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Regulatory T and B cells in peripheral blood of subcutaneous immunotherapy-treated Japanese cedar pollinosis patients

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Aim: The aim of this study was to clarify whether there are more regulatory T (Treg) and regulatory B (Breg) cells, and higher levels of IL-10-related transcription factors in subcutaneous immunotherapy (SCIT)-treated pollinosis patients than in non-SCIT-treated patients. **Methods:** Japanese cedar pollinosis patients undergoing SCIT had received treatment for at least 2.8 years. Peripheral blood mononuclear cells were used for flow cytometer analyses and mRNA measurement. **Results:** The numbers of type 1 regulatory T (Tr1)-like cells and Breg cells, and expression of *E4BP4* mRNA by peripheral blood mononuclear cells in SCIT-treated patients were higher than those in non-SCIT-treated patients. **Conclusion:** Tr1-like cells, Breg cells and *E4BP4* may be involved in the effectiveness of SCIT.

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Keywords allergies • cellular immunology • clinical immunology • immunological markers • immunotherapy

Allergen immunotherapy (AIT), such as subcutaneous immunotherapy (SCIT) and sublingual immunotherapy, using allergen extracts, has been reported to induce immune tolerance, and is used as curative therapy for allergic diseases [1–3]. Among these, SCIT has been frequently employed [4]. Japanese cedar pollen is the most common pollen allergen causing allergic rhinitis in Japan [5]. Japanese cedar pollen-induced allergic rhinitis is a typical seasonal allergy lasting approximately 3 months from February to April, during which time widely dispersed airborne cedar pollen causes allergic rhinitis and/or conjunctivitis in more than 30% of the Japanese population [6]. In clinical studies of SCIT for Japanese cedar pollen-induced allergic rhinitis, the efficacy and safety have been well demonstrated [7]. However, the mechanisms of SCIT for Japanese cedar pollinosis have not been fully elucidated.

More recently, it has become clear that the numbers of regulatory T (Treg) and regulatory B (Breg) cells are increased by AIT [8–12]. Treg cells are generally divided into two main groups: thymus-derived CD25⁺ forkhead box P3 (Foxp3)⁺ Treg cells [13] and peripherally derived Treg cells induced outside the thymus by antigenic stimulation. The thymus-derived CD25⁺ Foxp3⁺ CD4⁺ T cells are further separated into three groups: resting Treg (CD45RA⁺ Foxp3^{low} Treg), non-Treg (CD45RA⁺ Foxp3^{low} Treg) and effector Treg (CD45RA⁺ Foxp3^{high} Treg) cells. Among the three groups, effector Treg cells are terminally differentiated from resting Treg cells after antigen stimulation and become highly suppressive [14,15]. On the other hand, the peripherally derived Treg cells are classified into two subsets: peripherally induced Foxp3⁺ Treg cells [16] and type 1 regulatory T (Tr1) cells [17]. Tr1 cells secrete high levels of IL-10, but are negative for Foxp3 [18]. Although a significant correlation was reported between the improvement of symptoms and the increase in Tr1 cell numbers during immunotherapy for *Dermatophagoides pteronyssinus* (Der p)-sensitized children with allergic rhinitis [19], it is unclear whether the numbers of effector Treg cells and Tr1 cells are increased by AIT for Japanese cedar pollinosis. In contrast, it is well known that serum antigen-specific IgG4 levels are increased in AIT-treated patients [20]. IgG4 has been characterized as a blocking antibody that competes with IgE for antigen binding [21]. Veen *et al.* found that human Breg cells produced a

Table 1. Subject characteristics.			
Factor	Healthy subjects	Non-SCIT-treated patients	SCIT-treated patients
N	10	10	15
Sex (male/female)	3/7	4/6	4/11
Age (years)	46 (21–56)	46 (21–57)	55 (33–80)
Treatment period (years)	–	–	5.8 (2.8–11.6)
Data are presented as medians (range). SCIT: Subcutaneous immunotherapy.			

large amount of IgG4 [11]. Breg cells may also play key roles in the induction of immune tolerance to allergens by suppressing allergen-mediated inflammation via IL-10 [11,21]. The frequency of antigen-specific Breg cells was increased by AIT in allergic patients [11]. However, there have been few studies regarding the roles of Breg cells in AIT compared with those of Treg cells.

IL-10 is an anti-inflammatory cytokine required for preventing inflammation that is produced from not only Treg cells and Breg cells but also from monocytes [22]. Indeed, it was previously demonstrated that CD14⁺ monocytes in human peripheral blood mononuclear cells (PBMCs) were capable of IL-10 synthesis [23]. Yamanaka *et al.* reported that the number of IL-10-producing monocytes in PBMCs was significantly increased during and after AIT [24]. However, further studies are needed to clarify the roles of IL-10-producing monocytes in AIT, including whether monocytes are increased by AIT.

The anti-inflammatory activity of Tr1 cells was found to be mediated via the production of IL-10 [25]. In addition to Foxp3, avian musculoaponeurotic fibrosarcoma oncogene homolog (Maf), early growth response 2 (Egr-2) and E4-binding protein 4 (E4BP4) were reported to be involved in the expression of IL-10 in CD4⁺ T cells [26–29]. As biomarkers for the effectiveness of AIT, detection of mRNA by real-time reverse transcription polymerase chain reaction (RT-PCR) is more convenient than detection of live cells. However, to our knowledge, whether mRNA expression of these IL-10-related transcription factors is affected by AIT has not been examined.

In this study, we comprehensively assessed whether the numbers of subsets of Treg cells, Breg cells and IL-10-producing monocytes, as well as expression levels of the IL-10-related transcription factors *Foxp3*, *Maf*, *Egr-2* and *E4BP4* were higher in SCIT-treated patients than in non-SCIT-treated Japanese cedar pollinosis patients.

Methods

Patient characteristics

This 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; the approval numbers are RIN-37 (1568-01) and 2015-032, respectively. The patient characteristics are summarized in Table 1. 10 healthy subjects, ten Japanese cedar pollinosis patients (non-SCIT-treated patients) and 15 patients receiving SCIT were recruited in May 2017. Healthy subjects were asymptomatic and were negative on the nasal provocation test described below. Although five of ten healthy subjects had an allergen-specific IgE level > 0.1 IUA/ml, they were asymptomatic and negative on the nasal provocation test. Pollinosis patients (non-SCIT-treated patients) were all symptomatic with a clear history of seasonal rhinitis symptoms, and had an allergen-specific IgE level > 0.1 IUA/ml or were positive on the nasal provocation test.

SCIT-treated patients were initially treated subcutaneously with 0.02 ml of 20 JAU/ml of Japanese cedar pollen extract (Torii Pharm. Co. Ltd, Tokyo, Japan). The dose was increased by 1.5- or 2-fold every week. Then, doses were further increased to a maintenance dose of 0.2 ml of 2000 JAU/ml once a month. Patients undergoing SCIT had received treatment for at least 2.8 years. Subjective symptoms in all SCIT-treated patients were alleviated during the pollen season in 2017.

The nasal antigen provocation test was performed to evaluate sensitivity to a specific antigen. All participants underwent the nasal provocation test by placing a 2 × 2-mm filter paper containing Japanese cedar pollen extract on the inferior turbinate mucosa for 5 min in accordance with the practical guidelines for the management of allergic rhinitis in Japan (PG-MARJ) [30]. From 24 h before the test, the subjects were not allowed to take any antiallergy drugs. 5 min after removal of the filter, allergic nasal symptoms comprising sneezing or nose itching, aqueous rhinorrhea, or swelling and pallor of the inferior turbinate mucosa were evaluated (–: none, ±: 1 symptom,

Table 2. Nasal provocation test.

	Healthy subjects	Non-SCIT-treated patients	SCIT-treated patients [†]	p-value
Negative	10	3	11	0.022
Positive	0	7	3	

Categorical variables for non-SCIT-treated patients and SCIT-treated patients were compared using Fisher's exact test. The p-value represents comparison of effectiveness between non-SCIT-treated patients and SCIT-treated patients, and was regarded as significant.

[†] One of the 15 patients refused to undergo the nasal provocation test.

SCIT: Subcutaneous immunotherapy.

1+: 2 symptoms, 2+: 3 symptoms and 3+: 3 symptoms and sneezing more than six times). Scores of – or ± were considered to be negative for Japanese cedar pollen allergy, and scores of 1+, 2+ or 3+ were considered positive.

All healthy subjects were negative on the test (Table 2). Although seven of ten patients without SCIT treatment were positive, only 3 of 14 SCIT-treated patients were positive (Table 2). The difference between non-SCIT-treated patients and SCIT-treated patients was significant at $p = 0.022$ (Table 2).

Measurement of allergen-specific IgE, IgG & IgG4 in the serum

Amounts of total IgE and allergen-specific IgE, IgG and IgG4 in the 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).

FACS analyses

PBMCs were isolated from heparinized blood by centrifugation over Histopaque®-1077 (Sigma–Aldrich, MO, USA). The obtained PBMCs were suspended in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, 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 1×10^6 cells/ml.

For analyses of IL-10 producing cells, the PBMCs were incubated with phosphate-buffered saline (PBS), Cry j 1 (10^{-5} g/ml, Hayashibara, Okayama, Japan), oligonucleotide (ODN) 2006 (Toll-like receptor 9 [TLR9] agonist, 1 µM, InvivoGen, CA, USA), lipopolysaccharides (LPS, *Salmonella Minnesota* strain Re 595, 100 ng/ml, Sigma–Aldrich), 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, CA, USA). For the detection of CD25⁺ Foxp3⁺ CD4⁺ T cells and CD45RA[–] Foxp3^{high} CD4⁺ T cells, the PBMCs were not treated by the above-mentioned culture conditions.

To exclude dead cells, the cells were stained with eFluor® 780-conjugated Fixable Viability Dye (Thermo Fisher Scientific). After washing with 2% FBS-containing PBS, the cells were treated with Human BD Fc Block™ (BD Biosciences, CA, USA) to reduce potential nonspecific antibody binding, followed by staining with fluorescein isothiocyanate-conjugated antihuman CD3 (HIT3a), phycoerythrin (PE)-conjugated antihuman CD14 (M5E2), PE-conjugated antihuman CD19 (HIB19), peridinin chlorophyll protein (PerCP)-conjugated antihuman CD4 (OKT4), phycoerythrin-cyanin 7 (PE/Cy7)-conjugated antihuman CD45RA (HI100; all from BioLegend, CA, USA), and/or allophycocyanin-conjugated antihuman CD25 (BC96) (Thermo Fisher Scientific) at 4°C for 20 min. After staining the cell surface, the cells were fixed and permeabilized by fixation/permeabilization concentrate (Thermo Fisher Scientific) at room temperature for 1 h. After fixation and permeabilization, the cells were washed with permeabilization buffer (Thermo Fisher Scientific), followed by staining with allophycocyanin-conjugated antihuman IL-10 (JES3-9D7; all from BioLegend) and/or PE-conjugated antihuman Foxp3 (236A/E7) (Thermo Fisher Scientific) at room temperature for 1 h. After washing with 2% FBS-containing PBS, the cells were analyzed using FACS Aria™ Fusion (Becton Dickinson, CA, USA).

Measurement of mRNA expression in PBMCs

The isolated PBMCs were suspended in 0.5 ml of RNAiso Plus® (Takara, Ohtsu, Japan). Total RNA was extracted by isopropyl alcohol precipitation after extraction with chloroform. Reverse transcription was performed using ReverTra Ace® (Toyobo, Osaka, Japan) in the presence of dNTP Mix (Promega, Tokyo, Japan), random primers (Promega), and RNase inhibitor at 30°C for 10 min, 42°C for 40 min and 99°C for 5 min. Quantitative PCR was performed with Fast SYBR™ Green Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions with the StepOne™ Real-Time PCR System (Thermo Fisher Scientific). Transcript amounts were

Table 3. Analyses of antibody production in sera.			
	Healthy subjects	Non-SCIT-treated patients	SCIT-treated patients
Total IgE (IU/ml)	55 (12–251)	133 (57–264)	76 (7–515)
Ag-specific IgE (IUA/ml)	1.4 (0–41.3)*	26.9 (0–57.8)	8.8 (0.89–41.0)
Ag-specific IgG (mg/l)	7.3 (4.0–14.5)	8.1 (4.0–13.7)	36.7 (7.3–134.0)***
Ag-specific IgG4 (mg/l)	0	0	4.1 (0.3–50.0)***
The amounts of total IgE, allergen (Ag)-specific IgE, Ag-specific IgG and Ag-specific IgG4 in sera of healthy subjects (N = 10), non-SCIT-treated patients (N = 10) and SCIT-treated patients (N = 15) were measured by chemiluminescent enzyme immunoassay. Data are presented as medians (range). *p < 0.05 and ***p < 0.001 vs non-SCIT-treated patients. SCIT: Subcutaneous immunotherapy.			

normalized to that of *GAPDH*. Primers used for this reaction were as follows: *GAPDH*, *glyceraldehyde-3-phosphate dehydrogenase*, 5'-TGTTTCGTCATGGGTGTGAAC-3' and 5'-ACTGTGGTCATGAGTCCTTCC-3'; *Foxp3*, 5'-CACCTGGCTGGGAAAATGG-3' and 5'-GGAGCCCTTGTCGGATGAT-3'; *Maf*, 5'-TGGCATCAGAACTGGCAATG-3' and 5'-TCCACCGGTTCTTTTTTCAC-3'; *Egr-2*, 5'-CCGGAGATGGCATGATCAAC-3' and 5'-AGGGTCAATGGAGAACTTGC-3'; *E4BP4*, 5'-TTCTGCATGTCTGGAGGAAAC-3' and 5'-ATTCAGTCGACGCTTCTCAC-3'. The mixture was heated at 95°C for 20 s to activate Fast SYBR Green Master Mix. Denaturation (95°C, 3 s), annealing, and extension (60°C, 30 s) were repeated for 40 cycles. The melting curve of each primer set was then acquired by increasing the temperature from 60 to 95°C using the StepOne software version 2.3 (Thermo Fisher Scientific).

Statistical analyses

Statistical analysis for two related samples was performed using the Wilcoxon test. For two nonrelated samples, the Mann–Whitney test was conducted. To compare multiple groups, Steel’s multiple comparison test was performed. Categorical variables were compared using Fisher’s exact test. A p-value less than 0.05 was considered significant. Statistical analyses were performed with JMP® Pro (Version 11.2.0, SAS Institute Japan, Tokyo, Japan).

Results

Antibodies

As shown in Table 3, there was no significant difference in the total serum IgE level among the healthy subjects, non-SCIT-treated patients and SCIT-treated patients. On the other hand, the serum allergen-specific IgE level of non-SCIT-treated patients was higher than that of the healthy subjects. The allergen-specific IgE level of the SCIT-treated patients was slightly lower than that of the non-SCIT-treated patients (Table 3), but both allergen-specific IgG and IgG4 levels of SCIT-treated patients were significantly higher than those of non-SCIT-treated patients (Table 3).

Treg cells

As shown in Figure 1, Foxp3⁺ Treg cells were analyzed as CD25⁺ Foxp3⁺ CD4⁺ T cells (CD25⁺ Foxp3⁺ Treg cells) and CD45RA[−] Foxp3^{high} CD4⁺ T cells (effector Treg cells), respectively. Almost all of the effector Treg cells were positive for CD25 (data not shown). There was no significant difference in the numbers of CD25⁺ Foxp3⁺ Treg cells and effector Treg cells in PBMCs among the three groups (Figure 1B & D).

Foxp3 is constitutively expressed by effector Treg cells even without stimulation, whereas Tr1 cells are known as Foxp3[−] cells producing a large amount of IL-10 under stimulation [25]. Thus, Tr1-like cells were analyzed as Foxp3[−] CD4⁺ T cells that produced IL-10 in response to Cry j 1 or anti-CD3/CD28 antibody. As shown in Figure 2B, under stimulation with Cry j 1, the numbers of Tr1-like cells in PBMCs were not significantly different among the three groups. However, under stimulation with anti-CD3/CD28 antibody, the number of Tr1-like cells in non-SCIT-treated patients was lower than that in healthy subjects (Figure 2C). Of note, in SCIT-treated patients, the number of Tr1 cells was significantly higher than that in non-SCIT-treated patients (Figure 2C).

Breg cells

Breg cells in PBMCs were considered to be CD19⁺ cells that produced IL-10 in response to Cry j 1 or the TLR9 agonist ODN2006 (Figure 2D). Irrespective of stimuli, the number of Breg cells in non-SCIT-treated patients was significantly lower than that in healthy subjects (Figure 2E). Moreover, there were more Breg cells in SCIT-treated patients than in non-SCIT-treated patients (Figure 2E & F).

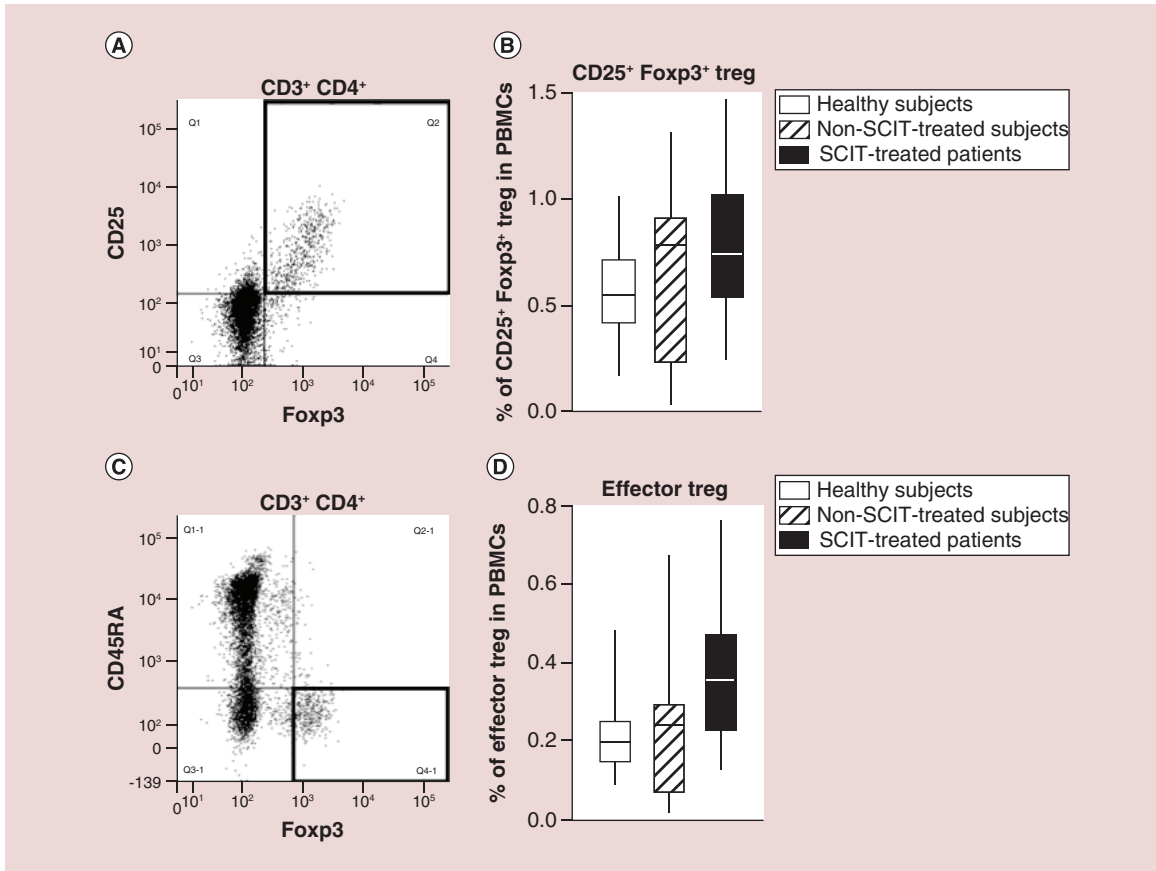


Figure 1. Analyses of Foxp3⁺ regulatory T (Treg) cells. FACS profiles of CD25⁺ Foxp3⁺ regulatory T (Treg) cells (A) and CD45RA⁺ Foxp3^{high} Treg, effector Treg cells (C), and box plot analyses of the numbers of CD25⁺ Foxp3⁺ Treg cells (B) and effector Treg cells (D) in PBMCs of healthy subjects (N = 10), non-SCIT-treated patients (N = 10) and SCIT-treated patients (N = 15). PBMCs were isolated from heparinized blood. PBMC: Peripheral blood mononuclear cell; SCIT: Subcutaneous immunotherapy.

IL-10-producing monocytes

IL-10-producing monocytes in PBMCs were analyzed following stimulation with Cry j 1 or lipopolysaccharides (Figure 2G). After stimulation, there was no difference in the number of IL-10-producing monocytes among the three groups (Figure 2H & I).

IL-10-related transcription factors

mRNA levels of four IL-10-related transcription factors, *Foxp3*, *Maf*, *Egr-2* and *E4BP4* in PBMCs were assessed. The expression of *Foxp3*, *Maf* and *Egr-2* in PBMCs of SCIT-treated patients was higher than that in non-SCIT-treated patients, but the differences were not significant (Table 4). On the other hand, the *E4BP4* mRNA level was significantly higher in SCIT-treated patients than in non-SCIT-treated patients (Table 4).

Discussion

In this study, there was no significant difference in the number of CD25⁺ Foxp3⁺ Treg cells in PBMCs among healthy subjects, non-SCIT-treated-patients and SCIT-treated patients. The number of effector Treg cells (CD45RA⁺ Foxp3^{high} Treg cells) in SCIT-treated patients was also not larger than that in non-SCIT-treated patients. To date, studies reported that the number of Foxp3⁺ Treg cells was increased by SCIT [31,32], whereas other studies have yielded controversial results [33,34]. In our present study as well as in these reports [31–34], the Foxp3⁺ Treg cells were analyzed in PBMCs. As it has been reported that effector Treg cells have highly suppressive function, future experiments are needed to analyze whether the Foxp3⁺ Treg cells are functionally altered by SCIT.

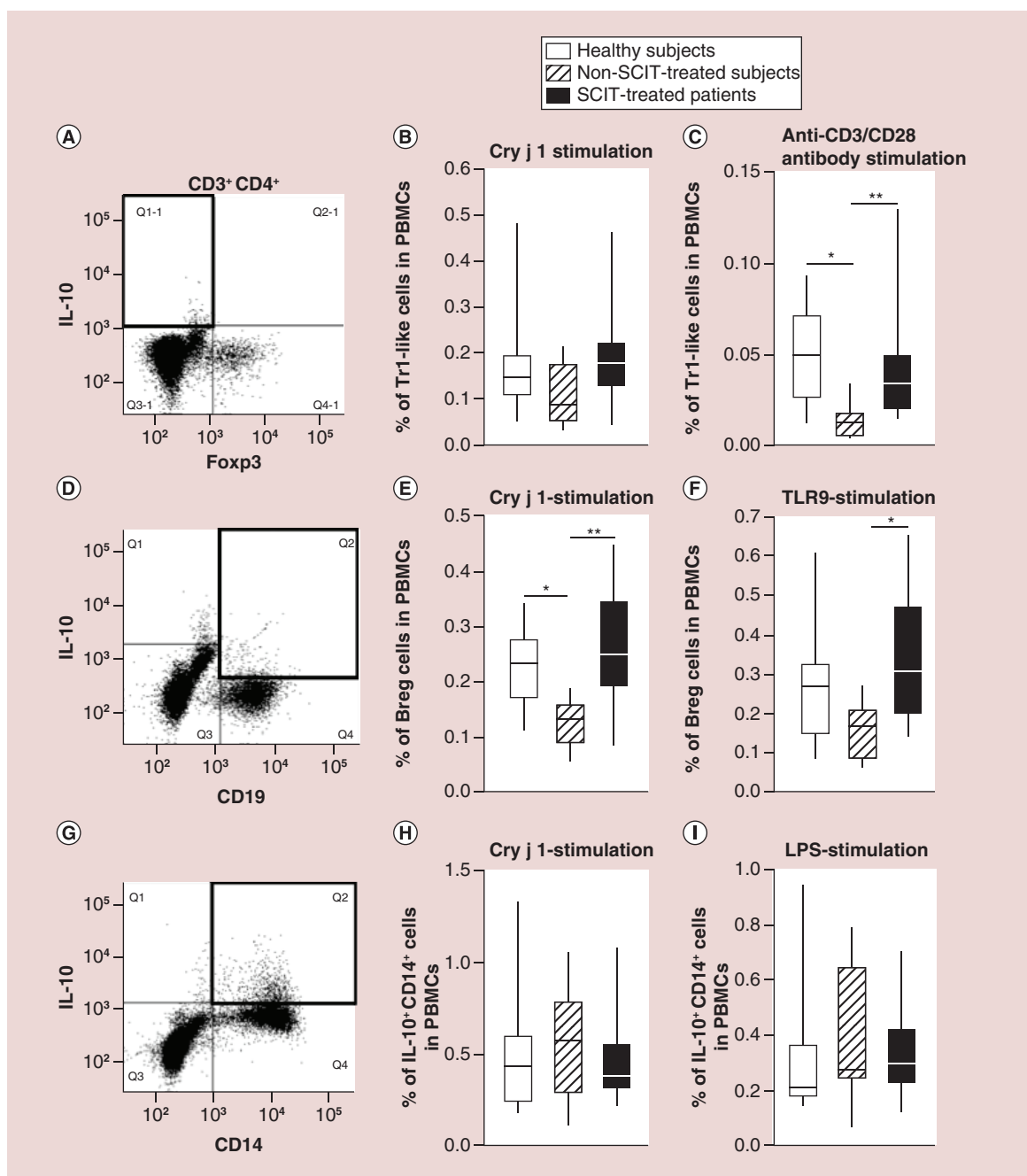


Figure 2. Analyses of IL-10-producing cells. FACS profiles of IL-10-producing forkhead box P3 (Foxp3)⁺ CD4⁺ T cells (A), IL-10-producing B cells (D), and IL-10-producing monocytes (G). Box plot analyses of the numbers of IL-10-producing Foxp3⁺ CD4⁺ T cells after stimulation with Cry j 1 (B) or anti-CD3/CD28 antibody (C), numbers of IL-10-producing B cells after stimulation with Cry j 1 (E) or TLR9 agonist (F), and numbers of IL-10-producing monocytes after stimulation with Cry j 1 (H) or LPS (I) in PBMCs of healthy subjects (N = 10), non-SCIT-treated patients (N = 10) and SCIT-treated patients (N = 15). PBMCs were isolated from heparinized blood, followed by stimulation with Cry j 1, anti-CD3/CD28 antibody, TLR9 agonist or LPS. *p < 0.05 and **p < 0.01. LPS: Lipopolysaccharide; PBMC: Peripheral blood mononuclear cell; SCIT: Subcutaneous immunotherapy; TLR9: Toll-like receptor 9.

Table 4. Analyses of mRNA levels of IL-10-related transcription factors.

	Ratio of mRNA levels		
	Healthy subjects	Non-SCIT-treated patients	SCIT-treated patients
<i>Foxp3</i>	0.0090 (0.0041–0.054)	0.012 (0.007–0.027)	0.019 (0.0017–0.241)
<i>Egr-2</i>	0.013 (0.0010–0.44)	0.0077 (0.0010–0.12)	0.041 (0.0012–3.7)
<i>Maf</i>	0.16 (0.072–0.50)	0.12 (0.016–0.41)	0.24 (0.030–6.6)
<i>E4BP4</i>	0.079 (0.033–0.86)	0.068 (0.0061–0.64)	0.50 (0.046–3.2) [†]

The mRNA levels of the IL-10-related transcription factors *Foxp3*, *Egr-2*, *Maf* and *E4BP4* in PBMCs of healthy subjects (N = 10), non-SCIT-treated patients (N = 10) and SCIT-treated patients (N = 15) were measured by real-time reverse transcription polymerase chain reaction (RT-PCR). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood. Each value represents transcript amounts normalized to that of *GAPDH*. Data are presented as medians (range).

[†]p < 0.05 vs non-SCIT-treated patients.

In contrast, the number of IL-10-producing Foxp3⁺ T cells, Tr1-like cells, was significantly higher in SCIT-treated patients than in non-SCIT-treated patients, suggesting that suppressive roles of Tr1-like cells are important for the clinical effectiveness of SCIT. Of note, even in healthy subjects, Tr1-like cells were able to be stimulated by the specific antigen, Cry j 1, to produce IL-10 (Supplementary Table 1). These results suggest that antigen-responsive Tr1-like cells exist even in healthy subjects, and may exert regulatory functions in asymptomatic conditions. Consistent with this, Akdis *et al.* also reported that antigen-responsive Tr1-like cells exist in healthy subjects [35]. Furthermore, Tr1-like cells may be a candidate biomarker to predict the efficacy of SCIT.

Recently, we successfully developed a murine airway allergic model to assess the effects of SCIT [36]. Ovalbumin (OVA) + Al(OH)₃-sensitized BALB/c mice received subcutaneous dosages of OVA at 1 mg/animal for SCIT, and then were intratracheally challenged with OVA at 5 µg/animal [37]. Similar to the present human study, the number of Tr1-like cells was significantly increased in the lungs by SCIT, whereas that of Foxp3⁺ Treg cells was not increased in this model. Moreover, by other previous experiments, we demonstrated that Tr1-like cells can be induced by 7-day culture of spleen cells isolated from OVA-sensitized mice with OVA and several cytokines, and that adoptive transfer of the induced Tr1-like cells effectively suppressed eosinophilic airway inflammation and airway epithelial mucus plugging [37]. These findings indicate that Tr1-like cells exert anti-inflammatory functions *in vivo*. Our present results in human studies suggest that suppressive roles of Tr1-like cells are important for the clinical effectiveness of SCIT for Japanese cedar pollinosis.

On the other hand, there were significantly more IL-10 producing B cells, Breg cells, in SCIT-treated patients than in non-SCIT-treated patients. The IL-10 production by B cells in each group was not significantly increased by Cry j 1 or the TLR9 agonist (ODN2006) (Supplementary Table 2), whereas our data suggested that the constitutive IL-10 production by B cells was augmented by SCIT. Breg cells can inhibit the activation of antigen-presenting cells by secreting IL-10 and promoting Treg cell expansion [21]. Veen *et al.* found that human IL-10-producing B cells produced a large amount of IgG4, and were designated as B regulatory 1 (Br1) cells [11]. In this study, the allergen-specific IgG4 level was markedly increased in the serum of SCIT-treated patients. Therefore, the increase in Breg cells observed in this study may be closely related to the marked elevation of allergen-specific IgG4 in the serum. Moreover, the number of Breg cells in healthy subjects was significantly higher than that in non-SCIT-treated patients. These data imply that Breg cells in healthy subjects exert regulatory functions, resulting in an asymptomatic condition even though sera of some of the healthy subjects were positive for allergen-specific IgE.

Expression of IL-10 in CD4⁺ T cells is mediated by the transcription factors Foxp3, Maf, Egr-2 and E4BP4 [26–29]. In this study, among the four factors, only *E4BP4* mRNA expression in SCIT-treated patients was significantly higher than that in non-SCIT-treated patients. Consistent with the present result, Motomura *et al.* demonstrated that the expression of IL-10 was upregulated in chronically antigen-stimulated CD4⁺ T cells via increased expression of *E4BP4* mRNA in murine studies [29]. To our knowledge, the present study is the first to report that the expression of *E4BP4* mRNA was increased by AIT in humans. In this study, significant differences in IL-10 production between SCIT-treated patients and nontreated patients were noted in the numbers of Tr1-like cells and Breg cells. Thus, the high expression of *E4BP4* may be associated with the increases in numbers of the IL-10-producing cells in SCIT-treated patients.

As reviewed by Shamji *et al.* [38], the clinical management of patients receiving AIT may be enhanced by predictive biomarkers. At present, the use of allergen-specific IgG4 as a biomarker is recommended. This study suggested that the Tr1-like cells and Breg cells can be also cellular biomarkers to predict effectiveness. However, it is difficult to standardize the methods for detection of the cells. In addition, *E4BP4* may be a candidate molecular biomarker

other than allergen-specific IgG4. The detection of *E4BP4* mRNA by RT-PCR can be relatively easily standardized. However, there were variations among SCIT-treated patients in the level of *E4BP4* mRNA. Thus, further studies are required to confirm that the increases in Tr1-like cells, Breg cells and *E4BP4* are associated with the clinical effectiveness of AIT.

Conclusion

The numbers of Tr1-like cells and Breg cells, and expression of *E4BP4* in SCIT-treated patients were higher than those in non-SCIT-treated patients, suggesting that induction of these IL-10-producing cells is associated with the immune tolerance to the antigen. In addition, Tr1-like cells, Breg cells and *E4BP4* may be candidates for biomarkers to predict the efficacy of SCIT for Japanese cedar pollinosis.

Summary points

- The number of Tr1-like cells (IL-10⁺ Foxp3⁺ CD4⁺ T cells) in peripheral blood mononuclear cells (PBMCs) of subcutaneous immunotherapy (SCIT)-treated patients was significantly larger than that in the non-SCIT-treated patients.
- The number of IL-10-producing B cells (Breg cells) in PBMCs of SCIT-treated patients was also markedly larger than that in non-SCIT-treated patients.
- The expression of *E4BP4*, a transcription factor for IL-10 production, by PBMCs of SCIT-treated patients was significantly higher than that in non-SCIT-treated patients.

Author contributions

Masaya Matsuda, Tetsuya Terada, Ryo Kawata, and Takeshi Nabe were involved in the study design, data collection, data interpretation, data analysis, and writing the manuscript. Nau Tsujimoto, Yuki Morie, Tomoki Ishida, Hiromu Takahashi, Junpei Hamaguchi, Yuki Tabuchi, and Kana Doi were involved in data collection, data interpretation, and data analysis. Keiki Noro, Yusuke Kikuoka, Shuji Omura, Takuya Yoshida, Yusuke Ayani, Manabu Suzuki, Takahiro Ichihara, Yuko Inaka, and Takaki Inui were involved 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.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Informed consent disclosure

The authors state that they have obtained verbal and written informed consent from the patient/patients for the inclusion of their medical and treatment history within this case report.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/imt-2018-0170

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