (OKT4) (all from BioLegend, San Diego,

CA, USA), and/or allophycocyanin (APC)-conjugated anti-human CD25 (BC96) (Thermo Fisher Scientific) at 4 °C for 20 min. After staining the cell surface, cells were fixed and permeabilized by Fixation/Permeabilization Concentrate (Thermo Fisher Scientific) at room temperature for 1 h. After fixation and permeabilization, cells were washed with Permeabilization Buffer (Thermo Fisher Scientific), followed by staining with APC-conjugated anti-human IL-10 (JES3-9D7) (all from BioLegend) and/or PE-conjugated anti-human Foxp3 (236A/E7) (Thermo Fisher Scientific) at room temperature for 1 h. After washing with 2% FBS-containing PBS, cells were analyzed using FACSAria™ Fusion (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Two related samples were analyzed using the paired t test. A bivariate correlation was performed using a correlation analysis. A p value < 0.05 was considered to be significant. Statistical analyses were performed with JMP® Pro (Version 13.1.0, SAS institute Japan, Tokyo, Japan).

Results

Effects of SLIT on the visual analogue scale

SLIT was initiated for 7 Japanese cedar pollinosis patients in 2014 or 2015 and continued until May 2019. The clinical effects of SLIT were evaluated in May 2017 and May 2019 using the visual analogue scale (VAS). VAS was significantly higher in 2019 than in 2017 (Fig. 1a, $p = 0.037$), indicating that clinical symptoms in 2017 were ameliorated by a further 2 years of SLIT.

On the other hand, as shown in Fig. 1b, airborne pollen concentrations were higher in May 2019 than in May 2017, suggesting that the increase observed in the clinical effects of SLIT in 2019 was not associated with airborne pollen concentrations.

Effects of SLIT on antibody production

Total IgE, allergen-specific IgE, allergen-specific IgG, and allergen-specific IgG4 levels in the sera of SLIT-treated pollinosis patients were measured in 2017 and 2019 (Fig. 2). As shown in Fig. 2, no significant differences were observed in total IgE, allergen-specific IgE, or allergen-specific IgG levels between 2017 and 2019 (Fig. 2a–c). However, allergen-specific IgG4 levels were markedly higher in 2019 than in 2017 (Fig. 2d, $p = 0.0072$).

Fig. 2 Changes in total IgE (a), allergen-specific IgE (allergen-IgE, b), allergen-specific IgG (allergen-IgG, c), and allergen-specific IgG4 (allergen-IgG4, d) levels in sera of patients receiving sublingual immunotherapy (SLIT) between 2017 and 2019. Each point represents values of respective patients. Each bar represents the mean. $**p < 0.01$

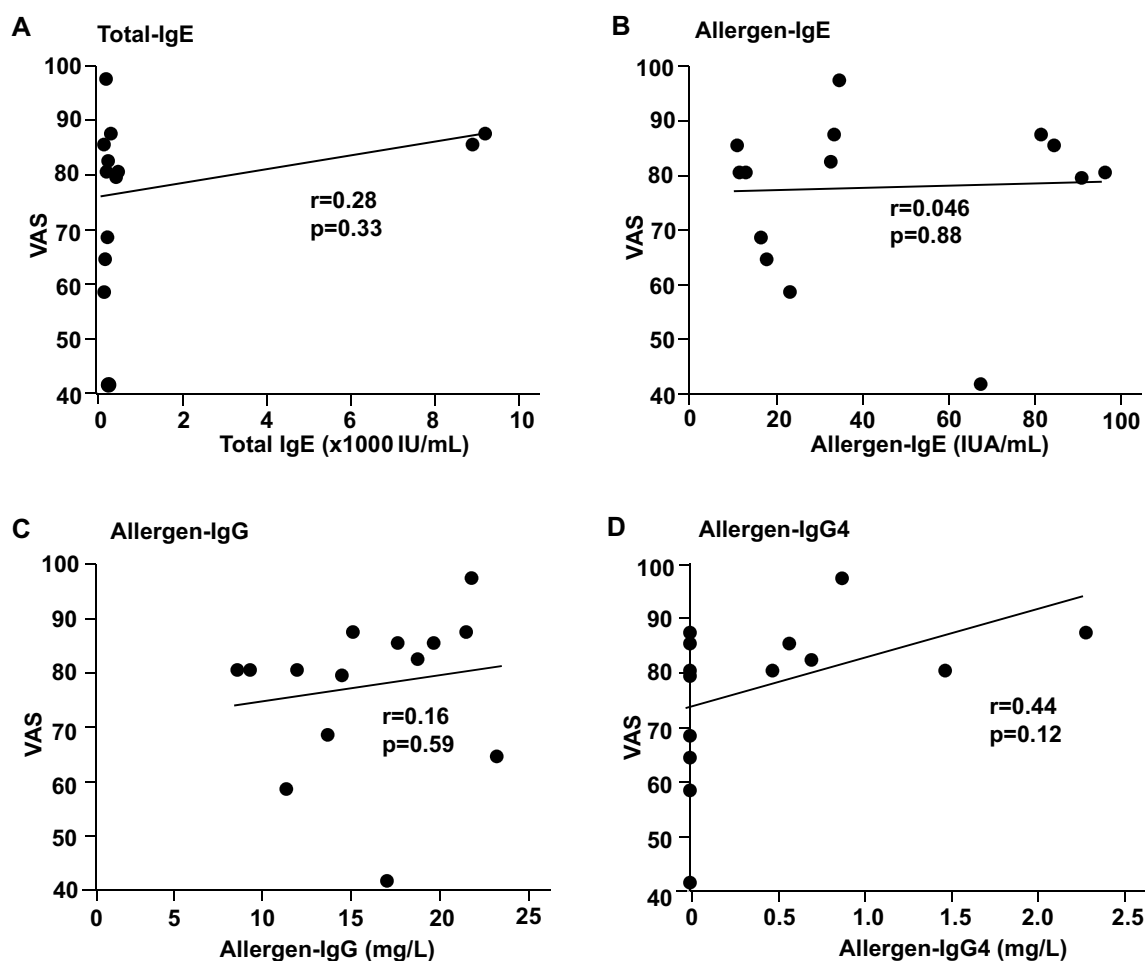
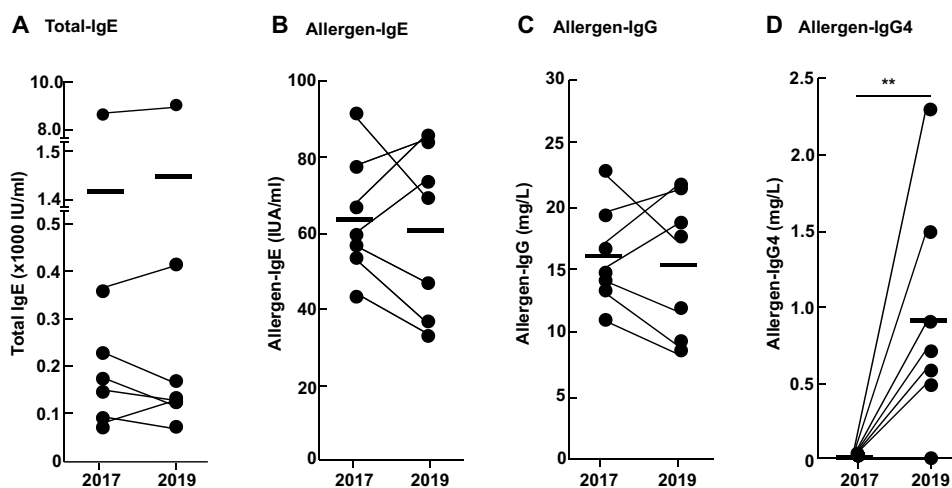


Fig. 3 Relationships between total IgE (a), allergen-specific IgE (allergen-IgE, b), allergen-specific IgG (allergen-IgG, c) and allergen-specific IgG4 (allergen-IgG4, d) levels and the visual analogue scale (VAS). Each point represents values of respective patients in 2017 and 2019

Relationships between antibody levels and VAS in SLIT-treated pollinosis patients

The relationships between antibody levels in 2017 and 2019 and VAS in SLIT-treated pollinosis patients were examined. As shown in Fig. 3a–c, total IgE, allergen-specific IgE, and allergen-specific IgG levels did not correlate with VAS. On the other hand, allergen-specific IgG4 levels were associated with VAS (Fig. 3d).

Effects of SLIT on percentages of Foxp3⁺ Treg cells, Tr1 cells and Breg cells in peripheral blood mononuclear cells

Foxp3⁺ Treg cells were detected as CD25⁺ Foxp3⁺ CD4⁺ T cells (Fig. 4a). The percentage of Foxp3⁺ Treg cells in peripheral blood mononuclear cells (PBMCs) was significantly higher in 2019 than in 2017 (Fig. 4d, $p = 0.0001$). Tr1 cells were detected as IL-10-producing Foxp3⁺ CD4⁺ T cells under an anti-CD3/CD28 stimulation (Fig. 4b). The percentage of Tr1 cells also markedly increased during the additional 2 years of treatment (Fig. 4e, $p = 0.0010$). Breg cells were detected as IL-10-producing CD19⁺ cells under an TLR9 agonist (ODN2006) stimulation (Fig. 4c). The percentage of Breg cells significantly increased during the additional 2 years of treatment (Fig. 4f, $p = 0.0020$). The absolute numbers of Foxp3⁺ Treg cells, Tr1 cells and Breg cells in peripheral bloods also significantly increased during

the additional 2 years of treatment (Supplemental Fig. 1a–c, $p = 0.0040$, 0.0027 and 0.030 , respectively).

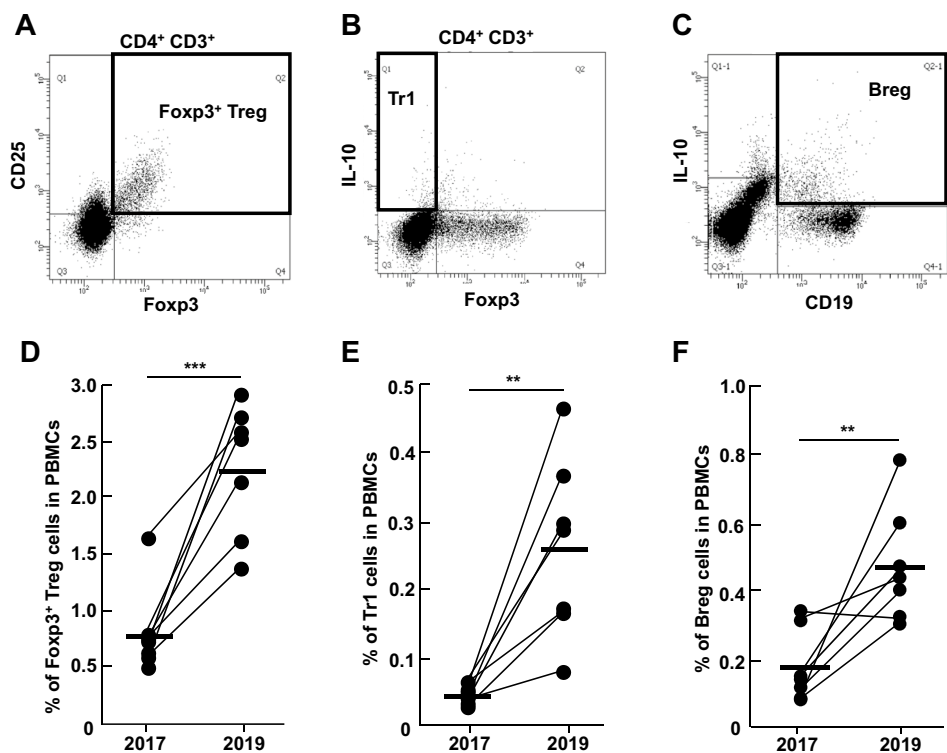
Relationships between regulatory cells and VAS in SLIT-treated pollinosis patients

The relationships between the percentages of Foxp3⁺ Treg cells, Tr1 cells, and Breg cells in PBMCs in 2017 and 2019 and VAS were investigated (Fig. 5). The percentage of Foxp3⁺ Treg cells in PBMCs positively correlated with VAS (Fig. 5a, $p = 0.0078$), whereas those of Tr1 cells and Breg cells did not (Fig. 5b, c). The absolute number of Foxp3⁺ Treg cells in peripheral bloods in 2017 and 2019 also positively correlated with VAS (Supplemental Fig. 1d, $p = 0.0043$), whereas those of Tr1 cells and Breg cells did not (Supplemental Fig. 1e, f).

Relationships between Treg cells and Breg cells in SLIT-treated pollinosis patients

Bianchini et al. [21] previously reported that allergen-IgG4 complexes induced Treg cells. Thus, the relationship between Treg cells and Breg cells in SLIT-treated pollinosis patients was also examined. The percentages of Foxp3⁺ Treg cells and Tr1 cells both positively correlated with allergen-specific IgG4 levels (Fig. 6a, b, $p = 0.0067$ and 0.016 , respectively). The absolute numbers of the cells also significantly correlated with allergen-specific IgG4 levels

Fig. 4 Changes in percentages of Foxp3⁺ Treg cells, Tr1 cells, and Breg cells in peripheral mononuclear cells (PBMCs) of patients receiving sublingual immunotherapy (SLIT) between 2017 and 2019. FACS profiles of Foxp3⁺ Treg cells (a), Tr1 cells (b), and Breg cells (c). Analyses of percentages of Foxp3⁺ Treg cells (d), Tr1 cells after a stimulation with an anti-CD3/CD28 antibody (e), and Breg cells after a stimulation with a TLR9 agonist (f) in PBMCs of patients ($N=7$) receiving sublingual immunotherapy (SLIT). PBMCs were isolated from heparinized blood. Each point represents values of respective patients. Each bar represents the mean. ** and *** $p < 0.01$ and 0.001



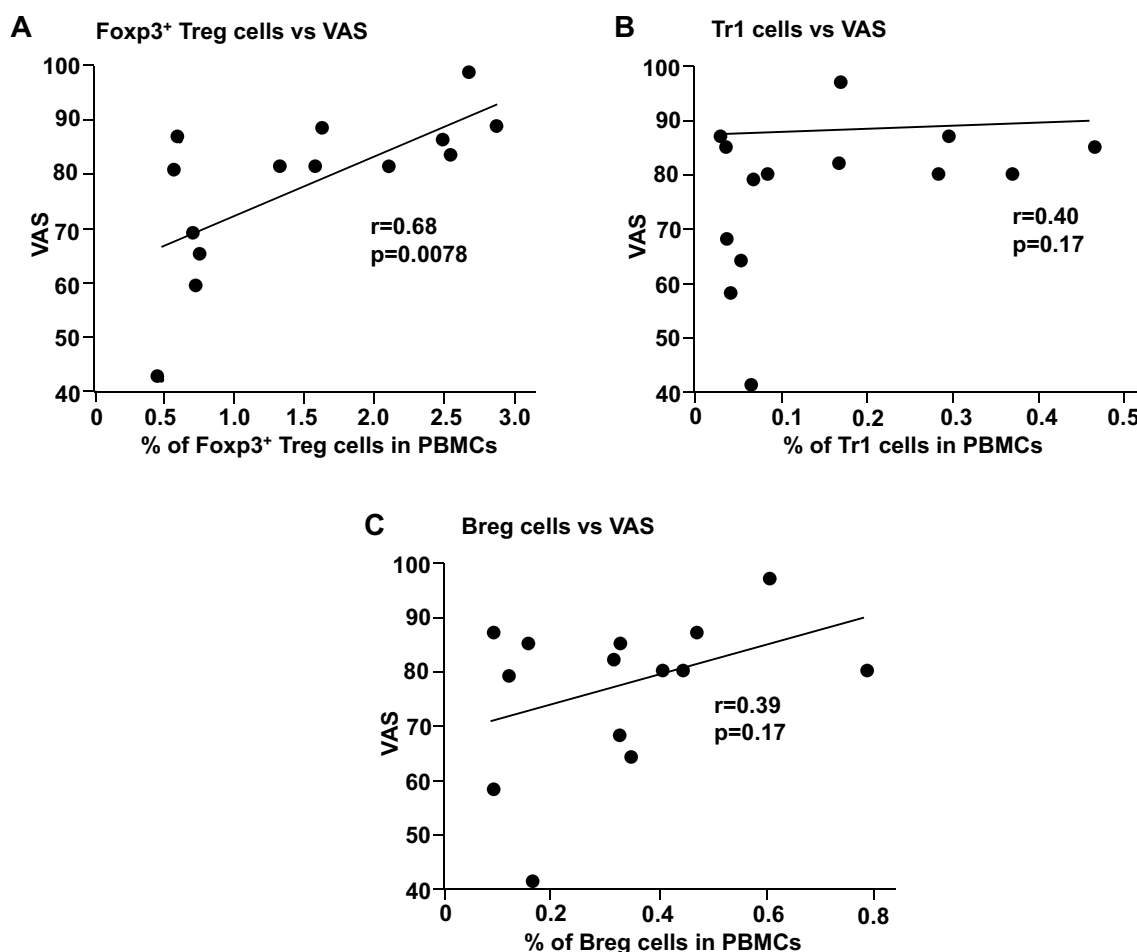


Fig. 5 Relationships between percentages of Foxp3⁺ Treg cells (a), Tr1 cells (b) and Breg cells (c) in peripheral mononuclear cells (PBMCs) and the visual analogue scale (VAS). Each point represents

values of respective patients. Each point represents values of respective patients in 2017 and 2019. The line represents a regression line

(Supplemental Fig. 2a,b, $p = 0.034$ and 0.038 , respectively). Moreover, the percentage and absolute number of Foxp3⁺ Treg cells positively correlated with the number of Breg cells (Fig. 6c and supplemental Fig. 2c, $p = 0.047$ and $p=0.034$). The percentage and absolute number of Tr1 cells was associated with the number of Breg cells (Fig. 6d and supplemental Fig. 2d).

Discussion

In the present study, (1) the clinical effects of SLIT on nasal symptoms in 2017 were significantly strengthened by an additional 2 years of treatment, (2) the numbers of Foxp3⁺ Treg cells, Tr1 cells, and Breg cells and allergen-specific IgG4 levels in peripheral blood were also increased by the additional 2 years of treatment, and (3) the number of Foxp3⁺ Treg cells correlated with clinical effects. These results suggest that more than 4 years of SLIT is needed

to significantly change immunological parameters, such as increases in Foxp3⁺ Treg cells, which were closely associated with the induction of the clinical effects of SLIT.

Marogna et al. [11] suggested that 4 years of SLIT is the optimal duration of treatment, because it induces long-lasting clinical improvements, similar to those observed with a 5-year course and greater than that with a 3-year vaccination. The present results demonstrated that the numbers of Foxp3⁺ Treg cells, Tr1 cells, and Breg cells as well as allergen-specific IgG4 levels were markedly increased by 4 or 5 years of SLIT. Therefore, the present results are consistent with previous findings [11] showing that a SLIT duration of more than 4 years is appropriate. In contrast, the results obtained in 2017 indicated that 2 or 3 years of SLIT was insufficient to induce the immunological changes associated with the suppression of clinical symptoms. These results will contribute to establishing the appropriate duration of SLIT. As for our study, the increase in allergen-specific IgG4 level was observed after 4 or 5 years of SLIT, but there was no

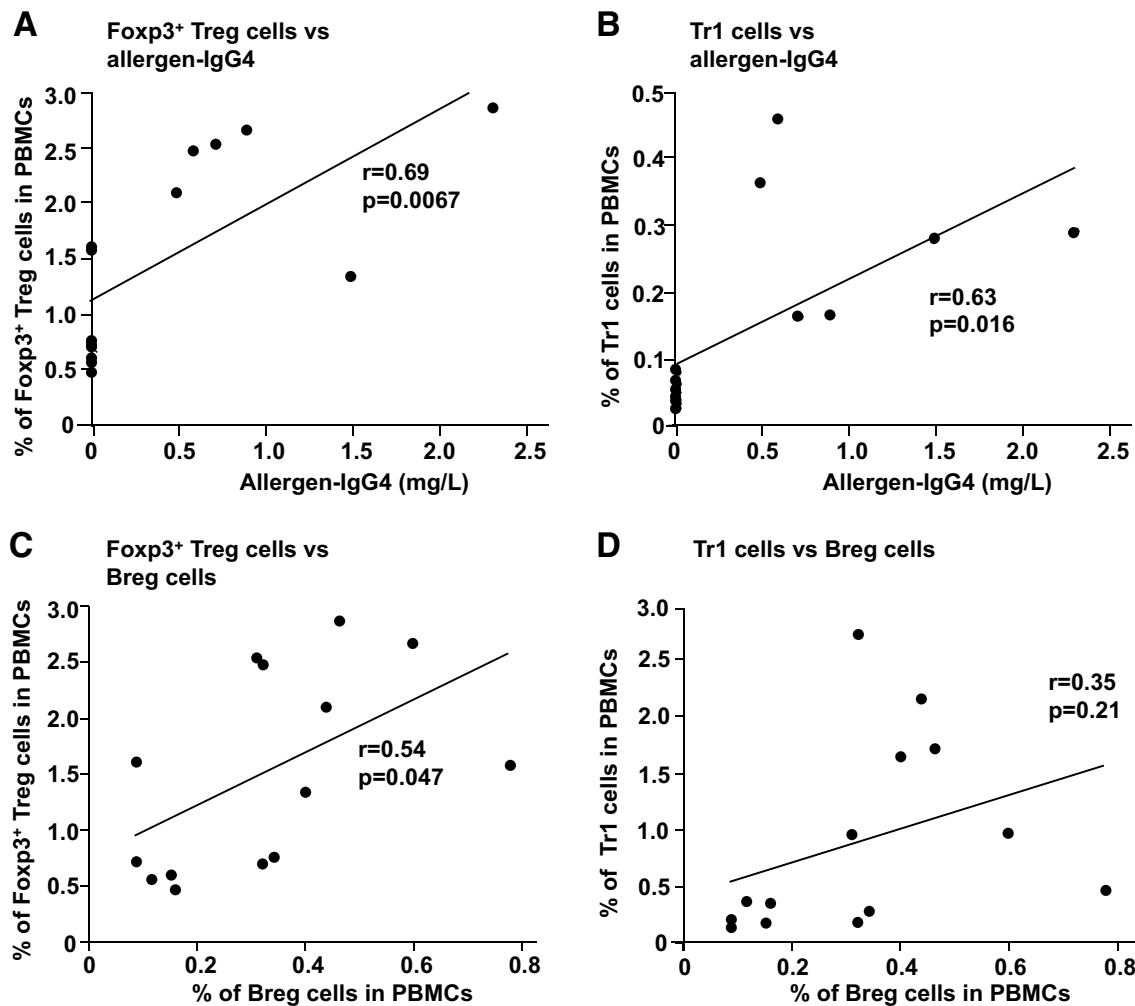


Fig. 6 Relationships between percentages of Foxp3⁺ Treg cells (a, c) and Tr1 cells (b, d) in peripheral mononuclear cells (PBMCs) and allergen-specific IgG4 (allergen-IgG4) or Breg cells. Each point represents values of respective patients in 2017 and 2019. The line represents a regression line

correlation between the IgG4 level and clinical efficacy (data not shown). Although previous literatures have suggested that allergen-specific IgG4 in peripheral blood has potential as a biomarker to predict the efficacy of SLIT [22–24], according to our findings together with those of others [25, 26], the level of allergen-specific IgG4 may be just an immunological phenomenon, not directly correlating with clinical effects.

Among the regulatory cells examined in the present study, only the number of Foxp3⁺ Treg cells correlated with clinical effects. Previous studies [27, 28] also reported that the number of Foxp3⁺ Treg cells in the peripheral blood of allergic patients was significantly increased by SLIT, and correlated with symptom medication scores. Kearley et al. [29] demonstrated in a murine study that the adoptive transfer of Foxp3⁺ Treg cells markedly suppressed airway

hyperresponsiveness in ovalbumin (OVA)-induced airway inflammation. Therefore, the present study confirmed that the suppressive roles of Foxp3⁺ Treg cells are important for the clinical effectiveness of SLIT.

The mechanisms underlying the induction of Foxp3⁺ Treg cells by SLIT currently remain unclear. Bianchini et al. [21] reported that allergen-IgG4 complexes induced Foxp3⁺ Treg cells via the generation of regulatory macrophages. Immune complexes consisting of antigens and IgG4 changed inflammatory M2a macrophages into regulatory M2b-like macrophages [21]. These macrophages abundantly produced Treg-induction factors, such as IL-10 and CCL1 [21]. Veen et al. [19] also demonstrated that IgG4 production was confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. In the present study, the number of Breg cells

and IgG4 levels correlated with the numbers of Foxp3⁺ Treg cells. Therefore, increases in IgG4-producing Breg cells are important for establishing a tolerogenic microenvironment via the induction of Foxp3⁺ Treg cells.

On the other hand, the present study revealed that not only the number of Foxp3⁺ Treg cells, but also that of Tr1 cells were markedly increased by SLIT. We previously showed that the number of Tr1 cells in PBMCs derived from SCIT-treated Japanese cedar pollinosis patients was significantly higher than in that from non-SCIT-treated patients [30]. Moreover, we established a murine allergic model to evaluate the effects of SCIT [31]. In the intratracheal OVA-induced asthma model of OVA + Al(OH)₃-sensitized BALB/c mice, OVA was subcutaneously injected for SCIT [31]. Similar to the findings obtained in a human study [30], the number of Tr1 cells was significantly increased in the lungs by SCIT [31]. Moreover, we previously demonstrated that Tr1 cells were induced by a culture of spleen cells isolated from OVA-sensitized mice with OVA, IL-21, IL-27, and TGF- β [32], and also that the adoptive transfer of the Tr1 cells induced effectively suppressed eosinophilic infiltration into the lungs and airway hyperresponsiveness [33]. These findings indicated that Tr1 cells contribute to the development of a tolerogenic environment. On the other hand, the percentage of Tr1 cells in PBMCs did not correlated with VAS in the present study. Although we did not examine differences in the types of Treg cells induced between SLIT and SCIT in murine allergy models, Foxp3⁺ Treg cells rather than Tr1 cells may have contributed to the clinical effects of SLIT for Japanese cedar pollinosis. This is supported by a prior study, in which SLIT induced the increase in Foxp3⁺ Treg cells associated with oral mucosal immune tolerance, leading to clinical improvements [34]. In contrast to SCIT, the administration route of allergens through the mucosal surface by SLIT may involve different immunological mechanisms for the induction of Treg cells.

In conclusion, the present results demonstrated that the numbers of Foxp3⁺ Treg cells, Tr1 cells, and Breg cells were markedly increased by SLIT within 4–5 years, and also that the number of Foxp3⁺ Treg cells correlated with VAS. Therefore, Foxp3⁺ Treg cells may be important for the efficacy of SLIT for Japanese cedar pollinosis. In addition, 4–5 years of SLIT appears to be appropriate immunotherapy for Japanese cedar pollinosis.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00011-021-01460-3>.

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with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Author's contributions Tetsuya Terada, Masaya Matsuda, Kazuyuki Kitatani, Ryo Kawata, and Takeshi Nabe were involved in the study design, data collection, data interpretation, data analysis, and writing the manuscript. Miki Inaba, Junpei Hamaguchi, Naoki Takekoto, Harumi Sakae, Kennosuke Hashimoto and Hayato Shimora were involved in data collection, data interpretation, and data analysis. Yusuke Kikuoka and Yuko Inaka were involved in data collection. Takeshi Nabe has full access to the data, and is responsible for the integrity of data and final decision to submit this manuscript. All authors have approved the final version of this manuscript for submission.

Declarations

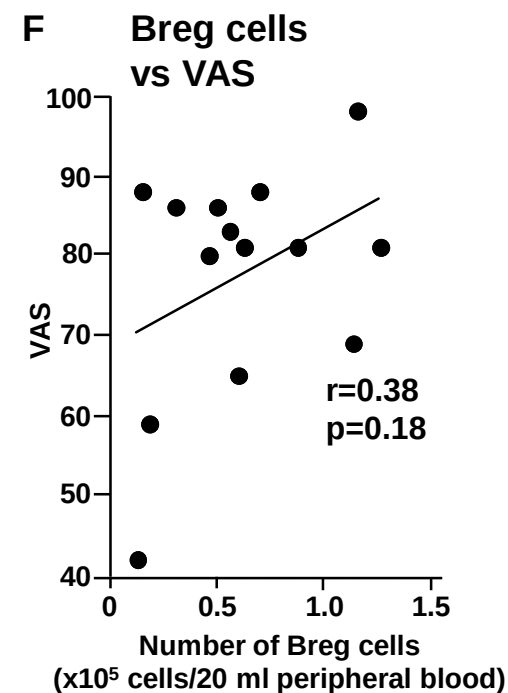
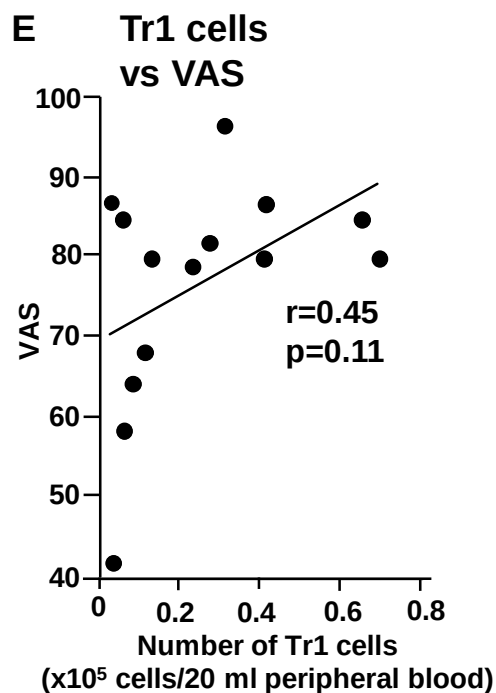
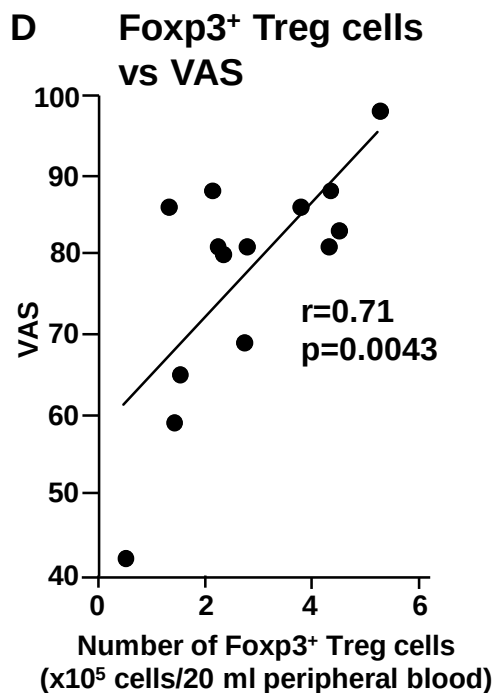
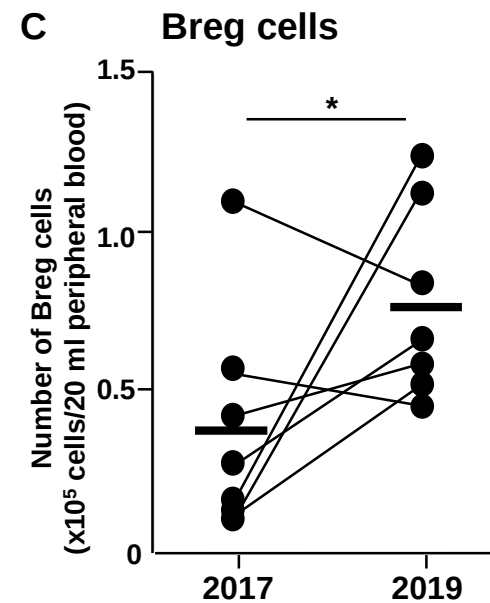
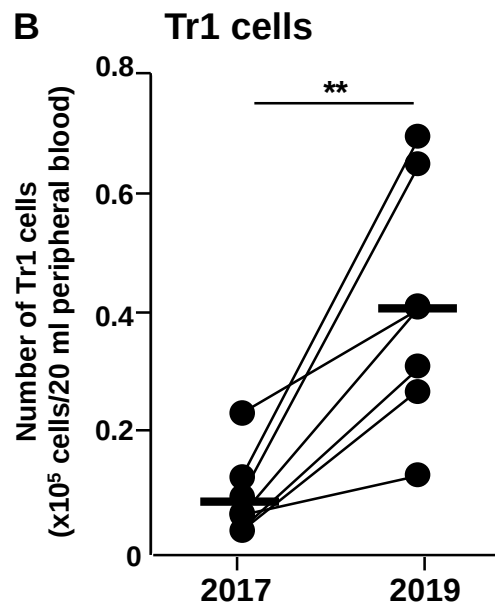
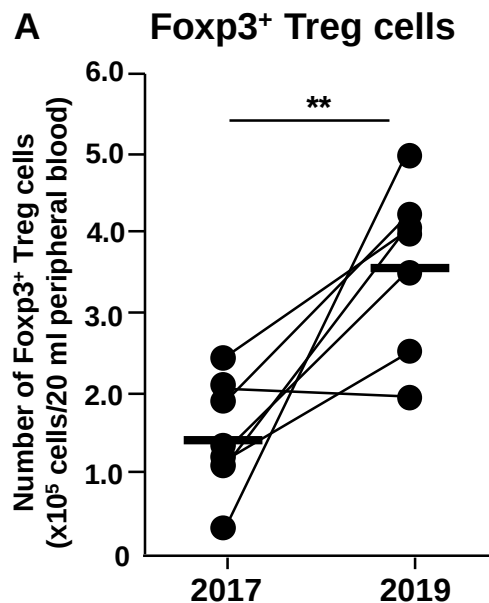
Conflict of interest All authors have no conflict of interests.

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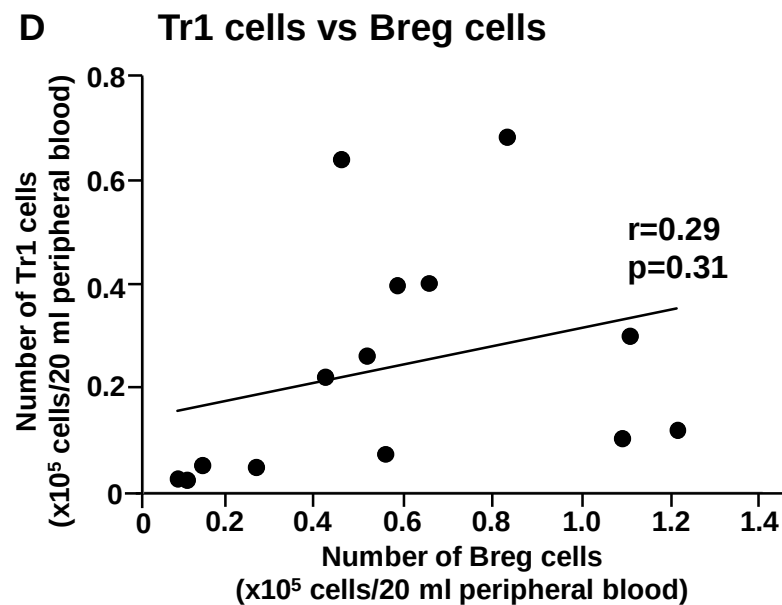
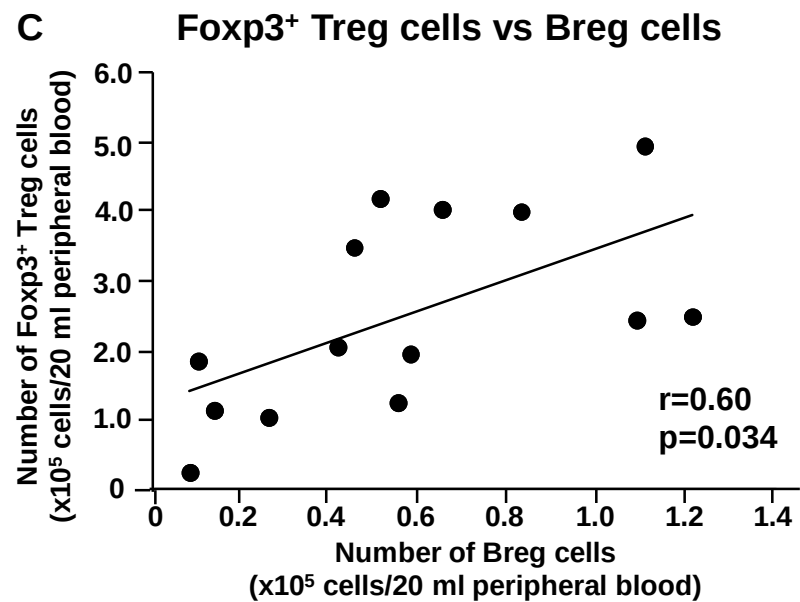
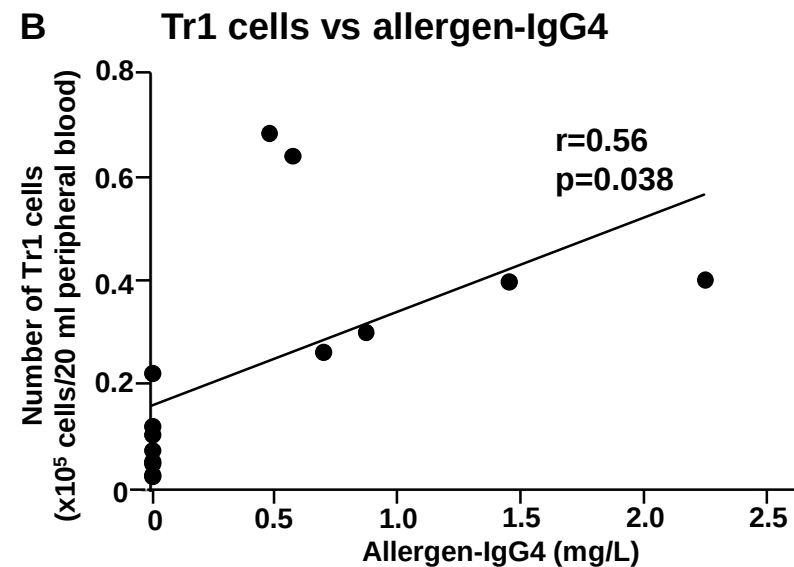
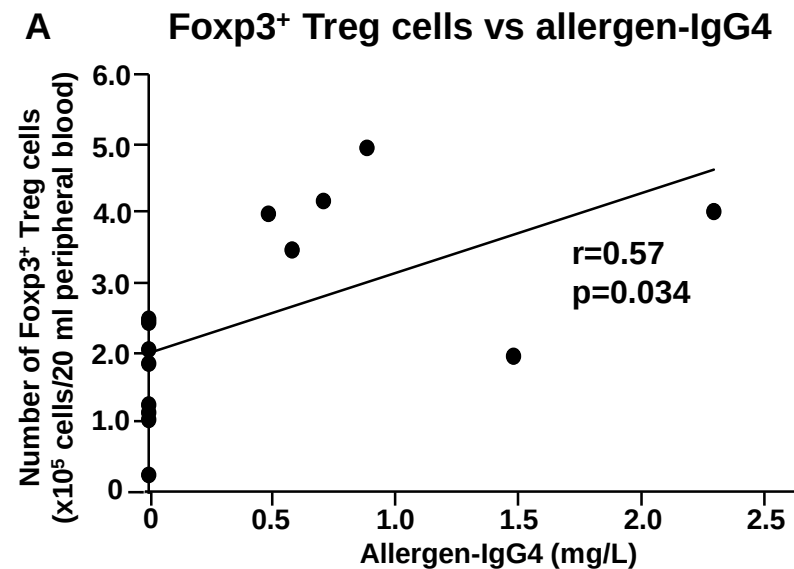
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Supplemental figure 1



Supplemental figure 2

Supplemental figure legends

Supplemental fig. 1 Changes in absolute numbers of Foxp3⁺ Treg cells (A), Tr1 cells (B), and Breg cells (C) in peripheral bloods of patients receiving sublingual immunotherapy (SLIT) between 2017 and 2019. Relationships between absolute numbers of Foxp3⁺ Treg cells (D), Tr1 cells (E) and Breg cells (F) in peripheral bloods and the visual analogue scale (VAS). Each point represents values of respective patients. Each point represents values of respective patients in 2017 and 2019. Each bar represents the mean. The line represents a regression line. * and **: $p < 0.05$ and 0.01 .

Supplemental fig. 2 Relationships between absolute numbers of Foxp3⁺ Treg cells (A, C) and Tr1 cells (B, D) in peripheral bloods and allergen-specific IgG4 (allergen-IgG4) or Breg cells. Each point represents values of respective patients. Each point represents values of respective patients in 2017 and 2019. The line represents a regression line.