

ORIGINAL ARTICLE

Responses of CD4⁺CD25⁺Foxp3⁺ and IL-10-secreting type I T regulatory cells to cluster-specific immunotherapy for allergic rhinitis in children

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Keywords

allergic rhinitis; peripheral tolerance; regulatory T cells; specific immunotherapy; Tr1 cells.

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Abstract

We investigated the effects of cluster specific immunotherapy (SIT) with *Dermatophagoides pteronyssinus* (Der p) on CD4⁺CD25⁺Foxp3⁺ Treg cells and IL-10-secreting type I T regulatory (Tr1) cells in Der p-sensitized children with allergic rhinitis (AR). We performed a prospective randomized study involving 46 children (aged 8–13 yr), of whom 25 children received Der p-SIT + pharmacotherapy and 21 received only pharmacotherapy, over a period of 1 yr. Prior to and at end of treatment, CD4⁺CD25⁺Foxp3⁺ Treg cells and allergen-specific IL-10⁺IL-4⁺, IFN- γ ⁺IL-4⁺, and IL-4⁺IFN- γ -CD4⁺ T cells were measured by flow cytometry. Similarly, IL-4, IFN- γ , and IL-10 in supernatants from allergen-stimulated peripheral blood mononuclear cell (PBMC) cultures were measured by ELISA, and the suppressive effect of CD4⁺CD25^{high} T cells on cell proliferation and cytokine release was estimated from both groups. Allergen-specific serum IgE and IgG4 were also assessed at the beginning and end of treatment by RAST and ELISA, respectively. The levels of allergen-specific Tr1 cells, IgG4, and allergen-induced IL-10 synthesis from PBMC cultures were significantly increased after SIT for 1 yr compared with baseline levels ($p < 0.001$ for all), with significant correlation between increased levels of Tr1 cells and improvements in nasal symptoms ($r = 0.48$, $p < 0.05$). In contrast, the levels of CD4⁺CD25⁺Foxp3⁺ T cells, allergen-specific Th1 and Th2 cells, the production of IL-4 and IFN- γ , and the function of CD4⁺CD25^{high} T cells were not altered in either group at the end of treatment. These data suggest that the up-regulation of Tr1 cells may play an important role in SIT and be a useful marker of successful SIT in AR patients.

Allergic rhinitis (AR) is a highly prevalent inflammatory disease affecting 10–40% of the global population (1, 2). The cornerstones of AR treatment are allergen avoidance, symptomatic pharmacotherapy, and allergen-specific immunotherapy (allergen-SIT). SIT is recognized as an effective therapeutic practice and recommended as an integrated part of an allergy management strategy in the treatment of allergic diseases especially in children (3).

Given the importance of T cells in the allergic response and the demonstration that T regulatory (Treg) cells play a crucial role in the modulation of the T-cell response in allergic disease (4, 5), there has been increased interest in the effect of SIT on the modulation of the Treg cells. In particular, the naturally occurring CD4⁺CD25⁺Foxp3⁺ Treg cells,

which possess high levels of CD25 and express FOXP3, a key transcription factor required for development, maintenance, and function of Treg cells (6, 7), and the inducible IL-10-secreting type I T regulatory (Tr1) cells, which synthesize high levels of IL-10 and TGF- β , but not IL-4, and are distinct from and modulate the activity of T helper (Th) 1 and Th2 cells (5, 8, 9), have been greatly investigated in studies of immune tolerance to allergens (10). It has been demonstrated that the immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific Tr1 and Th2 cells (5) or a higher IL-22 and IL-17 mRNA levels (11) and that the frequencies of CD4⁺CD25⁺ Treg cells and Tr1 cells are lower in peripheral blood from allergic patients (5). Moreover, there is evidence that IL-10⁺

CD4⁺CD25⁺ T cells may be induced by SIT (12, 13). Although CD4⁺CD25⁺ Treg and Tr1 cells have been shown to be distinct subsets (14), few studies have investigated different Treg cell subset responses to SIT. In view of these data, we hypothesized that effective SIT induced Tr1 and/or CD4⁺CD25⁺Foxp3⁺ Treg cell production and a shift from a Th2 to a regulator/suppressor T-cell response in allergic individual. The objective of our study was to investigate the effects of cluster SIT with *Dermatophagoides pteronyssinus* (Der p) on the incidence of Tr1 and CD4⁺CD25⁺Foxp3⁺ Treg cells in Der p-sensitized children with AR.

Methods

Patients

The study included 46 patients (aged 8–13 yr old) of Han Chinese ethnic origin, recruited from the Department of Otolaryngology Head and Neck Surgery allergy outpatient clinic at Beijing TongRen Hospital, Beijing, China. All patients had a documented history of house dust mite (HDM)-induced moderate to severe rhino-conjunctivitis for at least 3 yr and demonstrated a positive skin prick test result for Der p, as indicated by a wheal diameter of at least 6 mm. Furthermore, all patients demonstrated a positive test for specific immunoglobulin E (IgE) to Der p, as indicated by a RAST value of at least 0.7 kU/l. Patients with a history of asthma or atopic dermatitis were excluded.

Study design

This was a 1-yr open-label parallel group study in which 25 (aged 9–13 yr) patients were randomized to receive SIT with Der p extract (Alutard SQ; ALK-Abello, Hørsholm, Denmark) in addition to standard pharmacotherapy and 21 (aged 8–13 yr) patients randomized to receive standard pharmacotherapy (control group). Blood samples were collected for analysis of cell types, cytokines, allergen-specific serum IgE and IgG4, and cell culture, at baseline prior to treatment and at the end of the 1-yr treatment period and processed by investigators blinded to the sample status (i.e., from SIT or control subjects). Over the course of treatment, all patients recorded nasal symptom severity and medication use (each administration or variation of the initial drug therapy) in daily diary cards.

The study was approved by the Ethics Committee of Beijing Tongren Hospital, and written informed consent was obtained from all subjects/guardians prior to entry into the study. This study was registered with ClinicalTrials.gov (identifier number: NCT01291381).

Immunotherapy protocol

Children undergoing SIT were given Der p extract according to a cluster protocol that we have described previously (15). During the first 6 wk, each patient returned to the clinic at weekly intervals, during which they underwent a routine examination and received two injections 1 h apart with an increasing dose every week, reaching the highest concentra-

tion with an allergenic activity of 100,000 SQ/ml (equivalent to 9.8 µg/ml) Der p. After week 6, the children attended the clinic at one monthly interval, during which they were injected with the highest concentration of 100,000 SQ/ml Der p for the rest of the treatment period (Table 1). Over the course of the study, persistent rhinitis in all patients was managed with intranasal steroids at a standard dose and oral antihistamines as required.

Clinical evaluation

All patients rated the severity of the symptoms of rhinorrhoea, sneezing, nasal congestion, and nasal and ocular pruritus on a 4-point scale of 0–3 (0 = none; 1 = slight; 2 = heavy; 3 = severe), which were added to provide Total 5 Symptom Score (T5SS).

Medication use was scored as described previously (16), with arbitrary scores attributed to the drugs used (0.75 points for one puff of nasal corticosteroids and 1 point for one tablet of antihistamine). Patients were instructed to use only intranasal steroids and antihistamines were to be used additionally when there were no improvements in their symptoms. Patients were also instructed to stop drugs at least 7 days before blood sampling.

Assessment of T-cell subsets

Peripheral blood was obtained by venous puncture and collected into preservative-free heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by means of Ficoll-Plaque Plus density gradient centrifugation (Amersham Biosciences, Piscataway, NJ, USA).

Peripheral blood mononuclear cells (2×10^6 cells/ml) were stained at room temperature for 20 min with anti-CD4-PerCP, anti-CD25-PE, or isotype control (mouse IgG1-PerCP or IgG1-PE) (all from Beckman Coulter, Brea, CA, USA). Following incubation, cells to be investigated for intracellular Foxp3 were fixed with Foxp3 fixation/permeabilization buffer

Table 1 Cluster schedules of immunotherapy

Week	Cluster schedule	
	Visit	Dose (SQ-U)
0	1	10
	1	100
	1	1000
1	2	2000
	2	4000
2	3	5000
	3	10,000
3	4	10,000
	4	20,000
4	5	20,000
	5	40,000
5	6	40,000
	6	60,000
6	7	100,000

1 ml and permeabilized with Foxp3 permeabilization buffer 1 ml, prior to incubation for 30 min at room temperature with anti-human Foxp3-FITC or isotype control (IgG1-FITC) (all from BioLegend, CA, USA). The stained cells were then analyzed by flow cytometry for different T-cell subsets, including (i) Th1 cells: IFN- γ ⁺IL-4⁻CD3⁺CD8⁻ T cells, (ii) Th2 cells: IL-4⁺IFN- γ ⁻CD3⁺CD8⁻ T cells, and (iii) Tr1 cells: IL-10⁺IL-4⁻CD3⁺CD8⁻ T cells, by collecting at least 20,000 events/subset in the lymphocyte gate, using a FACS Calibur flow cytometer (BD Biosciences, NJ, USA) (17). The CD3⁺CD8⁻ subset was considered to be equivalent to the CD4⁺ subset, as a previous study demonstrated that the CD4 molecule rapidly down-modulates in response to PMA (18).

Assessment of cytokines

Intracellular and released cytokines were assessed in Der p1 (*Dermatophagoides pteronyssinus* major allergen 1, Endotoxin ≤ 0.03 EU/ μ g; Indoor Biotechnologies, Manchester, UK)-stimulated PBMCs, by flow cytometry and ELISA, respectively.

In the case of intracellular cytokines, PBMCs (2×10^6 cells/ml) were incubated with 10 μ g/ml Der p1 over a period of 24 h at 37°C, during which 25 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μ g/ml ionomycin, and 1.7 μ g/ml monensine (all from Sigma, St. Louis, San Diego, MO, USA) were added for the final 4 h of the incubation period. At the end of incubation, the cells were washed with PBS and then incubated with anti-CD3-APC and anti-CD8-PerCP for 20 min at room temperature. At the end of incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde/PBS and permeabilized with FACS Permeabilizing Solution (BD Pharmingen, CA, USA). The permeabilized cells were incubated for 30 min at room temperature with anti-IL-4-PE plus anti-IFN- γ -FITC, or anti-IL-10-PE plus anti-IL-4-FITC, or a matched antibody with a respective isotype IgG1 as control (all from BD Pharmingen) and then assessed by flow cytometry as mentioned earlier.

In the case of released cytokines, PBMCs of >90% viability, as assessed by trypan blue staining, were resuspended at 2×10^6 cells/ml in RPMI-1640 medium and incubated with 10 μ g/ml Der p1 for 6 days at 37°C in a humidified incubator under a 5% CO₂ in air atmosphere. At the end of incubation, the supernatant from each culture was collected and frozen at -20°C, until analysis for the presence of IFN- γ , IL-4, and IL-10, using commercial ELISA kits according to the supplier's instructions (R&D Systems, Minneapolis, MN, USA).

Allergen-specific serum antibodies analysis (specific IgE and IgG4)

Serum samples were collected from each patient at baseline and at the end of 1-yr treatment and stored at -20°C until analysis. Der p-specific IgE was measured using the UniCAP 100 system (Pharmacia Diagnostics, Uppsala, Sweden), and Der p-specific IgG4 was measured using commercial ELISA kits according to the supplier's instructions (ALK Laboratories, Hørsholm, Denmark).

Isolation of CD4⁺CD25^{high} T cells

Heparinized blood (30 ml) was obtained from SIT group (n = 8) and controls group (n = 8) for cell isolation and functional analysis. In this study, the isolation of CD4⁺CD25^{high} T cells was performed in a two-step procedure using the CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells were purified from the PBMCs by negative selection (by depletion of CD8, CD14, CD16, CD19, CD36, CD56, and CD123 positive cells). The purified total CD4⁺ T-cell subset [mean purity 95% (range = 93–97%)] was further incubated with anti-CD25 Micro Beads (2- μ l anti-CD25 beads/10⁷ cells) (19) and purified Foxp3⁺CD4⁺CD25^{high} T cells [mean purity 76% (range = 72–88%)] isolated by positive selection.

Suppressive capacity of CD4⁺CD25^{high} T cells

To evaluate the suppressive capacity of CD4⁺CD25^{high} T cells, cell proliferation and cytokines (IFN- γ , IL-4, and IL-10) were analyzed. CD4⁺CD25⁻ T cells were cultured alone or as co-cultures of CD4⁺CD25⁻ T cells and CD4⁺CD25^{high} T cells in a ratio of 2:1, in 200 μ l RPMI-1640 medium, containing 10 μ g/ml Der p1. Autologous irradiated PBMCs (3000 rad) were added at a concentration of 2×10^5 antigen-presenting cells/culture, and all cultures were incubated for 6 days at 37°C in a humidified incubator under a 5% CO₂ in air atmosphere. Negative controls were included in triplicate by incubating culture medium alone under the same culture conditions on each occasion the experiment was conducted. At the end of culture, 100 μ l supernatant was removed for analysis of specific cytokines, using ELISA kits (R&D Systems). Cell proliferation of CD4⁺CD25⁻ T cells was further assessed according to the manufacturers' protocols for WST-8 (modified tetrazolium salt) cell proliferation kit (Cell Counting Kit-8; Dojin, Kumamoto, Japan) and the results expressed as absorbance at 450 nm for the medium from each culture.

Statistics

Statistical significance between groups was analyzed using a Mann–Whitney test. The significance of intra-group pre- and post-therapy change was determined using the non-parametric Wilcoxon matched pairs test. The correlation coefficient r was assessed using the Pearson correlation. All analyses were performed using SPSS version 13.0 statistical software (SPSS Inc. Chicago, IL, USA). A 5% significance level was used ($\alpha = 0.05$), power (1- β) equal to 90%. All tests were two-tailed, and p-values of <0.05 were considered statistically significant.

Results

Patient demographics

Overall 50 patients were recruited to the study; 25 randomized to SIT group and 25 to control group, of whom four children in the control group (two boys, two girls) did not

return for follow-up visits, and therefore, any data from these patients were not included in the final analysis. The baseline characteristics of the subjects who completed the study were comparable in both treatment groups (Table 2).

Clinical efficacy of immunotherapy

The results for clinical efficacy of treatment based on T5SS and medication use scores evaluated by the patients at the beginning and over the course of the treatment period are shown in Fig. 1. The symptom scores (T5SS) in both SIT-treated and control patients at 3, 6, and 12 months were significantly reduced from baseline ($p < 0.05$ for both), with no significant difference between the two treatments at any time point (Fig. 1a). In contrast, only medication use scores were significantly decreased from baseline values at 3, 6, and 12 months of treatment in the SIT group ($p < 0.05$), with significant differences noted between the two treatments at all time points ($p < 0.05$) (Fig. 1b).

Frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in peripheral blood

Figure 2 shows the number of $CD4^+CD25^+Foxp3^+$ Treg cells detected by flow cytometry in the peripheral blood of Der p-sensitized children in SIT and control groups. Assessment of the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in $CD4^+$ T cells in the two treatment groups demonstrated that these were not significantly different in the SIT and control groups and not significantly altered from baseline levels in either treatment group at the end of the 1-yr treatment period (Fig. 2b).

Frequencies of Tr1, Th1, and Th2 cells in peripheral blood

Figure 3a shows the $CD3^+CD8^-$ cells (considered to be equivalent to the $CD4^+$ subset), Fig. 3b shows the Th1 ($IFN-\gamma^+IL-4^-$) and the Th2 ($IL-4^+IFN-\gamma^-$) cells, and Fig. 3c shows the Tr1 ($IL-10^+IL-4^-CD4^+$) cells, as detected by flow cytometry. Comparison of the frequencies of these cell types before and treatment for 1 yr with SIT or control therapy indicated no significant changes in the frequencies of Th1 and Th2 in either group before or after treatment (Fig. 3d,e). In contrast, Tr1 cells were significantly increased from baseline in the SIT-treated group ($p < 0.0001$), with significant difference between the treatment groups also noted at the end

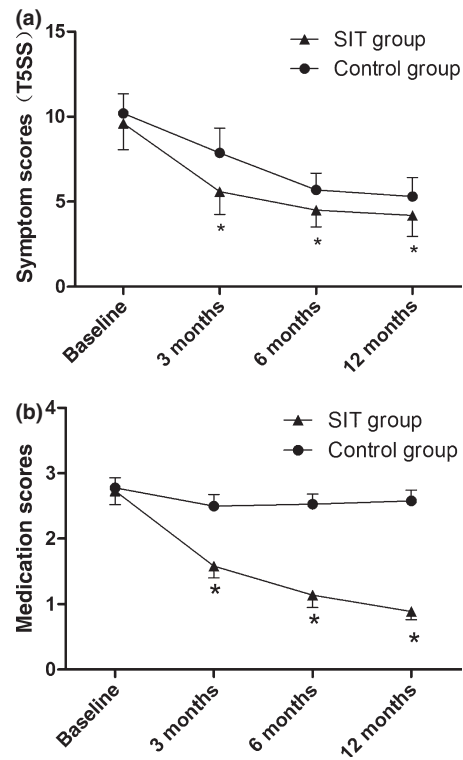


Figure 1 Effect of treatment on (a) T5SS (b) and medication use scores over the course of 1-yr treatment (* $p < 0.05$ vs. baseline; SIT, allergen-specific immunotherapy; T5SS, total 5 symptoms score).

of treatment (Fig. 3f). Furthermore, the ratio of Tr1/Th2 cells was also significantly increased from baseline in the SIT treatment group, but not in the control treatment group ($p < 0.001$; Fig. 3g).

Correlation of nasal symptom score with the change of Tr1 cells

Assessment of any association between clinical severity and number of Tr1 cells demonstrated that increased numbers of Tr1 cells were significantly correlated with improved nasal symptoms (i.e., decrease in T5SS) in SIT group by the end of treatment for 1 yr ($r = 0.48$, $p < 0.05$, Fig. 4). There was no

Table 2 Demographic and clinical characteristics of study participants on baseline

	SIT (n = 25)	Controls (n = 21)	p-value
Age (yr)	12 (9–13)	11 (8–13)	0.842*
Sex (male/female)	12/13	10/11	0.979†
Total IgE (KU/l)	187 (56–1024)	145 (73–1295)	0.553*
Specific IgE to Der p (KU/l)	5.7 (2.3–18)	4.2 (1.5–166)	0.483*
Specific IgG to Der p (AU/l)	2691 (568.5–5968)	2875 (656.2–4985)	0.494*
Nasal symptom score (T5SS)	9 (7–12)	10 (6–12)	0.423*

Der p, *Dermatophagoides pteronyssinus*; SIT, specific immunotherapy; T5SS, total 5 symptoms score. Data are median (range) or number of patients. *Mann–Whitney U-test. †Chi-squared test.

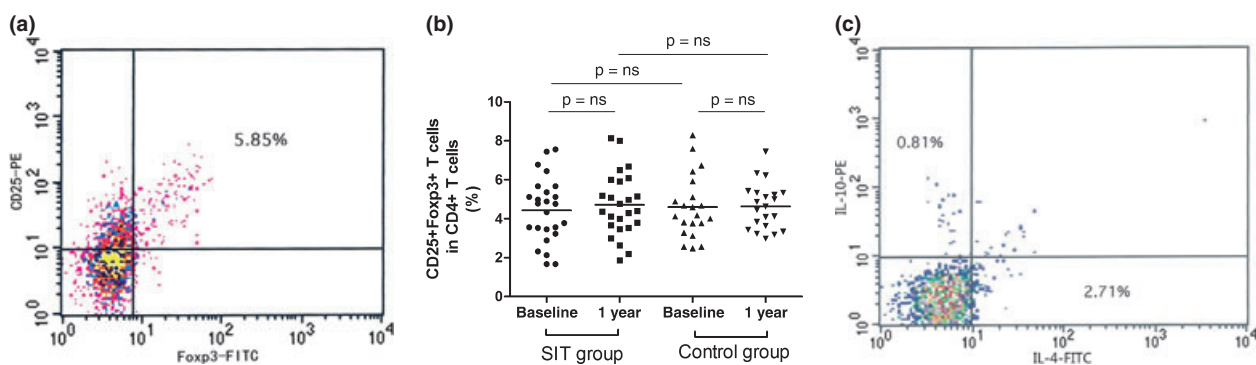


Figure 2 Flow cytometric evaluation of CD4⁺CD25⁺Foxp3⁺ T cells. (a) Percentages of different cell subsets in total CD4⁺ T cells, with scatter dots in the right upper quadrant representing CD4⁺CD25⁺Foxp3⁺ T cells in CD4⁺ T cells gate. (b) Percentage of CD4⁺CD25⁺Foxp3⁺ T cells in CD4⁺ T cells from the SIT group (n = 25) and control group (n = 21) (horizontal lines represent means; ns, not significant; SIT, allergen-specific immunotherapy).

change in the number of Tr1 before and after treatment in the control group; therefore, there was no association between clinical severity and number of Tr1 cells in the control group ($r = -0.19$, $p > 0.05$).

Release of IL-10, IL-4, and IFN- γ from Der p1-stimulated PBMCs in culture

The levels of IFN- γ and IL-4 released in comparable samples (culture supernatants from the same patient, stimulated with the same doses of allergen performed at baseline and 1 yr, n = 10) were unchanged in both treatment groups after treatment for 1 yr (Fig. 5a,b). By contrast, the levels of IL-10 were significantly increased in the SIT treatment group, but not in the control group (Fig. 5c).

Changes of allergen-specific IgE and IgG4 after SIT

Assessment of allergen-specific serum IgE and IgG4 demonstrated that the concentrations of allergen-specific IgE were not significantly altered from baseline in either treatment group after 1 yr (Fig. 6a). In contrast, the concentration of IgG4 was significantly increased from baseline in the SIT-treated, but not the control treatment group, after treatment for 1 yr ($p < 0.001$; Fig. 6b).

Suppressive capacity of CD4⁺CD25^{high} T cells after allergen stimulation

The suppressive capacity of CD4⁺CD25^{high} T cells on responsive T cells was assessed in peripheral blood samples collected from eight patients each in the SIT and control groups before and after treatment for 1 yr. CD4⁺CD25^{high} T cells significantly suppressed Der p1-induced CD4⁺CD25⁺ T-cell proliferation and IFN- γ synthesis in cells at baseline in both SIT and control groups (Fig. 7a,b). In contrast, IL-4 (Fig. 7c) synthesis was not significantly suppressed by CD4⁺CD25^{high} T cells, and IL-10 (Fig. 7d) synthesis was not changed at baseline in either SIT or control groups.

The suppressive effect of CD4⁺CD25^{high} T cells on Der p1-induced CD4⁺CD25⁺ T-cell proliferation (Fig. 7a) or IFN- γ synthesis (Fig. 7b) was not significantly altered from baseline after treatment for 1 yr in either treatment group. And indeed IL-4 synthesis was not significantly suppressed by CD4⁺CD25^{high} T cells in both group (Fig. 7c), but IL-10 (Fig. 7d) synthesis was significantly increased in the SIT group after 1-yr treatment.

Discussion

In this study of cluster allergen-SIT with Der p extract, we found that the immunological changes involved in the effective desensitization of pediatric patients included a higher frequency of allergen-specific Tr1 cells and increased production of IL-10 and IgG4. The increase in allergen-specific Tr1 cells correlated with the decline of clinical symptom scores over the course of the study. However, SIT did not increase either the frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells or the suppressive capacity of CD4⁺CD25^{high} T cells.

Although previous studies have suggested an increase in Treg cell response in asthmatic children receiving pollen- or HDM-SIT, as indicated by an increase in CD4⁺CD25⁺ T cells (12, 20), recent evidence suggests that CD25 is an activation marker of T lymphocytes and not a specific marker of Treg cells (21). In contrast, Foxp3 has been shown to play a key role in the development and function of Treg cells and is therefore considered to be a more specific marker for Treg cells (22). The finding in this study for the lack of any significant change in circulating CD4⁺CD25⁺Foxp3⁺ Treg cells following Der p-SIT in children with AR after 1 yr, however, is in accordance with the findings of a recent SIT study of HDM-SIT in HDM-allergic asthmatic children (23). Similarly, in another study, Ajduk et al. (24) investigated the CD4⁺ Treg cells (CD69⁺CD45RO⁺CTLA-4⁺ and CD3⁺CD4⁺CD25⁺FOXP3⁺) in peripheral blood from 16 HDM-allergic asthmatic children following 1 yr of subcutaneous SIT. The authors demonstrated that despite improvements in clinical variables; including symptom score, medication

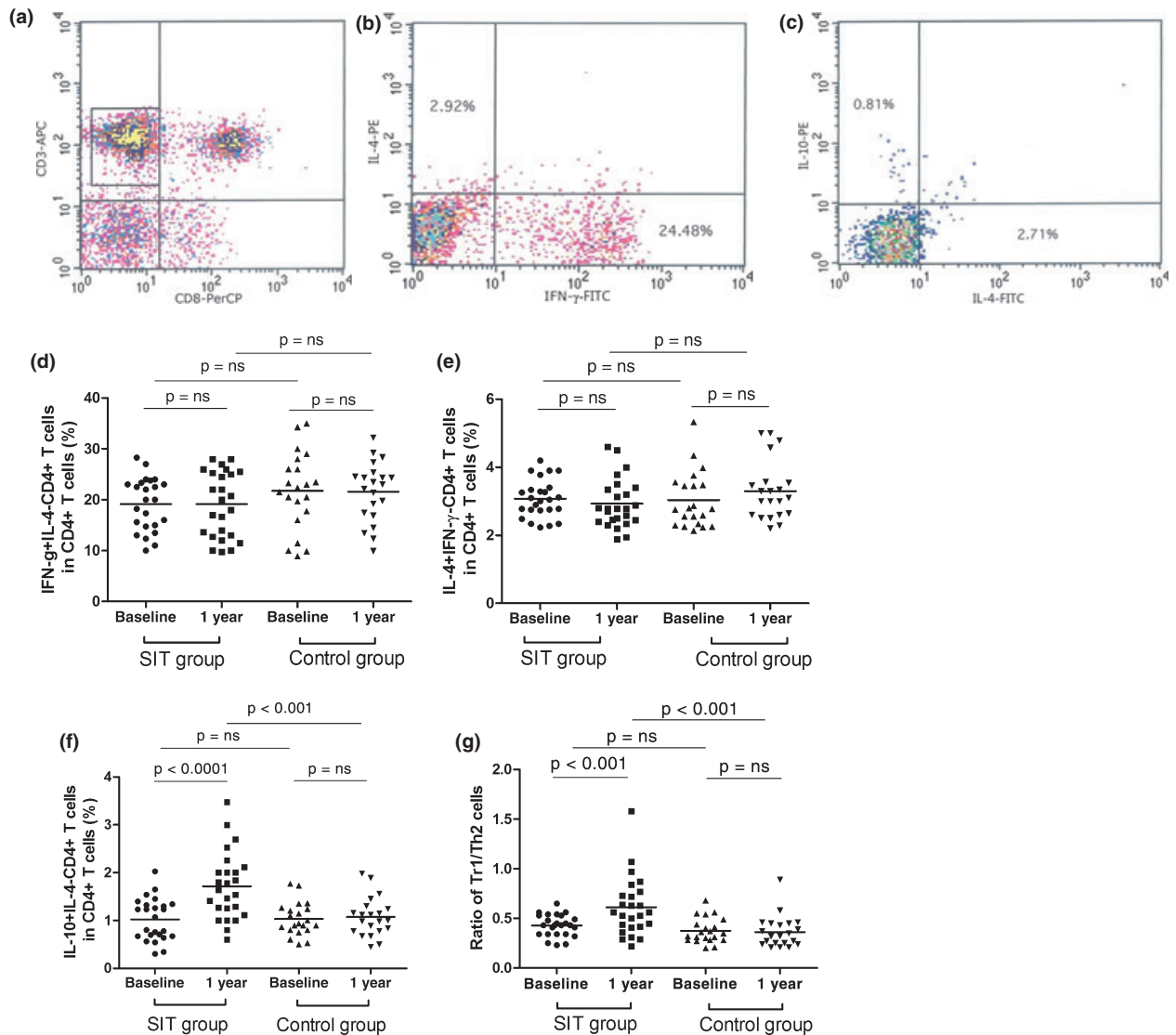


Figure 3 Flow cytometric analysis of intracellular cytokine-positive cells in PBMCs stimulated with Der p1 from 0 to 24 h and PMA plus ionomycin from 20 to 24 h. (a) CD3⁺CD8⁻ T cells (i.e., CD4⁺ T cells, the scatter dots in the left upper quadrant) were gated in PBMCs. (b) left upper quadrant: IL-4⁺IFN-γ⁻CD4⁺ T cells (Th2 cells); right lower quadrant: IFN-γ⁺IL-4⁻CD4⁺ T cells (Th1 cells). (c) left upper quadrant: IL-10⁺IL-4⁻CD4⁺ T cells (Tr1 cells). Frequencies of allergen-specific Th1 (d), Th2 (e), and Tr1 (f) cells in CD4⁺ T cells in the SIT group (n = 25) and control group (n = 21). (g) Ratios of Der p1-specific Tr1/Th2 cells. (Values in dot plots indicate percentages of different cell subsets in total CD4⁺ T cells; horizontal lines represent means; ns, not significant; PBMCs, peripheral blood mononuclear cells; SIT, allergen-specific immunotherapy).

requirements, forced expiratory volume in 1 s, peak expiratory flow rate, and serum IgE levels, the percentage of CD4⁺CD25⁺CD69⁻CD45RO⁺ Treg cells FOXP3⁺ Treg cells did not change after 1 yr of immunotherapy. In contrast, Aslam et al. (25) demonstrated that wasp venom immunotherapy for 3–5 wk could induce FOXP3⁺ allergen-specific T cells. Similarly, in another study, Radulovic et al. (26) found that the numbers of FOXP3⁺CD25⁺CD3⁺ cells were increased in the nasal mucosa of grass pollen allergic patients

following grass pollen immunotherapy for 2 yr. These findings raise the possibility that SIT may preferentially induce CD4⁺CD25⁺FOXP3⁺ Treg cells in the local tissue, but not in the peripheral blood. Nevertheless, there are limitations to accurately evaluating the expression of FOXP3 in the suppressive activity of Treg cells, and there is evidence that non-regulatory human CD4⁺ and CD8⁺ T cells can be induced to transiently express FOXP3, but without suppressive activity (27).

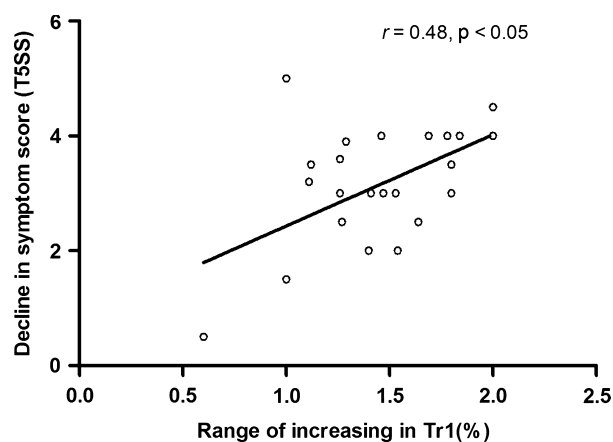


Figure 4 Correlation between Tr1 cells and clinical symptoms following SIT for 1 yr ($r = 0.48$, $p < 0.05$; SIT, allergen-specific immunotherapy).

The expression of Foxp3, however, is closely correlated with the function of $CD4^+CD25^+$ T cells in mice and $CD4^+CD25^{high}$ T cells in humans (28). To further demonstrate the role of $CD4^+CD25^+Foxp3^+$ Treg cells in HDM-SIT, we compared the suppressive capacity of $CD4^+CD25^{high}$ T cells obtained from patients in SIT and control groups and demonstrated that SIT did not significantly alter the function of $CD4^+CD25^{high}$ T cells in suppression of Th2 cells. This finding was further supported by the demonstration that IL-10, which is known to be synthesized by $CD4^+CD25^+$ T cells (29) and plays an important role in the suppression of T-cell proliferation and cytokine production (30), was not found to be increased in co-cultures of $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ T cells compared with $CD4^+CD25^-$ T cells alone, from SIT or control patients, either at baseline or after treatment for 1 yr. However, we found that SIT induced IL-10 synthesis in co-cultures of $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ T cells and $CD4^+CD25^-$ T cells alone after 1-yr treatment compared with baseline and control group. Our finding for the lack of allergen-specific SIT on the suppressive function of

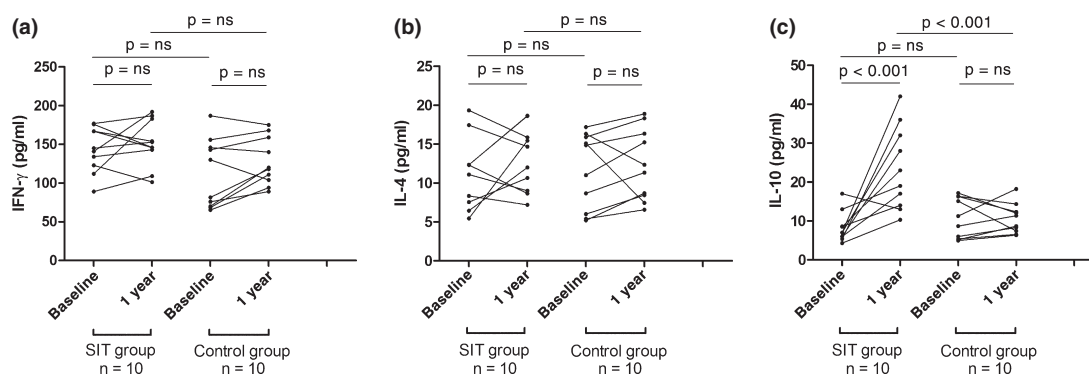


Figure 5 Der p1-induced synthesis of (a) IFN- γ , (b) IL-4, and (c) IL-10 in cultures of PBMCs obtained from SIT and control groups, at baseline and after treatment for 1 yr (ns, not significant; PBMCs, peripheral blood mononuclear cells; SIT, allergen-specific immunotherapy).

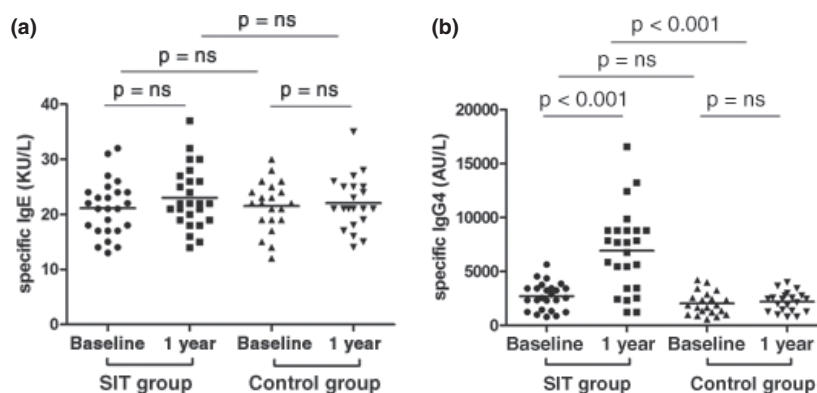


Figure 6 Allergen-specific serum IgE (a) and IgG4 (b) concentrations in SIT group ($n = 25$) and control group ($n = 21$) after treatment for 1 yr (ns, not significant; SIT, allergen-specific immunotherapy).

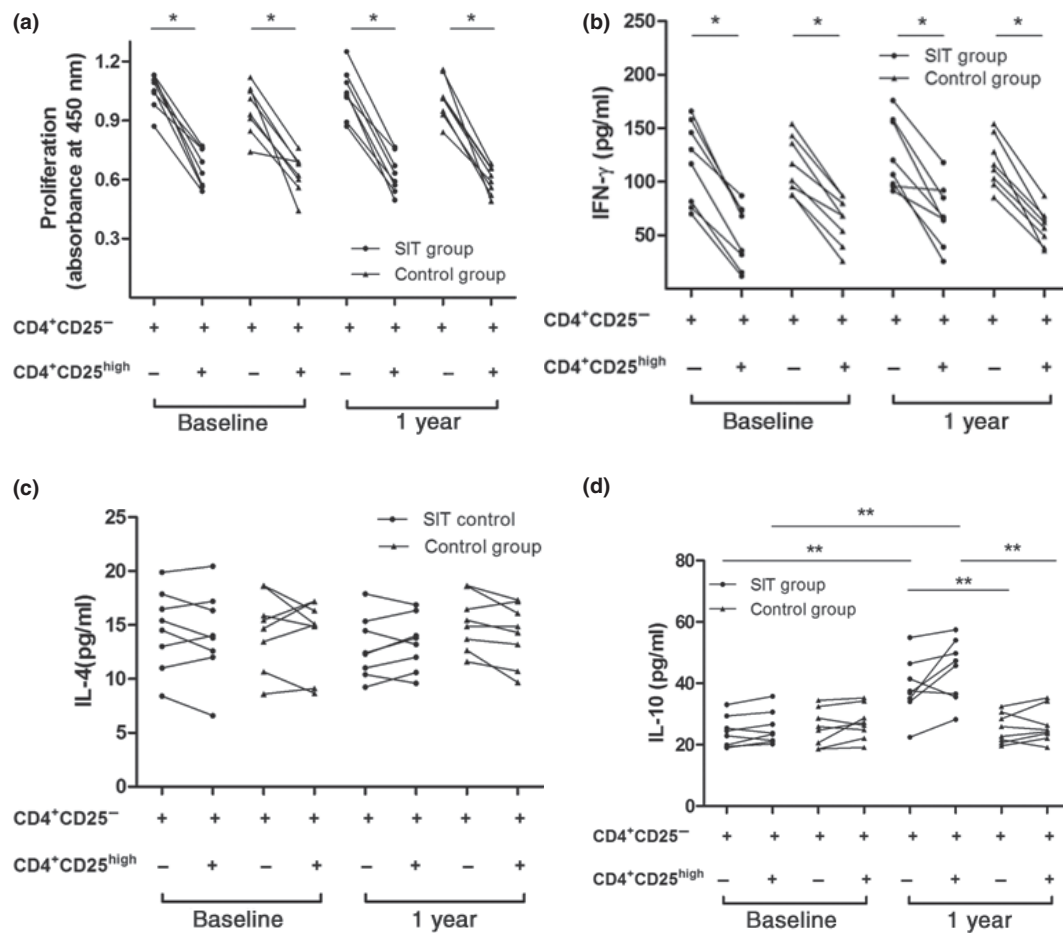


Figure 7 Effect of $CD4^+CD25^{high}$ T cells on Der p1-induced (a) $CD4^+CD25^-$ T-cell proliferation, (b) IFN- γ synthesis, (c) IL-4 synthesis, and (d) IL-10 synthesis at baseline and before and after treatment for 1 yr in SIT group and control group (* $p < 0.05$, ** $p < 0.001$; ns, not significant; SIT, allergen-specific immunotherapy).

$CD4^+CD25^{high}$ T cells, however, is in accordance with the finding of Smith et al. (31) who also found that cat allergen peptide-SIT did not enhance the suppression of Th2 cells by $CD4^+CD25^+$ T cells. Collectively, these results suggest that $CD4^+CD25^+Foxp3^+$ Treg cells in peripheral blood may play a limited role in allergen-SIT. It is possible that allergen-specific $CD4^+CD25^+Foxp3^+$ Treg cells are recruited to sites of local organs such as the nasal mucosa as for AR, resulting in the subsequent change regarding the number or function of $CD4^+CD25^+Foxp3^+$ Treg cells may change in the nasal mucosa.

In this study, the cytokine alterations were also detected in culture supernatants of Der p1-stimulated PBMCs. We demonstrated that IL-10 was significantly increased, but the levels of IL-4 and IFN- γ were unchanged after 1-yr SIT. This result supports the suggestion that a switch toward an IL-10-secreting Tr1 cell response may occur during SIT, as cells suppress effective T cells in an IL-10-dependent manner (8). Unfortunately, it was not possible to specifically assess the suppressive activity of IL-10 in Der p1-stimulated cultures blocked for IL-10 activity in this study, because of difficulties in

collecting blood samples of sufficient size from each child. However, we have recently investigated the suppressive effect of IL-10-secreting cells in adult patients with AR, by assessing cell proliferation and synthesis of IL-4 and IFN- γ in parallel Der p1-stimulated cultures of PBMCs and PBMCs depleted of IL-10-producing cells (17). This study that PBMCs depleted of IL-10-secreting T cells displayed enhanced proliferation and IL-4 and IFN- γ compared with un-depleted PBMCs.

Previous studies, however, have reported inconsistent results in the cytokine profiles secreted by allergen-stimulated PBMCs. Dehlink et al. (32) reported that levels of IL-4, IFN- γ , and IL-10 secreted by allergen-specific PBMCs after 12 days of culture did not change significantly after 8 wk of SIT in children. In contrast, Cosmi et al. (33) demonstrated that Der p1-driven IFN- γ and IL-10, but not IL-4, were significantly increased in cultured PBMCs from patients treated for 6 months, in comparison with those detected at the beginning of SIT. Differences in the cytokine profiles may relate to allergens, duration of SIT, age of patient, method of administration, as well as to the type and status of atopic disease.

However, from this study, we can conclude that the immune response in Der p-SIT children is skewed toward a suppressor cell response.

A previous study demonstrated that in healthy individuals, Tr1 cells represented the dominant subset for common environmental allergens, whereas a high frequency of allergen-specific IL-4-secreting T cells (Th2-like) was found in allergic individuals (5). Thus, a change in the dominant subset might lead to the recovery of allergy. In this study, although no significant alteration in $CD4^+CD25^+Foxp3^+$ Treg cells was evident, an obvious increase in Der p1-specific Tr1 cells was found in the SIT children. In addition, an increase in Tr1 cells was correlated with the decline of clinical symptom scores during 1-yr SIT. Our data thus suggest that SIT may preferentially result in an allergen-specific Treg cell response characterized by an increased number of Tr1 cells, but not $CD4^+CD25^+Foxp3^+$ Treg cells; which is in line with our previous finding of a deficiency of allergen-specific Tr1 cells in AR patients (17). Although the early induction of allergen-specific IL-10-secreting cells after 3 months of SIT (induction phase) has been reported (13, 34), data on their persistence during the maintenance phase are rare. Möbs et al. (35) reported that Bet v-1-specific Tr1 cells increased continuously from the end of the induction phase throughout the first year of SIT compared with pretreatment values in adult patients allergic to birch pollen. The results are consistent with our study, despite the different methods were used in the two studies: the frequency of Tr1 cells was analyzed by ELISPOT assay in Möbs' study (35), whereas flow cytometry was used in this study. Thus, the level of Tr1 cells in peripheral blood might be considered a marker of successful SIT in the future.

In contrast to other studies reporting a decrease in allergen-specific Th2 cells after SIT (36), our study has demonstrated that the frequency of Th2 cells was similar in both the SIT and untreated groups. On the other hand, the Der p1-specific Tr1/Th2 cell ratio was increased significantly in the SIT group, likely because of increased number of $IL-10^+$ cells. This finding suggests that the balance between allergen-specific Th2 cells and Tr1 cells, rather than the absolute number of Th2 cells, may be critical for the development of allergen tolerance (5). This finding, however, is consistent with the findings of Meiler et al. (37), who demonstrated that *in vivo* venom antigen-specific Th1 and Th2 cells show a switch toward IL-10-secreting Tr1 cells in beekeepers after multiple bee stings.

It has been shown that IL-10 can regulate specific isotype formation and skews the specific response from an IgE to an IgG4 dominated phenotype (38). This study demonstrated that SIT increased IgG4 production, but without a concur-

rent decrease in IgE levels. This is in accordance with the findings of Jutel et al. (13), who reported that HDM-SIT did not change specific IgE levels after 70 days of treatment, but significantly increased specific IgG4. Indeed, in one study, Akdis and Akdis (22) showed that SIT frequently induced a transient increase in serum-specific IgE, followed by a gradual decrease over a period of months or years of treatment. IgG4 is thought to capture the allergen before reaching the effector cell-bound IgE, and thus, increased IgG4 prevents the activation of mast cells and basophils (39) and attenuates the AR symptoms.

In conclusion, our data demonstrate that 1 yr of cluster SIT with Der p does not increase the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells or enhance the suppressive capacity of $CD4^+CD25^{high}$ T cells. However, it does increase the frequency of Tr1 cells and the production of IL-10 in PBMCs, with a simultaneous increase in IgG4 production, which play a mainly role in attenuating AR symptoms. In addition, the ratio of Tr1/Th2 cells is also increased as a consequence of increased number of Tr1 cells. Moreover, the increase in allergen-specific Tr1 cells is correlated with decreased clinical symptoms scores over the course of treatment. In this respect, this study is somewhat limited in that all immunological analyses could not be performed similarly in a time dependent manner over the course of treatment, primarily because of ethical aspects and disagreement on the part of children/guardians to be subjected to a multiple blood sampling protocol. Despite this limitation, collectively our findings suggest that an increased level of peripheral blood Tr1 cells might be a useful and valid marker of successful SIT in subjects with AR.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Bousquet J, Van CP, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; **108** (Suppl. 5): S147–334.
2. Warner JO. Allergic rhinitis is an extremely common problem, but prevalence rates widely vary between countries. *Pediatr Allergy Immunol* 2010; **21** (1 Pt 2): e105–6.
3. Pifferi M, Baldini G, Marrazzini G, et al. Benefits of immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract in asthmatic children: a three-year prospective study. *Allergy* 2002; **57**: 785–90.
4. Ling EM, Smith T, Nguyen XD, et al. Relation of $CD4^+CD25^+$ regulatory T cell suppression of allergen-driven T cell activation to atopic status and expression of allergic disease. *Lancet* 2004; **363**: 608–15.
5. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic

- individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004; **199**: 1567–75.
6. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25) Break-down of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**: 1151–64.
 7. Yagi H, Nomura T, Nakamura K, et al. Crucial role of FOXP3 in the development and function of human CD25⁺CD4⁺ regulatory T cells. *Int Immunol* 2004; **16**: 1643–56.
 8. Groux H, O'Garra A, Bigler M, et al. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; **389**: 737–42.
 9. Xu D, Liu H, Komai-Koma M, et al. CD4⁺CD25⁺ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice. *J Immunol* 2003; **170**: 394–9.
 10. Nieminen K, Valovirta E, Savolainen J. Clinical outcome and IL-17, IL-23, IL-27 and FOXP3 expression in peripheral blood mononuclear cells of pollen-allergic children during sublingual immunotherapy. *Pediatr Allergy Immunol* 2010; **21** (1 Pt 2): e174–84.
 11. Farfariello V, Amantini C, Nabissi M, et al. IL-22 mRNA in peripheral blood mononuclear cells from allergic rhinitis and asthmatic pediatric patients. *Pediatr Allergy Immunol* 2011; **22**: 419–23.
 12. Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003; **111**: 1255–61.
 13. Jutel M, Akdis M, Budak F, et al. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003; **33**: 1205–14.
 14. Levings MK, Sangregorio R, Sartirana C, et al. Human CD25⁺CD4⁺ T suppressor cell clones produce TGF- β , but not IL-10 and are distinct from type 1T regulatory cells. *J Exp Med* 2002; **196**: 1335–46.
 15. Zhang L, Wang C, Han D, Wang X, Zhao T, Liu J. Comparative study of cluster and conventional immunotherapy schedules with *Dermatophagoides pteronyssinus* in the treatment of persistent allergic rhinitis. *Int Arch Allergy Immunol* 2008; **148**: 161–9.
 16. Colas C, Monzon S, Venturini M, et al. Double-blind, placebo-controlled study with a modified therapeutic vaccine of *Salsola kali* (Russian thistle) administered through use of a cluster schedule. *J Allergy Clin Immunol* 2006; **117**: 810–6.
 17. Han D, Wang C, Lou W, Gu Y, Wang Y, Zhang L. Allergen-specific IL-10-secreting type 1 T regulatory cells, but not CD4⁺CD25⁺Foxp3⁺ T cells, are decreased in peripheral blood of patients with persistent allergic rhinitis. *Clin Immunol* 2010; **136**: 292–301.
 18. Anderson SJ, Coleclough C. Regulation of CD4 and CD8 expression on mouse T cells active removal from the surface by two mechanisms. *J Immunol* 1993; **151**: 5123–34.
 19. Beyer M, Kochanek M, Darabi K, et al. Reduced frequencies and suppressive function of CD4⁺CD25^{hi} regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood* 2005; **106**: 2018–25.
 20. Gardner LM, Thien FC, Douglass JA, Roland JM, O'Hehir RE. Induction of T 'regulatory' cells by standardized house dust mite immunotherapy: an increase in CD4⁺CD25⁺ interleukin-10⁺ T cells expressing peripheral tissue trafficking markers. *Clin Exp Allergy* 2004; **34**: 1209–19.
 21. Sakaguchi S. Naturally arising Foxp3-expressing CD25CD4 regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**: 345–52.
 22. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2007; **119**: 780–9.
 23. Wei W, Liu Y, Wang Y, et al. Induction of CD4⁺CD25⁺Foxp3⁺IL-10⁺ T Cells in HDM-Allergic asthmatic children with or without SIT. *Int Arch Allergy Immunol* 2010; **153**: 19–26.
 24. Ajduk J, Marinic I, Aberle N, Rabatic S, Gagro A. Effect of house dust mite immunotherapy on transforming growth factor β 1-producing T cells in asthmatic children. *Ann Allergy Asthma Immunol* 2008; **100**: 314–22.
 25. Aslam A, Chan H, Warrell DA, Misbah S, Ogg GS. Tracking antigen-specific T-cells during clinical tolerance induction in humans. *PLoS ONE* 2010; **5**: e11028.
 26. Radulovic S, Jacobson MR, Durham SR, Nouri-Aria KT. Grass pollen immunotherapy induces Foxp3-expressing CD4⁺CD25⁺ cells in the nasal mucosa. *J Allergy Clin Immunol* 2008; **121**: 1467–72.
 27. Gavin MA, Torgerson TR, Houston E, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA* 2006; **103**: 6659–64.
 28. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005; **22**: 329–41.
 29. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor- β : the role of T regulatory cells. *Immunology* 2006; **117**: 433–42.
 30. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4⁺CD25⁺ regulatory T cells control Leishmania major persistence and immunity. *Nature* 2002; **420**: 502–7.
 31. Smith TRF, Alexander C, Kay AB, Larch M, Robinson DS. Cat allergen peptide immunotherapy reduces CD4⁺ T cell responses to cat allergen but does not alter suppression by CD4⁺CD25⁺ T cells: a double-blind placebo-controlled study. *Allergy* 2004; **59**: 1097–101.
 32. Dehlink E, Eiwegger T, Gerstmayr M, et al. Absence of systemic immunologic changes during dose build-up phase and early maintenance period in effective specific sublingual immunotherapy in children. *Clin Exp Allergy* 2006; **36**: 32–9.
 33. Cosmi L, Santarlasci V, Angeli R, et al. Sublingual immunotherapy with Dermatophagoides monomeric allergoid down-regulates allergen-specific immunoglobulin E and increases both interferon- γ and interleukin-10-production. *Clin Exp Allergy* 2006; **36**: 261–72.
 34. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007; **120**: 707–13.
 35. Möbs C, Slotosch C, Löffler H, Jakob T, Hertl M, Pfützner W. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol* 2010; **184**: 2194–203.
 36. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995; **154**: 4187–94.
 37. Meiler F, Zumkehr J, Klunker S, Rückert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med* 2008; **205**: 2887–98.
 38. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; **102**: 98–106.
 39. Ejrnaes AM, Svenson M, Lund G, Larsen JN, Jacobi H. Inhibition of rBe v 1-induced basophil histamine release with specific immunotherapy –induced serum immunoglobulin G: no evidence that Fc γ RIIB signalling is important. *Clin Exp Allergy* 2006; **36**: 273–82.