

The Induced Regulatory T Cell Level, Defined as the Proportion of IL-10⁺Foxp3⁺ Cells among CD25⁺CD4⁺ Leukocytes, Is a Potential Therapeutic Biomarker for Sublingual Immunotherapy: A Preliminary Report

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Key Words

Allergic rhinitis · Biomarker · Foxp3 · Immunotherapy · Interleukin-10 · Japanese cedar · Pollinosis · Regulatory T cell · Sublingual immunotherapy

Abstract

Background: Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most prevalent allergies in Japan. Recently, two reports described the positive effects of sublingual immunotherapy (SLIT) against Japanese cedar pollinosis. However, the therapeutic biomarkers for SLIT are still unclear. We performed this unblinded, nonrandomized, open-label study to identify therapeutic biomarkers for SLIT against Japanese cedar pollinosis. **Methods:** We performed an open-label study during one pollinosis season in 2007, enrolling 19 patients from in-house volunteers suffering from Japanese cedar pollinosis. Peripheral blood was obtained from all participants before SLIT treatment as well as before and after the pollen season. The plasma levels of an immunoglobulin

specific to a major allergen (Cry j 1) were determined. We analyzed the induction of regulatory T cells (iTregs), namely IL-10⁺Foxp3⁺ cells in CD25⁺CD4⁺ leukocytes, by flow cytometry. The Th2-type responses were analyzed by cytokine production from peripheral blood mononuclear cells after stimulation with Cry j 1. Clinical symptoms were estimated using a quality of life questionnaire in the middle of the pollen season. **Results:** The difference in numbers of iTregs between the medium-only control cell culture and cells stimulated with Cry j 1 was significantly decreased in the non-SLIT group but was unchanged in the SLIT group after the pollen season. The subgroup of the SLIT group with increased iTregs showed more attenuated Th2-type cytokine profiles, and symptom scores in the subgroup with increased iTregs were significantly lower than those in the subgroup with decreased iTregs. **Conclusion:** The antigen-specific iTreg level is a potential therapeutic biomarker that correlates with clinical pollinosis symptoms and may be involved in the therapeutic mechanisms of SLIT.

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Introduction

Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most prevalent allergies in Japan; a nationwide survey in 2008 found a prevalence of 26.5% [1]. Compared with the estimate of 13.1% in 2001, this more recent figure implies that the number of affected patients is rapidly increasing [2].

Recently, two reports have described the positive clinical effects of sublingual immunotherapy (SLIT) against Japanese cedar pollinosis [3, 4]. A randomized double-blind study reported that efficacy variable scores in an active treatment group were significantly lower than those in a placebo group, and the quality of life (QOL) symptom score of the active group was almost half that of the placebo group [3]. We also previously reported that active treatment significantly ameliorated symptom scores and symptom-medication scores compared with placebo in a randomized controlled trial. Furthermore, we reported that SLIT decreased the number of Th2 clones specific to CS712, namely recombinant protein-conjugated T cell epitopes from Cry j 1 and Cry j 2 [4, 5].

Several reports have suggested the involvement of Foxp3-positive regulatory T cells (Tregs) in the therapeutic mechanisms of immunotherapy. It was reported that the number of Tregs, namely CD25^{bright} and/or Foxp3⁺CD4⁺ T cells, was significantly increased during specific immunotherapy against bee venom [6]. It has also been reported that Foxp3⁺CD25⁺ and Foxp3⁺CD4⁺ cell levels were significantly increased in the nasal mucosa of patients receiving immunotherapy treatment with grass pollen [7]. mRNA expression of Foxp3 and IL-10 was reported to be induced after SLIT treatment, and the suppression of effector cell proliferation was IL-10-dependent [8]. The central mechanisms by which Tregs downregulate antigen-specific Th2 responses are suggested to be mediated by the production of the suppressor cytokine IL-10 in a soluble or membrane-bound form [9]. However, the therapeutic mechanisms and the relationship between clinical symptoms and Treg induction remain unclear.

In this study, we analyzed Cry j 1-specific Th2 responses and induced Tregs (iTregs), defined as CD25⁺CD4⁺ leukocytes positive for both IL-10 and Foxp3. We considered that antigen-specific iTregs would produce the suppressor cytokine IL-10 with Cry j 1 activation. These Cry j 1-specific iTregs (Cry j 1-iTregs) from the SLIT group were maintained after pollen season, whereas the Cry j 1-iTregs from the non-SLIT group decreased significantly after pollen season. Furthermore, the sub-

group of the SLIT group with increased iTregs showed a tendency for attenuated cytokine profiles compared to both the subgroup with decreased iTregs and the non-SLIT group. The subgroup with increased iTregs also showed lower clinical symptom scores than the subgroup with decreased iTregs. We propose that the level of antigen-specific iTregs is a suitable biomarker for the severity of symptoms and the therapeutic effects of SLIT.

Materials and Methods

Study Population

Nineteen in-house volunteers between 22 and 63 years of age, who were otherwise healthy but who had a clinical history of Japanese cedar pollinosis, were enrolled in this pilot study. The diagnosis of Japanese cedar pollinosis was based on clinical history and IgE specific to Japanese cedar pollen of at least class 2 status (CAP-RAST method, Phadia, Tokyo, Japan). Patients who had a history of any immunotherapy, had a current diagnosis of asthma or were pregnant were excluded. The patients in the non-SLIT group were statistically older than those in the SLIT group; however, there was no statistical difference between the groups with regard to the period of suffering from Japanese cedar pollinosis (3–10 years). All patients had showed moderate or severe symptoms in the previous pollen season [10]. Antigen-specific IgE titers for orchard grass, Japanese cypress and house dust mites were also evaluated by the CAP-RAST method. The protocol was approved by the Ethics Committee of Chiba University; written informed consent was obtained from each of the patients prior to participation in the study.

Clinical Protocols

Standardized Japanese cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) was used for SLIT [11]. The trial was performed from October 2006 to June 2007. The treatment protocol consisted of graded courses of the extract in 50% glycerol, followed by maintenance therapy [4]. The extract was graded in 3 strengths: 20, 200 and 2,000 Japanese Allergy Units (JAU)/ml. The content of Cry j 1 in the 2,000 JAU/ml extract was 1.5–4.2 µg, as determined by enzyme-linked immunosorbent assay (ELISA) and as reported previously [12]. Patients received increasing doses with each vial, beginning with 0.2 ml from the 20 JAU/ml vial and increasing by 0.2 ml a day for 5 days per week; the vaccine was taken sublingually, kept for 2 min without a retention reagent and then spit out. The procedure was then repeated with each vial until the maximum dose (1.0 ml of 2,000 JAU/ml) was reached. The maintenance dose was 1.0 ml of 2,000 JAU/ml once a week until the end of the study. The non-SLIT group was administered neither the vaccine nor a placebo. All participants were allowed to take symptom-reducing drugs.

Blood Samples

Peripheral blood was obtained from each patient before the beginning of treatment, before the pollen season and after the pollen season. Peripheral blood mononuclear cells (PBMCs) were isolated from whole peripheral blood by Ficoll density gradient centrifugation using Lymphocyte Separation Medium (MP Bio-

medicals Inc., Solon, Ohio, USA). Isolated cells were counted and tested for viability by trypan blue exclusion prior to culture. The PBMCs were frozen at -80°C and stored in liquid nitrogen using a cell banker (Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan) until use.

Antigens for in vitro Stimulation and ELISA

Cry j 1 was purified from Japanese cedar pollen according to the method of Yasueda et al. [13] with some modifications. The concentration of purified Cry j 1 was determined by the Lowry method using a detergent-compatible protein assay reagent (Bio-Rad Laboratories Inc., Hercules, Calif., USA). CS712, which is a recombinant protein with 7 conjugated regions of T cell epitopes from Cry j 1 and Cry j 2 [4, 5], was kindly provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan).

Antigen-Specific Immunoglobulin Titer

The Cry j 1-specific IgE titer in the plasma was measured by the method of Yasueda et al. [14]. The Cry j 1-specific IgG4 titer was measured by ELISA as described previously [4].

Flow Cytometric Analysis

For intracellular staining of Foxp3 and IL-10, PBMCs were cultured with or without Cry j 1 for 3 days, followed by culture with 10 ng/ml phorbol 12-myristate 13-acetate, 1 μM ionomycin and 2 μM monensin for 6 h. The PBMCs were stained with phycoerythrin-anti-CD25 (eBioscience, San Diego, Calif., USA) and phycoerythrin-Cy7-anti-CD4 antibody (BD Biosciences, San Diego, Calif., USA) in PBS containing 1% FCS and 0.1% sodium azide for 20 min at 4°C . After surface staining, the PBMCs were stained with FITC-anti-Foxp3 (clone PCH101, eBioscience) and allophycocyanin-anti-IL-10 antibody (BD Biosciences) for 30 min at 4°C using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. The numbers of IL-10⁺Foxp3⁺ cells in 10^4 CD25⁺CD4⁺ leukocytes were calculated from the percentage of IL-10⁺Foxp3⁺ cells in CD25⁺CD4⁺ leukocytes.

Clinical Symptoms

The participants were instructed to fill in a QOL questionnaire in the middle of the 2007 pollen season. Japanese cedar pollen scattered from the middle of January to early May in 2007. The Japanese Allergic Rhinitis QOL Standard Questionnaire No. 1 was used for the assessment of QOL symptom scores for allergic rhinitis [15]. The total QOL symptom score was calculated as the sum of each score: none = 0; mild = 1; moderate = 2; severe = 3; very severe = 4. Nasal and ocular symptoms covered by the questionnaire included runny nose, sneezing, nasal congestion, itchy nose, itchy eyes and watery eyes [3].

Enzyme-Linked Immunospot Assay

The numbers of IL-4- and IL-5-producing cells stimulated with Cry j 1 or CS712 were determined by enzyme-linked immunospot assay. A 96-well sterile filter plate (Millipore Corp., Billerica, Mass., USA) was coated with monoclonal antibody to human IL-4 or IL-5 (Mabtech AB, Nacka Strand, Sweden), following preincubation with 35% ethanol. After washing with PBS, the plate was preincubated with AIM-V medium at 37°C for 1 h. The medium was discarded, then 3×10^5 PBMCs/well were cultured with fresh medium alone, 10 $\mu\text{g}/\text{ml}$ Cry j 1, 20 nM CS712 or 1 $\mu\text{g}/$

ml phytohemagglutinin as a positive control for 17 h at 37°C in AIM-V medium containing 5% human blood plasma fractions. The plates were then washed with PBS, incubated with biotinylated detection monoclonal antibody to human IL-4 or IL-5 for 2 h and then incubated with streptavidin-conjugated alkaline phosphatase for 1 h at room temperature. After washing with PBS, the plates were incubated with BCIP/NBT^{PLUS} (Mabtech AB) for 5 min at 37°C . The numbers of positive spots were automatically calculated by the ImmunoScanTM (Cellular Technology Ltd., Cleveland, Ohio, USA) using the same parameter settings throughout.

Assay of Cytokine Production from PBMCs

Isolated PBMCs were cultured at 2.5×10^6 cells/ml with or without 5 $\mu\text{g}/\text{ml}$ Cry j 1 for 3 days at 37°C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich Inc., St Louis, Mo., USA). After centrifugation at 300 g for 10 min, the supernatant was divided into aliquots and stored at -20°C until cytokine assay. The concentrations of IL-5, IL-10, IL-13 and IFN- γ cytokines were measured by means of the BDTM Cytometric Beads Assay Flex system (BD Biosciences) according to the manufacturer's instructions.

Data Representation

The Cry j 1-specific cytokine production and the numbers of Cry j 1-iTregs are presented as the difference between the value from Cry j 1-stimulated cells and that from the medium-only control cell culture. Each upregulation after pollen season was represented as the difference between the pre-pollen season and post-pollen season values.

Statistical Analysis

Results are presented as means $\pm$ SD. Two-group comparisons were performed using the Wilcoxon t test or Mann-Whitney U test to determine the significance of the difference. p values <0.05 were considered significant.

Results

Study Population and Adverse Events

Nineteen adult patients were recruited from in-house volunteers at Chiba University and Chiba University Hospital on the basis of a history of Japanese cedar pollinosis and positive specific IgE (CAP-RAST score over 2; table 1). The 12 subjects who accepted the vaccine were enrolled in the SLIT group, and the 7 individuals who hesitated to take the vaccine were enrolled in the non-SLIT group. Three patients had mild discomfort and 1 patient complained of mild itching in the mouth; however, these adverse events were not serious and did not present a reason to discontinue SLIT.

Immunoglobulin Production

The Cry j 1-specific IgE production in the SLIT group was not statistically significantly higher after the pollen

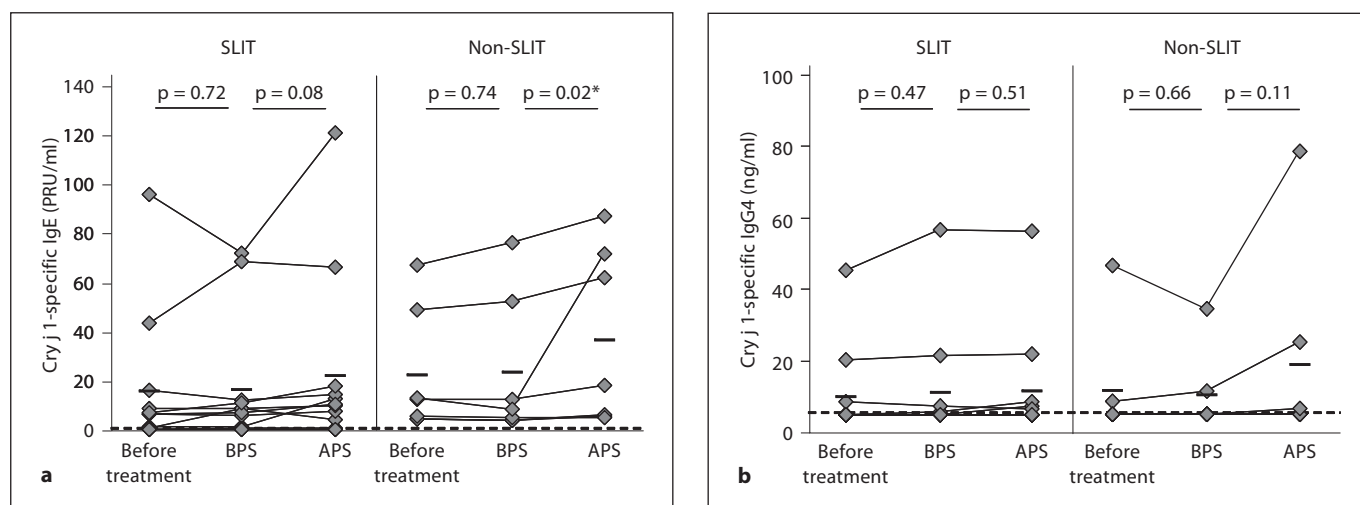


Fig. 1. Cry j 1-specific IgE (a) and IgG4 titer (b) from the SLIT and non-SLIT groups before treatment and before (BPS) and after the pollen season (APS). Bars show the group averages. The dashed line indicates the threshold for detection. PRU = Phadebas RAST unit. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$.

season compared with before the pollen season ($p = 0.08$); in contrast, IgE production in the non-SLIT group was statistically significantly increased after the pollen season compared with before the pollen season ($p = 0.02$; fig. 1a). Cry j 1-specific IgG4 production was not significantly changed after treatment in either the SLIT or non-SLIT group (fig. 1b).

Division of the SLIT Group According to the Change in iTregs

We analyzed a population of IL-10⁺Foxp3⁺ cells in CD25⁺CD4⁺ leukocytes as a marker of iTregs after stimulation with or without Cry j 1 (fig. 2a). The Cry j 1-iTreg levels, that is, the difference between those stimulated with Cry j 1 and the medium-only control, were significantly increased after treatment, and the difference in numbers of Cry j 1-iTregs before the pollen season was comparable to those after the pollen season in the SLIT group. However, we found that the difference in the non-SLIT group after treatment was comparable with that before treatment and significantly decreased after the pollen season compared to before the pollen season (fig. 2b). The upregulation between before and after the pollen season in the SLIT group (5 ± 42) was higher than that in the non-SLIT group (-24 ± 20), although the difference in the levels between the groups was not statistically significant (fig. 2c).

In all but one participant from the non-SLIT group, the difference in the number of Cry j 1-iTregs was down-

Table 1. The characteristics of participants at the time the study started

	SLIT	Non-SLIT
Participants	12	7
Sex (M/F)	9/3	5/2
Age, years		
Mean $\pm$ SD	24.1 $\pm$ 2.0	37.5 $\pm$ 15.8
Range	22–30	21–63
IgE class ¹	3.5	4.1
Other allergy ²		
Orchard grass	6	4
Japanese cypress	12	7
House dust mite	3	5

¹ Specific IgE to Japanese cedar pollen; mean CAP allergy class.

² Numbers of subjects who had specific IgE of at least class 2.

regulated after pollen season; in contrast, half of the SLIT group had higher Cry j 1-iTreg levels and the other half had lower levels (fig. 2c). Therefore, we divided the SLIT group into two subgroups according to whether Cry j 1-iTreg levels increased or decreased after pollen season. The total symptom score from the QOL questionnaire in the SLIT group was comparable to that in the non-SLIT group before the division into two groups. After the division, we found that the symptom score in the subgroup

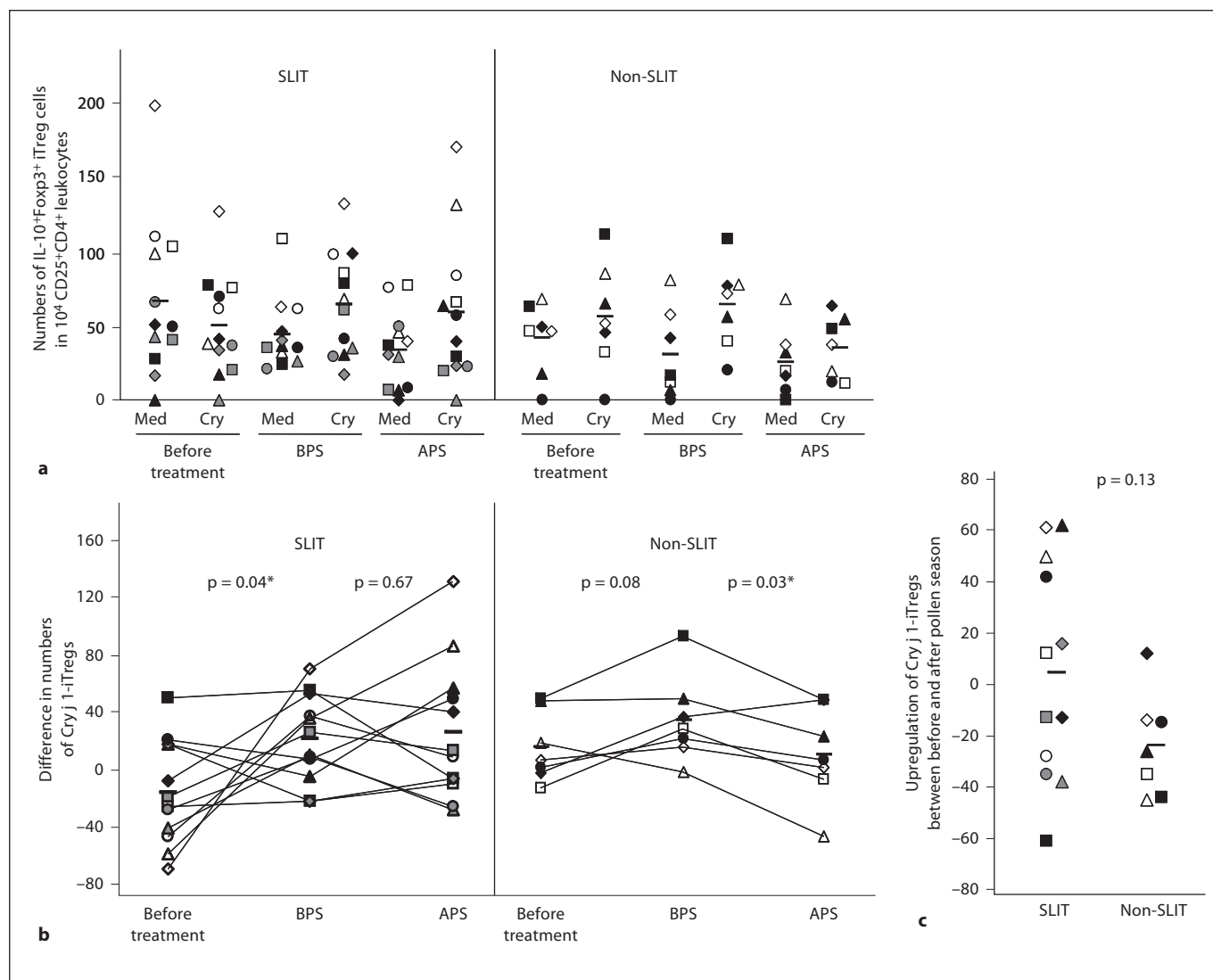


Fig. 2. **a** The numbers of IL-10⁺Foxp3⁺ cells in 10⁴ CD25⁺CD4⁺ leukocytes (iTregs) cultured with (Cry) or without (Med) Cry j 1 before treatment and before (BPS) and after the pollen season (APS). Each symbol in the SLIT group and the non-SLIT group represents an identical individual. Bars show the group averages. **b** The difference in numbers of Cry j 1-iTregs between medium-only control and cells stimulated with Cry j 1 was plotted for be-

fore treatment and before and after the pollen season in the SLIT and non-SLIT groups. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. **c** The upregulation of Cry j 1-iTregs between before and after the pollen season in the SLIT and non-SLIT groups. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * p < 0.05.

with increased iTregs was significantly lower than that in the subgroup with decreased iTregs (p = 0.03; fig. 3).

We also divided the SLIT group into severe and mild subgroups according to their total QOL symptom scores. We found that the upregulation of Cry j 1-iTregs between before and after pollen season in the mild-symptom subgroup was significantly higher than that in both the severe-symptom subgroup and the non-SLIT group (fig. 4).

Th2-Type Cytokine Profiles

We analyzed the numbers of Th2-type cytokine-producing cells and cytokine production after stimulation with native Cry j 1. The numbers of Cry j 1-specific Th2-type cytokine-producing cells were analyzed by enzyme-linked immunospot assay after stimulation with Cry j 1 or CS712 (fig. 5a and data not shown). The upregulation of both IL-4- and IL-5-producing cells in the SLIT group

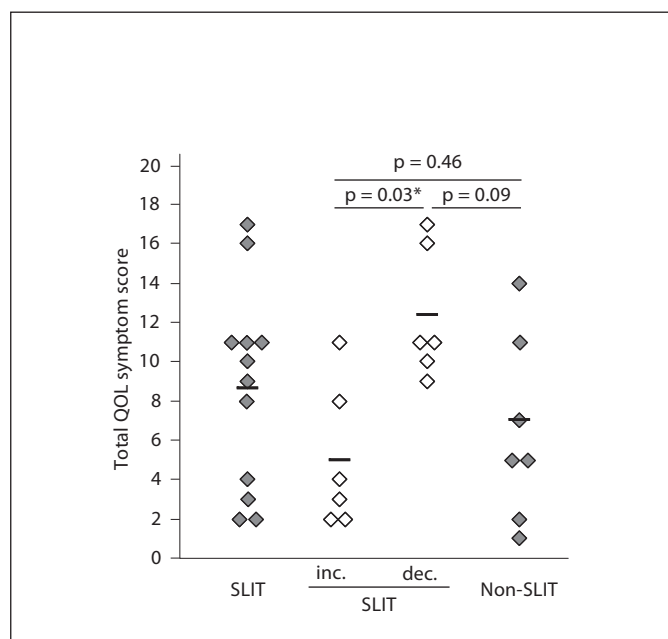


Fig. 3. Total symptom score from the QOL questionnaire was plotted for the SLIT and non-SLIT groups as well as for the subgroups from the SLIT group with increased (inc.) and decreased (dec.) iTregs. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

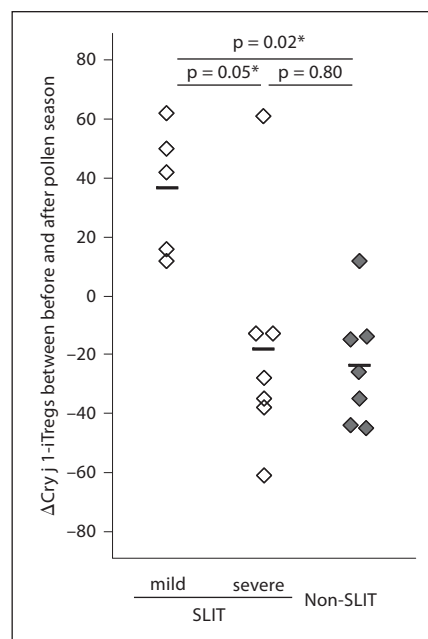


Fig. 4. The differences in numbers of Cry j 1-specific iTregs in 10^4 CD25⁺CD4⁺ leukocytes between before and after pollen season were plotted for the mild and severe subgroups of the SLIT group as well as for the non-SLIT group. The classification into severe and mild subgroups was based on the mean score of the SLIT group. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

(IL-4: 29 ± 33 ; IL-5: 23 ± 28) tended to be attenuated compared with that in the non-SLIT group (IL-4: 54 ± 38 ; IL-5: 43 ± 28). Furthermore, the upregulation in the subgroup with increased iTregs (IL-4: 19 ± 22 ; IL-5: 10 ± 11) was much lower than that in both the subgroup with decreased iTregs (IL-4: 40 ± 41 ; IL-5: 36 ± 35) and the non-SLIT group, although the difference in the levels between the groups was not statistically significant (fig. 5b). The same results were obtained using CS712 for stimulation (data not shown).

Cytokine production was analyzed in culture supernatant after 3 days of culture with Cry j 1 (fig. 6a). The upregulation of Th2-type cytokine production (IL-5 and IL-13), i.e. the differences between before and after pollen season, also tended to be attenuated in the SLIT group (IL-5: 94 ± 126 ; IL-13: 107 ± 134) compared to the non-SLIT group (IL-5: 178 ± 146 ; IL-13: 248 ± 222). We found that the Th2 cytokine profile in the subgroup with increased iTregs (IL-5: 54 ± 123 ; IL-13: 47 ± 110) also showed a strong tendency to be attenuated compared with that in the subgroup with decreased iTregs (IL-5: 134 ± 127 ; IL-13: 167 ± 137) and was significantly lower

than that in the non-SLIT group. The upregulation of IL-13 in the subgroup with increased iTregs showed statistically significant suppression compared with that in the non-SLIT group (fig. 6b). Upregulation of both IFN- γ and IL-10 production induced by Cry j 1 was almost the same in the SLIT (IFN- γ : -1.8 ± 22 ; IL-10: 0.8 ± 2.2) and non-SLIT groups (IFN- γ : -1.2 ± 32 ; IL-10: 1.2 ± 2.8 ; data not shown).

Discussion

We performed this pilot study to elucidate clinical biomarkers correlated with clinical symptoms in preparation for a future double-blind, placebo-controlled study of SLIT. Only one commercial standardized extract from Japanese cedar pollen is available for clinical use in Japan [11]. The cumulative dose of this major allergen after using the extract for 4 weeks is comparable to 2,500 SQ-standardized grass allergy immunotherapy tablet (SQ-T) in Europe [16]. In spite of the low dose, SLIT against Japanese cedar pollinosis has still been found to effectively

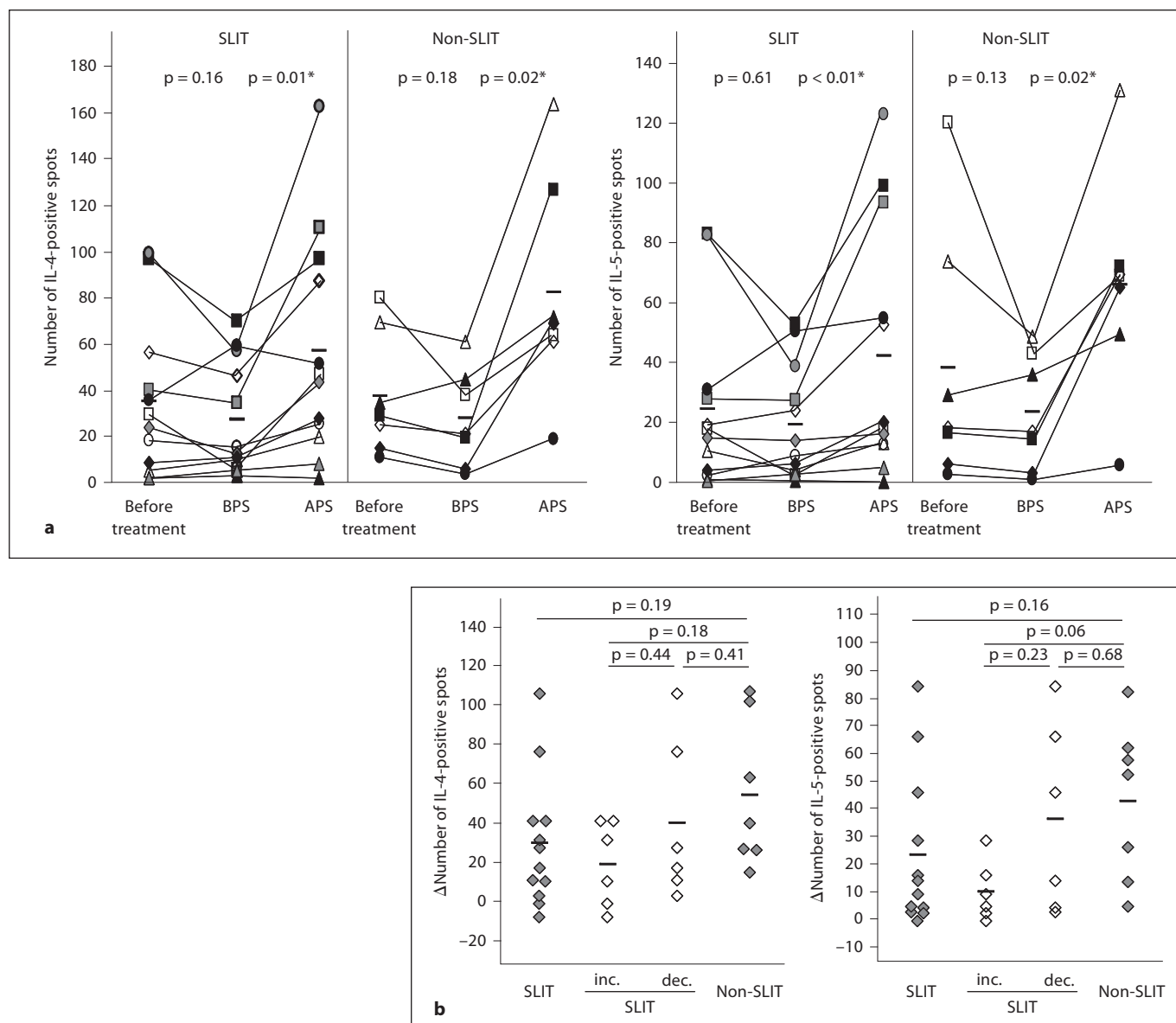


Fig. 5. a The numbers of Cry j 1-specific cytokine-producing cells before treatment and before (BPS) and after pollen season (APS). The numbers of positive cells for medium-only control were 1.3 ± 2.4 (SLIT) and 1.5 ± 3.1 (non-SLIT) for IL-4, and 1.0 ± 1.4 (SLIT) and 0.9 ± 1.6 (non-SLIT) for IL-5. Each symbol in the SLIT group or the non-SLIT group represents an identical individual. Bars show the group averages. Statistical analysis was performed using

the Wilcoxon t test. * $p < 0.05$. **b** The difference in numbers of Cry j 1-specific cytokine-producing spots is shown as the difference between values before and after the pollen season for each individual from the SLIT, the Cry j 1-iTreg-increased (inc.), the Cry j 1-iTreg-decreased (dec.) and the non-SLIT groups. The numbers of Cry j 1-specific spots were calculated as the difference between the medium-only control and the culture stimulated with Cry j 1.

ameliorate the QOL symptom score, medication score and symptom-medication score [3, 4]. Furthermore, SLIT attenuated antigen-specific Th2 responses and induced iTregs in some patients; this subgroup with increased iTregs showed greater amelioration of the Th2-type cytokine profile and their clinical symptoms.

In this clinical trial, significant induction of Cry j 1-specific IgG4 was not observed in the SLIT group. Our previous report showed induction of Cry j 1-specific IgG4 production with almost the same protocol; the previous study used a piece of bread to retain extract for sublingual administration [4]. The differences in the participants'

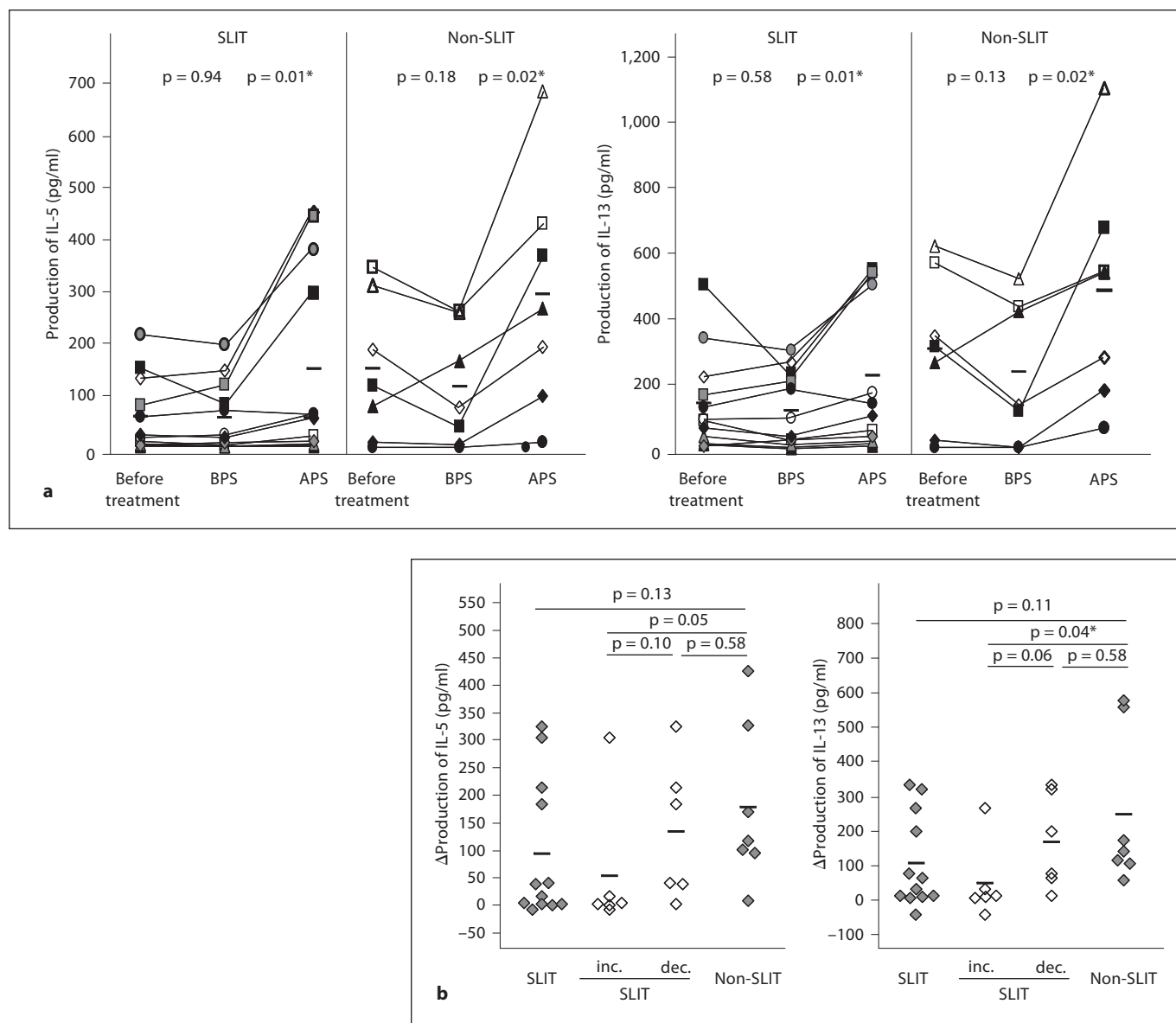


Fig. 6. a Th2-type Cry j 1-specific cytokine production before treatment and before (BPS) and after (APS) the pollen season. The cytokine production levels for medium-only control were 1.3 ± 2.0 (SLIT) and 3.4 ± 4.5 (non-SLIT) for IL-5, and 6.1 ± 9.6 (SLIT) and 10.6 ± 11.6 (non-SLIT) for IL-13. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$. **b** The upregulation of cytokine production induced by

Cry j 1 between before and after the pollen season from each individual from the SLIT, the Cry j 1-iTreg increased (inc.), the Cry j 1-iTreg decreased (dec.) and the non-SLIT groups is plotted on the y-axis. The cytokine production induced by Cry j 1 was calculated as the difference between medium-only control and the culture stimulated with Cry j 1. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

immunological backgrounds, methods of administration, period of administration and/or the amount of antigen absorbed in the oral mucosa may influence IgG4 induction. Antigen-specific IgG production was reported to be induced by high doses of extract, i.e. 25,000 SQ-T for 18 weeks or 75,000 SQ-T for 8 weeks [16]. This report

supports the hypothesis that the amount of antigen adsorbed by the oral mucosa affects the induction of antigen-specific IgG4.

We previously reported that SLIT significantly decreases the clone size of IL-4-producing T cells specific to epitopes from Cry j 1 and Cry j 2 [4]. Also, in the current

trial, SLIT attenuated Cry j 1-specific cytokine production, the numbers of IL-4- and IL-5-producing cells and IL-5 and IL-13 production in culture supernatant (fig. 5b, 6b). The difference in downregulation was not statistically significant between the SLIT and non-SLIT groups, but the results showing this tendency were reproducible. No significant difference in Th2-type cytokine production after stimulation with Cry j 1 was observed after sublingual administration of extract alone (i.e. the cytokine levels before and after pollen season in the SLIT group; fig. 5a, 6a). SLIT may attenuate the upregulation of antigen-specific Th2-type responses activated through natural exposure in pollen season. Therefore, the amount of scattering pollen may influence the degree of amelioration of Th2 responses by SLIT.

The upregulation of Cry j 1-specific iTregs, that is, the difference in levels of Cry j 1-iTregs between before and after pollen season, is suggested to be a suitable biomarker for clinical symptoms and therapeutic effects. The average difference in numbers of Cry j 1-iTregs after pollen season, that is, the difference in numbers of IL-10⁺Foxp3⁺ cells in CD25⁺CD4⁺ leukocytes between those stimulated with Cry j 1 and the medium-only control, was 24.8 in the SLIT group and 9.3 in the non-SLIT group in 10⁴ CD25⁺CD4⁺ leukocytes after 3 days' culture with Cry j 1 (fig. 2b). The number of antigen-specific Tr1 is reported to be estimated at 0.5–10 in 10⁴ whole peripheral CD25⁺CD4⁺ Tregs [17]. That estimate suggests the appropriateness of the numbers of Cry j 1-iTregs reported in this study. The subgroup with increased iTregs showed more attenuated Th2 cytokine profiles and a lower total QOL symptom score than the subgroup with decreased iTregs and the non-SLIT group (fig. 3, 5b, 6b). Several reports have shown that Foxp3-positive cells and/or IL-10-producing cells are induced by immunotherapy and that IL-10 is crucial for downregulation of inflammatory Th2 responses [6, 8, 18, 19]. Foxp3-expressing CD4⁺CD25⁺ cells are reported to be induced in the nasal mucosa after immunotherapy, and local induction of iTregs is suggested to be important to suppress local inflammation during pollen season [7]. Furthermore, the basal frequencies of Tregs defined as CD4⁺CD25^{bright}Foxp3⁺ cells in the peripheral blood of patients with severe allergic reactions to insect stings were reported to be lower than in individuals without a history of allergic diseases, and the Treg population was upregulated to a level comparable to that of nonallergic subjects after immunotherapy against bee venom [6].

The QOL symptom score was higher in the group with decreased Cry j 1-iTregs than in the group with increased

Cry j 1-iTregs. IL-17-secreting Foxp3⁺ Treg cells were recently identified in humans, and IL-17 mRNA expression was significantly correlated with poor clinical outcome after SLIT [20–22]. A low dose of SLIT may induce IL-17-secreting Tregs rather than IL-10-secreting Tregs for nonresponder populations and thereby worsen clinical symptoms.

We divided the SLIT group into two subgroups according to whether IL-10 or Foxp3 single-positive cells increased or decreased after pollen season. However, groups with IL-10 or Foxp3 single-positive cells showed no difference in Th2 cytokine profiles or symptom scores among the increased, decreased and non-SLIT groups (data not shown). We hypothesize that a population of Foxp3 or IL-10 single-positive cells may include many antigen-nonspecific Tregs and effector cells, whereas only antigen-specific iTregs are available as a therapeutic biomarker. These data suggest that IL-10 and Foxp3 double-positive cells rather reflect the antigen-specific iTregs that could be used as therapeutic biomarkers of SLIT.

Soluble IFN- γ and IL-10 production in culture supernatant was not upregulated by the SLIT treatment and did not differ between the SLIT group and the non-SLIT group (data not shown). This suggested that SLIT did not induce Cry j 1-specific Th1-type responses and that the membrane-bound form of IL-10 may be more important than soluble IL-10 for downregulation of Cry j 1-specific Th2 cells. Further investigation is needed to clarify the induction of Th1 cells and IL-10-mediated suppressive mechanisms by iTreg cells. In this trial, we failed to detect other regulatory molecules at the protein level, such as TGF- β from culture supernatant or cytotoxic T lymphocyte-associated protein-4 and glucocorticoid-induced TNF receptor on the surface of CD4⁺ T cells (data not shown). Further investigations are needed to analyze these regulatory molecules at the mRNA level. We are currently undertaking a transcriptome analysis of CD4⁺ cells from the SLIT and non-SLIT groups after stimulation with Cry j 1. On the other hand, the regulatory mechanisms of peripheral human Tregs were suggested to occur in a cell contact-dependent but cytokine-independent manner [23]. Furthermore, this report suggested that the regulatory function of human Tregs was independent of CD28, cytotoxic T lymphocyte-associated protein-4, TGF- β and IL-10 [23]. In order to elucidate the therapeutic mechanisms of SLIT, analysis of the regulatory function of iTregs is also important.

In this paper, we investigated antigen-specific iTregs as a therapeutic biomarker for SLIT. However, this study was a preliminary open-label study with a small population; therefore, a randomized, double-blind, placebo-controlled study of a large population will be needed to evaluate iTregs as a therapeutic biomarker for SLIT.

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