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Changes in CD4$^+$CD25$^+$FoxP3$^+$ Regulatory T Cells and Serum Cytokines in Sublingual and Subcutaneous Immunotherapy in Allergic Rhinitis with or without Asthma

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Keywords

Allergic rhinitis · Sublingual immunotherapy · Subcutaneous immunotherapy · Immunoglobulin G4 · Regulatory T cells · Cytokines

Abstract

Background: Few studies have directly compared the immunologic responses to specific subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). ***Objective:*** We aimed to directly compare clinical efficacy and immunological responses between SLIT and SCIT in allergic rhinitis (AR) sensitized to house dust mites. ***Methods:*** Sixty-seven patients (age 5–55 years) with moderate-severe *Dermatophagoides pteronyssinus* (Der-p) and *Dermatophagoides farinae* AR with or without asthma were randomized (2:2:1) into SLIT (n = 27), SCIT (n = 26) and placebo (n = 14) groups. Symptom and medication scores, visual analogue score, serum Der-p specific immunoglobulin G4 (Der-p-sIgG4), CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Tregs) and serum cytokines were measured. ***Results:*** After 1-year treatment, a significant improvement of total rhinitis score (TRS), total rhinitis medication score (TRMS) and visual analogue score occurred in both SLIT and SCIT. There were no differences in clinical efficacy except for TRMS (p = 0.026) when SLIT and SCIT were directly compared. CD4$^+$CD25$^+$FoxP3$^+$ Tregs had a trend towards upregulation in the 2 modes and inversely correlated with TRS (p = 0.024) only in SLIT. Der-p-sIgG4 significantly increased in SLIT and SCIT ($p < 0.05$), and it was 30 times higher in SCIT than SLIT after the treatment ($p < 0.05$). Serum interferon-γ significantly increased only in SCIT after 1 (p = 0.008), 6 (p = 0.007) and 12 (p = 0.008) months of treatment and inversely correlated with TRS (p = 0.032). ***Conclusion:*** While SCIT and SLIT have similar rates of clinical improvement, the 2 modes reveal heterogeneous changes of CD4$^+$CD25$^+$Foxp3$^+$ Tregs, sIgG4 and cytokines.

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Introduction

The prevalence of allergic rhinitis (AR) and asthma has increased dramatically in children and adults in China over recent decades [1, 2]. A recent survey in 18 major cities in mainland China revealed an average prevalence

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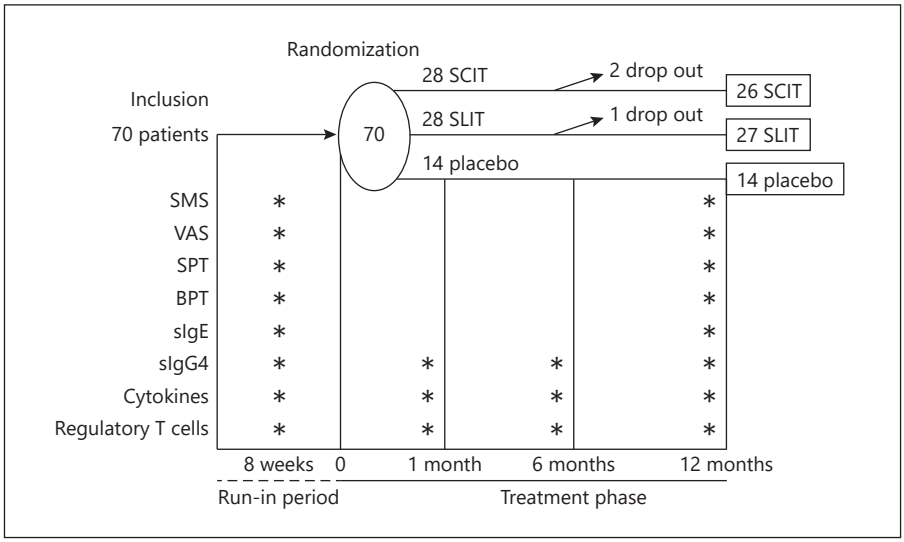

Fig. 1. Study design showing the flow of each stage. SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; SMS, symptom and medication score; VAS, visual analogue scale; sIgE, specific immunoglobulin E; sIgG4, specific immunoglobulin G4; SPT, skin prick test; BPT, bronchial provocation test.

of self-reported AR of 17.6%. Concurrently, the incidence of asthma was significantly higher among individuals with AR [1]. *Dermatophagoides pteronyssinus* (Der-p) and *Dermatophagoides farinae* (Der-f) are the most common allergens in patients with AR and asthma in China [3]. Pharmacotherapy offers clinical control of the disease but does not offer long-term benefit. Discontinuation of the medications results in relapse in symptoms and reduction of lung function [4, 5].

Allergen-specific immunotherapy (AIT) is the only treatment modality with the capacity to alter the natural course of allergic diseases [6]. Subcutaneous immunotherapy (SCIT) has been validated for the treatment of both asthma and AR and clinical improvement has been shown to persist for 3–10 years after discontinuation of the treatment [7, 8]. Compared to SCIT, sublingual immunotherapy (SLIT) appears to be associated with similar efficacy but with a lower incidence of systemic reactions [9, 10]. However, there is no conclusive evidence that one route is more cost effective or clinically effective [11].

Previous data showed that specific immunoglobulin G4 (sIgG4), T-cell and cytokines induced by AIT are regarded as immunological markers of clinical tolerance [12, 13]. But the immunological mechanisms underlying the clinical effects of SCIT and SLIT still remain debated. In this study, we directly compared the clinical efficacy and immunologic responses of both SCIT and SLIT in relation to immunologic changes for Der-p-sIgG4, $CD4^+CD25^+FoxP3^+$ Tregs and serum cytokines in patients with AR. The design was single centre, randomized, double-blinded, double-mimic, placebo controlled, 3 parallel grouped and prospectively followed for a period of 1 year.

Materials and Methods

Study Design

The study included 70 patients (34 females and 36 males, 5–55 years of age) diagnosed with mild-severe AR (with or without asthma) according to ARIA and GINA guidelines [4, 5], strictly sensitized to Der-p and Der-f as confirmed by a positive skin prick test and specific immunoglobulin E (sIgE) level of ≥0.35 kU/L. The patients with a clinical history of significant symptomatic seasonal or perennial AR caused by an allergen (e.g., pollens, cat, dog, cockroach… except house dust mites [HDMs]) to which the patient is regularly exposed and sensitized were excluded. Eligible patients underwent the 2-month run-in period to evaluate their baseline clinical conditions by means of symptom and medication scores, visual analogue scale (VAS), and histamine bronchial provocation test. According to the suggestion of Ethics Committee, patients were randomized into 3 groups (SLIT, SCIT and placebo, 2:2:1) by computer-generated method. The SLIT group received active sublingual drops and placebo subcutaneous injections, and the SCIT group received active subcutaneous injections and placebo sublingual drops, while the placebo group received placebo sublingual drops and placebo subcutaneous injections. Immunological parameters including total-IgE and sIgE were evaluated before and after 1-year treatment. Levels of Der-p-sIgG4, $CD4^+CD25^+FoxP3^+$ Tregs and serum cytokines including interferon-γ (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin (IL)-10 and IL-5 were evaluated before and 1, 6, and 12 months after the treatment (Fig. 1). The study was registered in the Chinese Clinical Trial Registry (ChiCTR-OOC-15006207) and the clinicaltrials.gov (NCT01603056) and approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (GYFYY-200908).

Treatment

The active ingredient of the trial allergen was a 50:50 mixture of Der-p and Der-f allergen extract administered as a glycerinated solution (SLIT, Pangramin, ALK-Abello, Spain) or Der-p extract adsorbed on aluminum hydroxide (SCIT, ALUTARDs. SQ, ALK-Abello). The SLIT was self-administered at home and included a

1-month induction phase (daily sublingual applications) followed by a maintenance phase (dose of 200 STU given 3 times a week), and the annual cumulative dose was 118.2 μg. The dose was placed under the tongue behind the teeth, for at least 3 min before swallowing. SCIT was administered in the clinic and included a 16-week induction phase (weekly subcutaneous injections) followed by a maintenance phase (dose of 100,000 Alutard SQ given every 6 weeks), and the annual cumulative dose was 81.2 μg (online suppl. Table 1; see www.karger.com/doi/10.1159/000503143 for all online suppl. material). The patients in the SLIT group received active drops and histamine dihydrochloride 0.01, 0.1, and 0.5 mg/mL for injections, patients in the SCIT group received active injection and glycerinated solution for the sublingual therapy, patients in the placebo group received both placebo injection and drops. The doctors carrying out the AIT had no knowledge of the randomization code, which was kept locked and sealed by a clinical coordinator not involved with the study. All patients were provided with rescue medication to relieve rhinitis as well as asthma symptoms during baseline or treatment period. The rescue medication was provided as open-labelled in a step-wise fashion depending on the persistency and severity of symptoms according to the patient diary card. For rhinitis symptoms: Step 1, short acting antihistamine; Step 2, nasal corticosteroid; Step 3, oral corticosteroid. For asthma symptoms: Step 1, short-acting β_2 agonists; Step 2, inhaled corticosteroid; Step 3, oral corticosteroid.

Symptom and Medication Scores

A 4-point scoring system (0: no symptoms, 1 point: mild symptoms, 2 points: moderate symptoms, 3 points: severe symptoms) was used to evaluate each rhinitis symptom (sneezing, nasal discharge, itching and nasal obstruction) and asthma symptom (wheezing, breathlessness, dyspnoea and cough). Total rhinitis/asthma scores (TRS/TAS) were defined as the sum of symptom score plus medication score [14]. Total rhinitis symptom score (TRSS) and total asthma symptom score represented all 4 rhinitis and asthma symptoms. Medication use was scored as follows: 1 point: short-acting β_2 agonists (salbutamol, 100 μg/puff); 2 points: steroids, budesonide nasal spray (64 μg/puff) or inhalation powder (200 μg/puff); 1.6 points: corticosteroid (prednisolone, 5 mg/tablet); 6 points: antihistamine (loratadine, 10 mg/tablet), and were calculated as total rhinitis medication score (TRMS) and total asthma medication score.

Visual Analogue Scale

A 10-cm line to grade severity of symptoms from "no symptoms" (0 cm) to "severe symptoms" (10 cm) was given to patients. The scale answers the question "How have your nasal complaints been today?" Patients were asked to record VAS from 0 to 10 before and after 12 months of treatment.

Histamine Challenge and Pulmonary Function

Lung functions were assessed using Cosmed Microquark Spirometer (Italy), which met the standards of the American Thoracic Society and European Respiratory Society [15]. Every subject took a 15-min rest before examination. FEV_1 was tested at least 3 times according to the quality control standard of forced vital capacity, and the difference value between the best 2 tests <150 mL was acceptable. Histamine dilutions, starting from 0.24 μmol, were increased in a doubling manner during bronchial provocation test. Bronchial hyper-responsiveness positive was defined as forced expiratory volume in 1 s/predicted value ratio (FEV_1%Pred) decreasing ≥20% of its baseline level when ≤7.8 μmol of cumulative dose of histamine is administered. All patients were not allowed to use short-acting β_2 agonist in 6 h or long-acting β_2 agonist in 12 h before the lung function test.

Immunoglobulin E and G4 levels

Serum IgE was measured by using ImmunoCAP system (Phadia 1000, ThermoFisher Scientific Inc., USA) according to the manufacturer's instructions and listed in kilo units per litre (kU/L). Der-p-sIgG4 was measured by a 4-layer sandwich ELISA [16]. The plate was coated with Der-p extract (ALK-Abello A/S) overnight at 4–8 °C. Then it was incubated with diluted patient serum for 2 h and mouse anti-IgG4 monoclonal antibody (ALK-Abello A/S) for 1 h. Peroxidase-labelled anti-mouse IgG (KPL Inc., USA) was used for detection. The IgG4 concentration was read at 450 nm (EL340, Bio-Tek Instruments Inc., USA). The sIgG4 levels were reported in arbitrary units (AU/mL).

Detection of $CD4^+CD25^+FoxP3^+$ Tregs

For the flow cytometric analysis, peripheral blood mononuclear cells were incubated to block non-specific binding and stained with each antibody. Cells were stained with fluorescein-isothiocyanate-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD25 (all from BD Biosciences), then fixed with Foxp3 fixation concentrate and permeabilized with permeabilization buffer, prior to incubation for 30 min at 4 °C with anti-Foxp3-PE. An EPICS XL flow cytometer (Beckman Coulter Inc., USA) was used for analysis. The percentage of $CD4^+CD25^+FoxP3^+$ Tregs in $CD4^+$ T cells was analysed by the gating strategy (online suppl. Fig. 1).

Analysis of Cytokines

Cytokines in serum were detected by the suspension chip method. A standard curve was set up and 50 μL MicroBeads was added to 96-well micro culture plates for 2 min and washed twice. The serum and standard sample (50 μL) were incubated for 40 min (washed with buffer 3 times) and then with 25 μL of test anti-body at 37 °C for 30 min. The streptavidin-PE was then added and incubated for 15 min. After washing, assay buffer (125 μL) was added, and the plates examined for cytokine levels (IL-5, IL-10, IFN-γ and TNF-α) by Lumine Bio-Plex 200 (Bio-Rad Laboratories, Inc., USA).

Data Analysis

Values are presented as mean ± SD or median (range). Comparisons for quantitative variables were performed by non-parametrical analysis, Mann-Whitney and Kruskal-Wallis tests for independent samples. Comparisons at 2 different time points were carried out using the Wilcoxon's test for related samples. Pearson's correlations were used to assess the relationships between clinical improvement and serum parameters. Significance was set at $p < 0.05$. SPSS 16.0 (SPSS Inc., USA) was used for analysis.

Results

Patients

Sixty-seven enrolled patients (3 dropped out) only sensitized to Der-p and Der-f were randomized (26 to

Table 1. Demographics and clinical characteristics of patients at screening

Characteristics	SCIT	SLIT	Placebo	*p* value
Patients, *n*	26	27	14	
Gender (female/male), *n*	11/15	15/12	5/9	ns
Age, years†	21.12±12.04	24.15±14.25	24.93±6.13	ns
Patients, *n* (%)				
With AR only	5 (19.2)	6 (22.2)	3 (21.4)	ns
With asthma and AR	21 (80.8)	21 (87.8)	11 (78.6)	ns
VAS†	5.00±1.96	5.35±2.01	4.40±2.06	ns
TRSS†	2.33±1.27	2.65±1.76	2.39±1.42	ns
TRMS†	5.12±4.53	4.40±2.60	3.35±2.50	ns
sIgE Der-p, kU/L†	50.22±37.29	47.89±35.95	49.59±35.93	ns
sIgE Der-f, kU/L†	52.96±34.35	51.83±34.09	53.63±38.08	ns
SPT Der-p†	11.25±3.91	11.02±4.53	11.27±5.49	ns
SPT Der-f†	12.00±3.71	10.19±4.38	11.39±4.18	ns
BHR positive, *n* (%)	17 (68)	13 (65)	5 (50)	ns

Comparison between groups using Kruskal-Wallis H. AR, allergic rhinitis; VAS, visual analogue scale; TRSS, total rhinitis symptom score; TRMS, total rhinitis medication score; SPT, skin prick test; BHR, bronchial hyperresponsiveness; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; NS, not significant, $p > 0.05$; Der-p, dermatophagoides pteronyssinus; Der-f, dermatophagoides farinae; sIgE, specific IgE. † Mean ± SD.

Table 2. Analysis of clinical efficacy among SCIT, SLIT and Placebo

	Placebo (mean ± SD)		SLIT (mean ± SD)		*p* value‡	SCIT (mean ± SD)		*p* value‡	*p* value†
	T0	T12	T0	T12		T0	T12		
TRS	5.74±3.05	6.72±2.80	7.05±3.72	3.75±2.68	0.045*	7.45±5.11	3.44±2.82	0.024*	0.207
TRSS	2.39±1.42	1.96±1.27	2.65±1.76	1.29±1.35	0.605	2.33±1.27	1.82±1.84	0.605	0.605
TRMS	3.35±2.50	4.76±2.44	4.40±2.60	2.46±1.90	0.977	5.12±4.53	1.62±2.52	0.026*	0.026*
TAS	0.78±1.20	0.75±1.08	1.83±2.50	1.44±1.95	0.042*	1.64±1.53	0.72±0.79	0.024*	0.386
TASS	0.56±0.99	0.61±1.03	0.92±0.89	0.59±0.87	0.362	0.57±0.68	0.43±0.51	0.362	0.362
TAMS	0.22±0.58	0.13±0.23	0.91±2.08	0.85±1.43	0.177	1.07±1.13	0.29±0.62	0.060	0.179
VAS	4.40±2.06	3.64±1.89	5.35±2.01	3.40±2.10	0.068	5.02±1.96	2.13±1.79	0.068	0.140

The *p* value of multiple tests was corrected by the BH method (Benjamini and Hochberg, 1995). TRS, total rhinitis score; TRSS, total rhinitis symptoms score; TRMS, total rhinitis medication score; TAS, total asthma score; TASS, total asthma symptoms score; TAMS, total asthma medical score; VAS, visual analogue scale (for allergy rhinitis); SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; T0, at baseline; T12, at 12 months after treatment. * $p < 0.05$; ‡ *p*, SCIT or SLIT vs. Placebo (change from 12 months to baseline); † *p*, SCIT vs. SLIT (change from 12 months to baseline).

SCIT, 27 to SLIT, 14 to the placebo group). The baseline levels of sIgE, symptom and medication scores and the wheal diameter of skin prick test were similar between the 3 groups, and no significant differences were observed in regard to demographic characteristics (Table 1).

Clinical Efficacy

After 1 year of treatment, a significant improvement of TRS, TRMS and VAS occurred in both SLIT and SCIT compared with baseline ($p < 0.05$). TRS ($p = 0.045$) and TAS ($p = 0.042$) were significantly reduced in SLIT when compared with the placebo group (Table 2). As for SCIT, the TRS ($p = 0.024$), TRMS ($p = 0.026$) and TAS ($p = 0.024$) were all significantly reduced when compared with the placebo group. A significant reduction of TRMS within the SCIT group was observed when directly compared with the SLIT group ($p = 0.026$), while other scores were no different (Table 2).

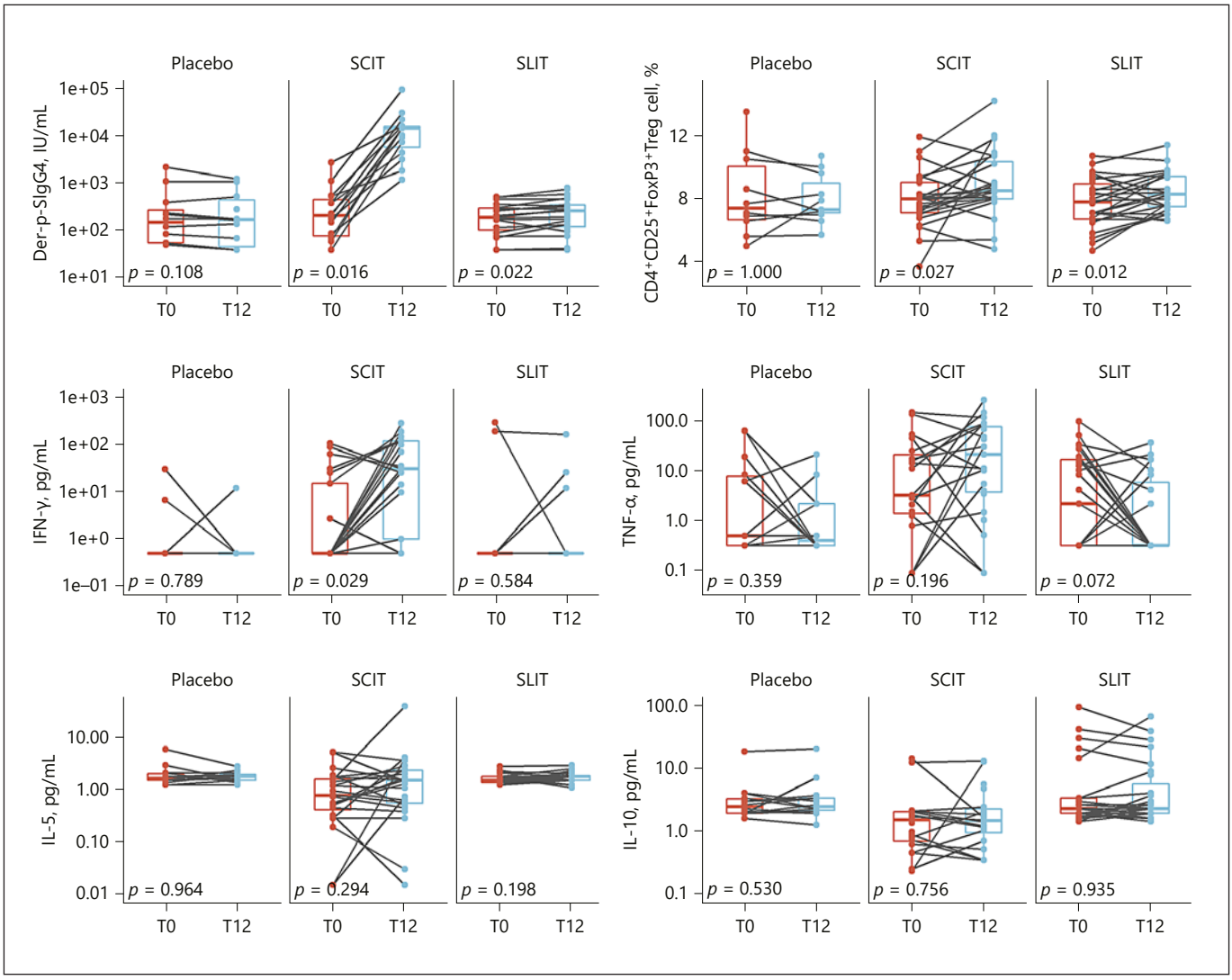

Fig. 2. The levels of biomarkers before and after 12 months of treatment. Paired values for Wilcoxon's signed-rank and Mann-Whitney U test. *p* value <0.05 is statistically significant. T0, at baseline; T12, at 12 months after treatment; $CD4^+CD25^+FoxP3^+$ Treg cell (%), the percentage of $CD4^+CD25^+FoxP3^+$ Treg cells in $CD4^+$ T cells. SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; Der-p, *Dermatophagoides pteronyssinus*; sIgG4, specific immunoglobulin G4; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; IL, interleukin.

Lung Functions and Der-p and Der-f sIgE Values

No difference was found for FEV_1%Pred among 3 groups at 12 months after treatment ($p > 0.05$). Compared with baseline (Table 1), significant increases in Der-p and Der-f sIgE levels were observed in all 3 groups after 12 months of treatment (Placebo, 57.70 ± 34.00 kU/L, p = 0.020, 60.59 ± 35.37 kU/L, p = 0.032; SLIT, 57.18 ± 35.79 kU/L, p = 0.033, 62.23 ± 33.34 kU/L, p = 0.009; SCIT, 60.28 ± 36.45 kU/L, p = 0.026, 63.45 ± 35.67 kU/L, p = 0.010), while no difference was found among the 3 groups.

Serum sIgG4 Values

A significant increase over baseline in Der-p sIgG4 in SCIT (p = 0.016) and SLIT (p = 0.022) groups after 1-year of treatment was observed (Fig. 2; online suppl. Fig. 2). The mean level of Der-p IgG4 in the SCIT group was almost 30 times higher than that of the SLIT group after therapy ($p < 0.05$; Fig. 2, 4). But no correlation was found between clinical improvement and the increase of Der-p sIgG4 in the SLIT or the SCIT group (Fig. 3).

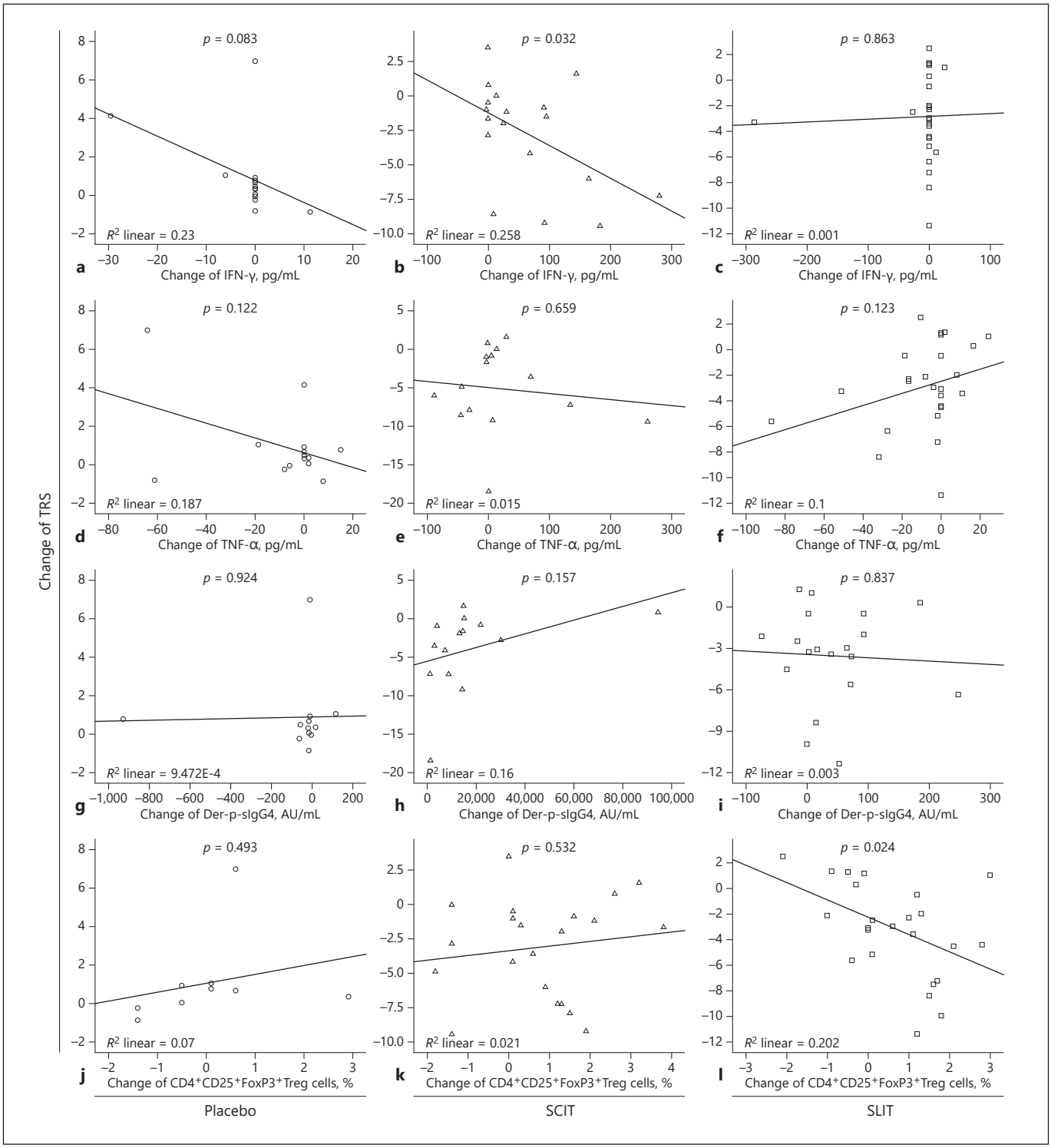

Fig. 3. The correlation analysis. **a–c** The correlation between change in IFN-γ and clinical improvement. **d–f** The correlation between change in TNF-α and clinical improvement. **g–i** The correlation between the change in Der-p-sIgG4 and clinical improvement. **j–l** The correlation between the change in rate of $CD4^+CD25^+FoxP3^+$ Treg cells and clinical improvement. Significance was evaluated by 2-tailed Pearson's correlations analysis. *p* value <0.05 is statistically significant. TRS, total rhinitis score; Der-p, *Dermatophagoides pteronyssinus*; sIgG4, specific immunoglobulin G4; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha.

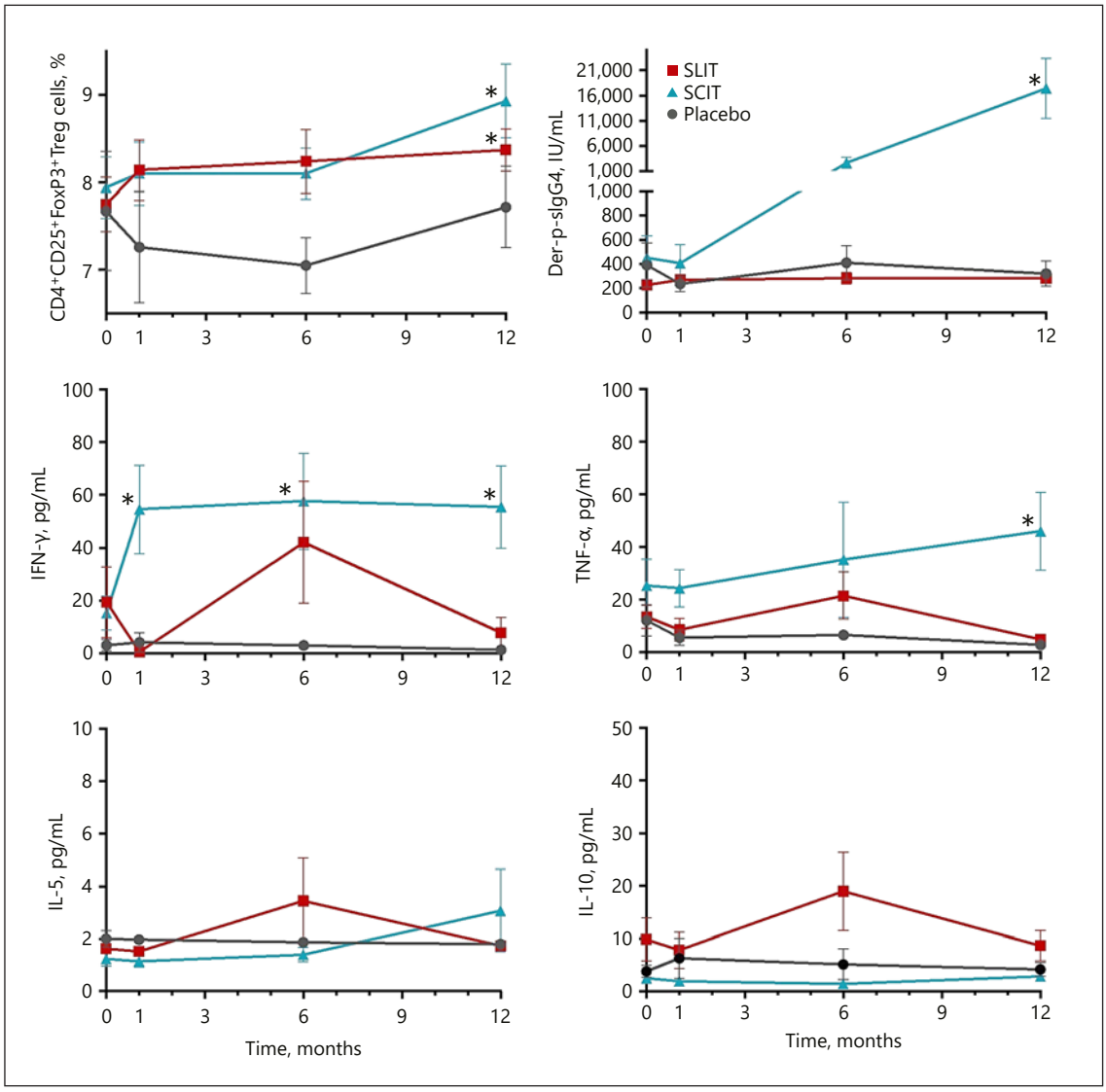

Fig. 4. The median values for levels of IFN-γ, TNF-α, IL-5 and IL-10 before and 1, 6, and 12 months after initiation of treatment. * *p* significant increases in serum level of biomarkers were observed in SCIT or SLIT at different points in time when compared with placebo group. SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; Der-p, *Dermatophagoides pteronyssinus*; sIgG4, subcutaneous immunotherapy G4; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor alpha; IL, interleukin.

Analysis of CD4⁺CD25⁺FoxP3⁺ Tregs

A significant increase in percentage of $CD4^+CD25^+$ $FoxP3^+$ Tregs in $CD4^+$ T cells occurred in subjects treated with SLIT ($p = 0.012$) and SCIT ($p = 0.027$) after 1 year when compared with the baseline (Fig. 2; online suppl. Fig. 2). Also, the percentage in ether SLIT or SCIT was significantly higher than the placebo group at 1 year (Fig. 4). The change of $CD4^+CD25^+FoxP3^+$ Tregs inversely correlated with TRS ($p = 0.024$) only in the SLIT group (Fig. 3).

Cytokines in Serum

A significant increase in serum level of IFN-γ was observed in SCIT after 1-, 6- and 12-month treatment ($p = 0.008$, $p = 0.007$, $p = 0.008$, respectively) compared with the placebo group (Fig. 4). Also, the change of IFN-γ inversely correlated with TRS ($p = 0.032$) only in the SCIT group (Fig. 3). Compared with the placebo group, significant increases in TNF-α were observed in SCIT ($p = 0.009$) at 12 months (Fig. 4), while no difference was found when compared with baseline (Fig. 2; online suppl.

Fig. 2). No significant increase was found for serum IL-5 and IL-10 among 3 groups after 1-year treatment (Fig. 2; online suppl. Fig. 2).

Discussion

Our study revealed that SCIT and SLIT had similar clinical efficacy and there was a trend towards the upregulation of $CD4^+CD25^+FoxP3^+$ Tregs in both SCIT and SLIT, while the Tregs correlated with clinical improvement only in SLIT. IFN-γ was induced at the early stage of treatment and correlated with clinical improvement in SCIT. And no significant changes of other cytokines such as IL-5, IL-10 and TNF-α were found among groups.

Both SCIT and SLIT have been accepted as treatment for mite-AR and asthma; however, some studies have yielded variable results [17–21]. Although direct comparative trials of 2 modes for efficacy are lacking, some data suggest that SCIT might have a slightly better efficacy profile [17, 22]. According to our direct comparative study, SCIT provided a little better outcome of TRMS than SLIT. But it was not enough to state that SCIT works better than SLIT. These 2 modes had similar clinical efficacy, while the total annual cumulative dosages of Der-p were different (SLIT, 118.2 μg; SCIT, 81.2 μg) according to pharmaceutical instructions. It was not clear whether the cumulative dosage was the main cause that affected clinical outcomes. The differences in clinical efficacy of our and other studies may result from different immune-mechanisms involving immunological parameters responses and methodological differences between the 2 therapeutic modes.

In this comparative study, an increase in Der-p and Der-f sIgE was observed in all 3 groups after 1-year therapy, which was in line with our previous study that levels of sIgE increased during 1-year treatment in SCIT [23]. Furthermore, perennial HDM allergens were detected in very high levels in household dust all year round in Guangzhou city [24], where all 3 groups of our patients exposed during the treatment could also explain their higher sIgE level. This finding is also consistent with some other studies [16, 25, 26]. However, other studies reported that the sIgE levels decreased the first year following AIT [27, 28]. The inconformity in sIgE levels of the studies may be due to the differences of geographical environments and immunologic responses in subjects of the studies.

Previous data demonstrated that increases in sIgG4 level correlated with the clinical improvement in SLIT [29] and the high level of sIgG4 in SCIT linked with an increase in serum inhibitory activity for sIgE binding to B cells [30, 31]. Also, one concept was that the counter-IgG responses induced by SCTI could lead to steric hindrance of IgE by the reactivity in the vicinity of IgE epitopes or the occupation of certain amino acid interacted with IgE [32]. As for our study, the increases in allergen-specific IgG4 level were observed in both SCIT and SLIT, and SCIT had a higher magnitude of sIgG4 than SLIT, but there was no correlation observed between sIgG4 and clinical improvement, which was consistent with some reports [27, 33]. AIT did not always induce cross-protective IgG antibodies [34] and our previous study also demonstrated that sIgG4 was not correlated with clinical efficacy in the early phase of SCIT [23]. According to our findings together with the findings of others, we suggested that the level of sIgG4 is just an immunological phenomenon, often not directly correlated with clinical outcomes. However, its absolute quantity in sera reflects the ability of immune-reactive but not functional levels in AIT.

There was a trend towards an increased percentage of $CD4^+CD25^+Foxp3^+$ Tregs in peripheral blood during peanut or pollen SLIT [35, 36]. Furthermore, our data showed a significant increase in $CD4^+CD25^+FoxP3^+$ Tregs in both HDMs SLIT and SCIT, which indeed correlated with clinical improvement only in SLIT. Though the Treg cells' response in SLIT has not been fully elucidated, the mechanism may be different from SCIT. It has been found that the percentage in $CD4^+CD25^+Foxp3^+$ Tregs did not change after 1 year of SCIT [37], while there was a significant reduction of follicular helper T cells in SCIT, which might relate to IL-2 production from allergen-specific T-cell responses [38]. It is also reported that SCIT may induce Th1 responses and reduce synthesis of Th2 cytokines [39], whereas in SLIT, induction of oral mucosal immune tolerance may be more important and oral dendritic cells are able to induce T-regulatory cells [40]. From these findings confirmed by us and others, we can speculate that SLIT and SCIT have different mechanisms for treatment and $CD4^+CD25^+Foxp3^+$ Tregs may play an important role in SLIT. However, we still need to further determine the details of follicular helper T, follicular regulatory T cells, subtypes of Tregs such as induced T-regulatory cells, Tr1 and transforming growth factor-β-producing $CD4^+$ T cells (Th3) in the 2 modes in a large sample size.

The roles of cytokines in AIT are under dispute. It has been reported that the level of serum IL-5 was significantly lower in SLIT compared with placebo, and no significant difference was found in IL-10 or IFN-γ levels in serum [17, 25]. In another randomized controlled trial, IL-10 significantly increased in pollen SLIT, whereas no changes were observed for IL-5 and IFN-γ [18]. The

seeming inconsistencies might result from methodological differences. In our double-blind randomized study, a significant increase in IFN-γ was observed, only in SCIT, after 1, 6, and 12 months of treatment. This is supported by a prior study, in which SCIT induced the increase in IFN-γ production in the peripheral blood [41]. As a Th1 cytokine, IFN-γ can lead to immune deviation from a Th2 to a Th1-driven response and promote IgM-producing B cells to class switch to IgG-producing B cells. Our results also showed that clinical improvement correlates with increased IFN-γ production in SCIT. According to our study and other studies, we considered that SCIT offers an earlier Th1 response, which leads to clinical improvements with earlier induction of IFN-γ than SLIT.

The limitations of the study were the unmatched sample size in the placebo group and the presence of different components of allergens between SCIT and SLIT. It was due to the 1-year duration of the study that the Ethics Committee of the hospital suggested that the protocol be changed to the manner of 2:2:1 to avoid non-active treatment in placebo patients for a comparatively longer period of time. However, we believed that the sample size of 14 in the placebo group still had enough power to give significant statistical analysis. Although there were 2 species of HDM (Der-p and Der-f) with 1:1 concentration for SLIT drops but only Der-p in SCIT injection, both allergens share similarity in allergenicity and immunogenicity and would be considered to induce similar clinical efficacy and immunologic responses [42].

Conclusions

Our study shows that despite the similar clinical efficacy, SCIT and SLIT have different immunological mechanisms with heterogeneous changes of $CD4^+CD25^+Foxp3^+$ Tregs, sIgG4 and cytokines. SCIT offers an earlier Th1 response with induction of IFN-γ than SLIT, while $CD4^+CD25^+$ $Foxp3^+$ Tregs associated with oral mucosal immune tolerance leads to clinical improvement during the early stage of SLIT. Further randomized comparative studies with a large-size population are needed to determine the long-term changes and the immunologic biomarkers with relevance to clinical improvement in these 2 modes of immunotherapy.

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Statement of Ethics

The study was registered in the Chinese Clinical Trial Registry (ChiCTR-OOC-15006207) and the clinicaltrials.gov (NCT01603056) and approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (GY-FYY-200908). Written consent was obtained from all adult patients or parents of children.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

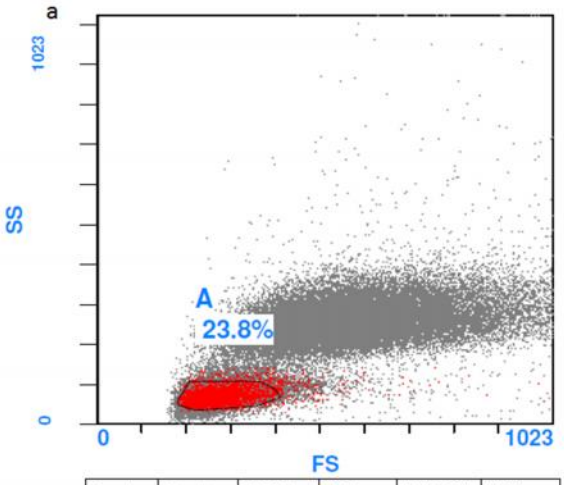
J.L. mainly designed the study. M.X. performed the survey, collected the data, performed the statistical analysis and drafted the manuscript. M.F., Y.D., N.W., and Q.S. mainly performed the clinical tests and collected the data.

References

1 Wang XD, Zheng M, Lou HF, Wang CS, Zhang Y, Bo MY, et al. An increased prevalence of self-reported allergic rhinitis in major Chinese cities from 2005 to 2011. Allergy. 2016 Aug;71(8):1170–80.
2 Li J, Wang H, Chen Y, Zheng J, Wong GW, Zhong N. House dust mite sensitization is the main risk factor for the increase in prevalence of wheeze in 13- to 14-year-old schoolchildren in Guangzhou city, China. Clin Exp Allergy. 2013 Oct;43(10):1171–9.
3 Li J, Sun B, Huang Y, Lin X, Zhao D, Tan G, et al.; China Alliance of Research on Respiratory Allergic Disease. A multicentre study assessing the prevalence of sensitizations in patients with asthma and/or rhinitis in China. Allergy. 2009 Jul;64(7):1083–92.
4 Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al.; AllerGen. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy. 2008 Apr;63 Suppl 86:8–160.
5 Global Initiative for Asthma (GINA). Global Strategy for Asthma Management and Prevention. Available from: http://www.ginasthma.org; revised 2014 Vancouver, 2014.
6 Alvarez-Cuesta E, Bousquet J, Canonica GW, Durham SR, Malling HJ, Valovirta E; EAACI, Immunotherapy Task Force. Standards for practical allergen-specific immunotherapy. Allergy. 2006;61(s82 Suppl 82):1–20.

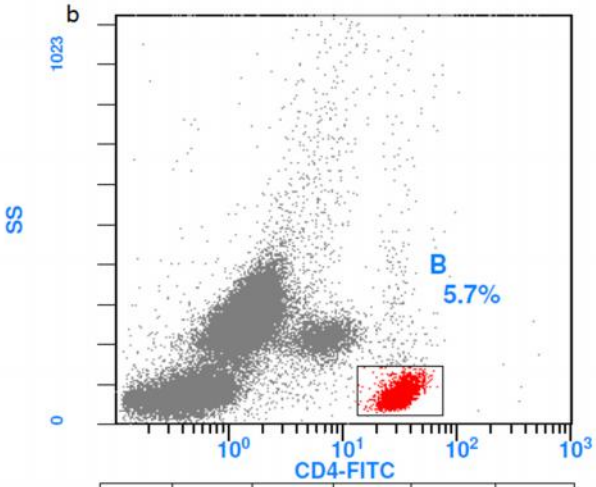
7 Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med. 1999 Aug;341(7):468–75.
8 Jacobsen L, Niggemann B, Dreborg S, Ferdousi HA, Halken S, Høst A, et al.; The PAT investigator group. Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. Allergy. 2007 Aug;62(8):943–8.
9 Wilson DR, Lima MT, Durham SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. Allergy. 2005 Jan;60(1):4–12.
10 Kim ST. Outcome of sublingual immunotherapy in patients with allergic rhinitis sensitive to house dust mites. Allergy Asthma Immunol Res. 2015 Mar;7(2):99–100.
11 Cox L. The role of allergen immunotherapy in the management of allergic rhinitis. Am J Rhinol Allergy. 2016 Jan-Feb;30(1):48–53.
12 Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy. J Allergy Clin Immunol. 2011 Jan;127(1):18–27.
13 Karakoc-Aydiner E, Eifan AO, Baris S, Gunay E, Akturk E, Akkoc T, et al. Long-Term Effect of Sublingual and Subcutaneous Immunotherapy in Dust Mite-Allergic Children With Asthma/Rhinitis: A 3-Year Prospective Randomized Controlled Trial. J Investig Allergol Clin Immunol. 2015;25(5):334–42.
14 Canonica GW, Baena-Cagnani CE, Bousquet J, Bousquet PJ, Lockey RF, Malling HJ, et al. Recommendations for standardization of clinical trials with Allergen Specific Immunotherapy for respiratory allergy. A statement of a World Allergy Organization (WAO) taskforce. Allergy. 2007 Mar;62(3):317–24.
15 Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. Eur Respir J. 2005 Nov;26(5):948–68.
16 Lai X, Li J, Xiao X, Liu E, Zhang C, Wang H, et al. Specific IgG4 production during house dust mite immunotherapy among age, gender and allergic disease populations. Int Arch Allergy Immunol. 2013;160(1):37–46.
17 Yukselen A, Kendirli SG, Yilmaz M, Altintas DU, Karakoc GB. Effect of one-year subcutaneous and sublingual immunotherapy on clinical and laboratory parameters in children with rhinitis and asthma: a randomized, placebo-controlled, double-blind, double-dummy study. Int Arch Allergy Immunol. 2012;157(3):288–98.
18 Khinchi MS, Poulsen LK, Carat F, André C, Hansen AB, Malling HJ. Clinical efficacy of sublingual and subcutaneous birch pollen allergen-specific immunotherapy: a randomized, placebo-controlled, double-blind, double-dummy study. Allergy. 2004 Jan;59(1):45–53.
19 Eifan AO, Akkoc T, Yildiz A, Keles S, Ozdemir C, Bahceciler NN, et al. Clinical efficacy and immunological mechanisms of sublingual and subcutaneous immunotherapy in asthmatic/rhinitis children sensitized to house dust mite: an open randomized controlled trial. Clin Exp Allergy. 2010 Jun;40(6):922–32.
20 Antúnez C, Mayorga C, Corzo JL, Jurado A, Torres MJ. Two year follow-up of immunological response in mite-allergic children treated with sublingual immunotherapy. Comparison with subcutaneous administration. Pediatr Allergy Immunol. 2008 May;19(3):210–8.
21 Mungan D, Misirligil Z, Gürbüz L. Comparison of the efficacy of subcutaneous and sublingual immunotherapy in mite-sensitive patients with rhinitis and asthma—a placebo controlled study. Ann Allergy Asthma Immunol. 1999 May;82(5):485–90.
22 Tabatabaian F, Casale TB. Selection of patients for sublingual immunotherapy (SLIT) versus subcutaneous immunotherapy (SCIT). Allergy Asthma Proc. 2015 Mar-Apr;36(2):100–4.
23 Feng M, Su Q, Lai X, Xian M, Shi X, Wurtzen PA, et al. Functional and Immunoreactive Levels of IgG4 Correlate with Clinical Responses during the Maintenance Phase of House Dust Mite Immunotherapy. J Immunol. 2018 Jun;200(12):3897–904.
24 Zhang C, Li J, Lai X, Zheng Y, Gjesing B, Spangfort MD, et al. House dust mite and storage mite IgE reactivity in allergic patients from Guangzhou, China. Asian Pac J Allergy Immunol. 2012 Dec;30(4):294–300.
25 Kim ST, Han DH, Moon IJ, Lee CH, Min YG, Rhee CS. Clinical and immunologic effects of sublingual immunotherapy on patients with allergic rhinitis to house-dust mites: 1-year follow-up results. Am J Rhinol Allergy. 2010 Jul-Aug;24(4):271–5.
26 Marcucci F, Sensi L, Incorvaia C, Dell'Albani I, Di Cara G, Frati F. Specific IgE response to different grass pollen allergen components in children undergoing sublingual immunotherapy. Clin Mol Allergy. 2012 Jun;10(1):7.
27 Chen J, Zhou Y, Wang Y, Zheng Y, Lai X, Westermann-Clark E, et al. Specific immunoglobulin E and immunoglobulin G4 toward major allergens of house-dust mite during allergen-specific immunotherapy. Am J Rhinol Allergy. 2017 May;31(3):156–60.
28 Moingeon P, Batard T, Fadel R, Frati F, Sieber J, Van Overtvelt L. Immune mechanisms of allergen-specific sublingual immunotherapy. Allergy. 2006 Feb;61(2):151–65.
29 Radulovic S, Wilson D, Calderon M, Durham S. Systematic reviews of sublingual immunotherapy (SLIT). Allergy. 2011 Jun;66(6):740–52.
30 Wachholz PA, Soni NK, Till SJ, Durham SR. Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. J Allergy Clin Immunol. 2003 Nov;112(5):915–22.
31 Ejrnaes AM, Svenson M, Lund G, Larsen JN, Jacobi H. Inhibition of rBet v 1-induced basophil histamine release with specific immunotherapy -induced serum immunoglobulin G: no evidence that FcgammaRIIB signalling is important. Clin Exp Allergy. 2006 Mar;36(3):273–82.
32 Gadermaier E, Flicker S, Aberer W, Egger C, Reider N, Focke M, et al. Analysis of the antibody responses induced by subcutaneous injection immunotherapy with birch and Fagales pollen extracts adsorbed onto aluminum hydroxide. Int Arch Allergy Immunol. 2010;151(1):17–27.
33 Aasbjerg K, Backer V, Lund G, Holm J, Nielsen NC, Holse M, et al. Immunological comparison of allergen immunotherapy tablet treatment and subcutaneous immunotherapy against grass allergy. Clin Exp Allergy. 2014 Mar;44(3):417–28.
34 Gadermaier E, James LK, Shamji MH, Blatt K, Fauland K, Zieglmayer P, et al. Epitope specificity determines cross-protection of a SIT-induced IgG4 antibody. Allergy. 2016 Jan;71(1):36–46.
35 Kim EH, Bird JA, Kulis M, Laubach S, Pons L, Shreffler W, et al. Sublingual immunotherapy for peanut allergy: clinical and immunologic evidence of desensitization. J Allergy Clin Immunol. 2011 Mar;127(3):640–6.e1.
36 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 Sep;120(3):707–13.
37 Ajduk J, Marinic I, Aberle N, Rabatic S, Gagro A. Effect of house dust mite immunotherapy on transforming growth factor beta1-producing T cells in asthmatic children. Ann Allergy Asthma Immunol. 2008 Apr;100(4):314–22.
38 Schulten V, Tripple V, Seumois G, Qian Y, Scheuermann RH, Fu Z, et al. Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells. J Allergy Clin Immunol. 2018 Feb;141(2):775–777.e6.
39 Passalacqua G, Durham SR; Global Allergy and Asthma European Network. Allergic rhinitis and its impact on asthma update: allergen immunotherapy. J Allergy Clin Immunol. 2007 Apr;119(4):881–91.
40 Novak N, Bieber T, Allam JP. Immunological mechanisms of sublingual allergen-specific immunotherapy. Allergy. 2011 Jun;66(6):733–9.
41 Savolainen J, Nieminen K, Laaksonen K, Laiho T, Jacobsen L, Lahesmaa R, et al. Allergen-induced in vitro expression of IL-18, SLAM and GATA-3 mRNA in PBMC during sublingual immunotherapy. Allergy. 2007 Aug;62(8):949–53.
42 Li J, Wu Y, Yang Y, Huang N, Li W, Zhang S, et al. The efficacy and safety of two commercial house dust mite extracts for allergic rhinitis: a head-to-head study. Int Forum Allergy Rhinol. 2019 Aug;9(8):876–82.

a



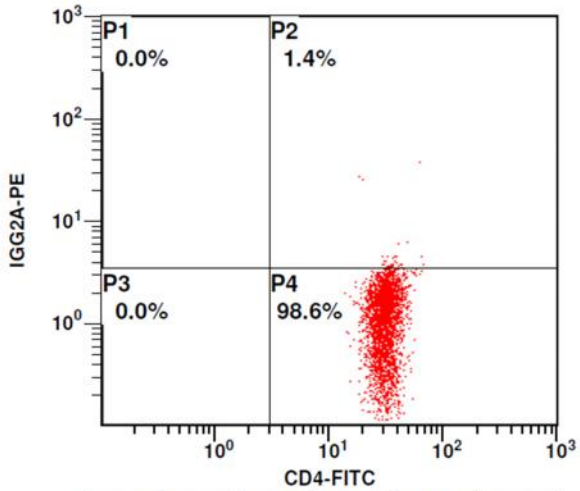
Region	Number	%Gated	X-Mean	X-HPCV	Y-Mean
A	11906	23.81	270.6	15.5	71.8

b



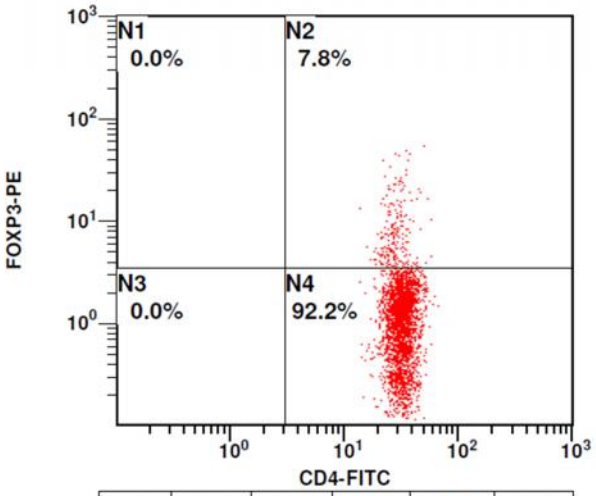
Region	Number	%Gated	X-Mean	X-HPCV	Y-Mean
B	2837	5.67	31.7	0.7	73.7

c



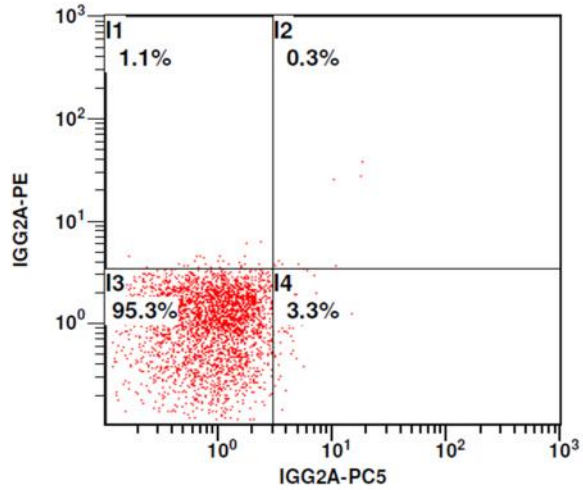
Region	Number	%Gated	X-Mean	X-HPCV	Y-Mean
P1	0	0.00	0.0	0.0	0.0
P2	40	1.36	37.4	0.1	6.0
P3	0	0.00	0.0	0.0	0.0
P4	2906	98.64	30.7	2.4	1.3

d



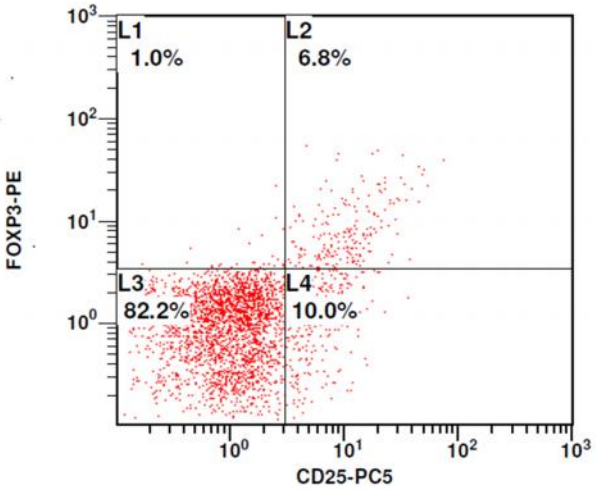
Region	Number	%Gated	X-Mean	X-HPCV	Y-Mean
N1	0	0.00	0.0	0.0	0.0
N2	222	7.83	29.0	0.1	10.0
N3	0	0.00	0.0	0.0	0.0
N4	2615	92.17	32.0	0.8	1.2

e



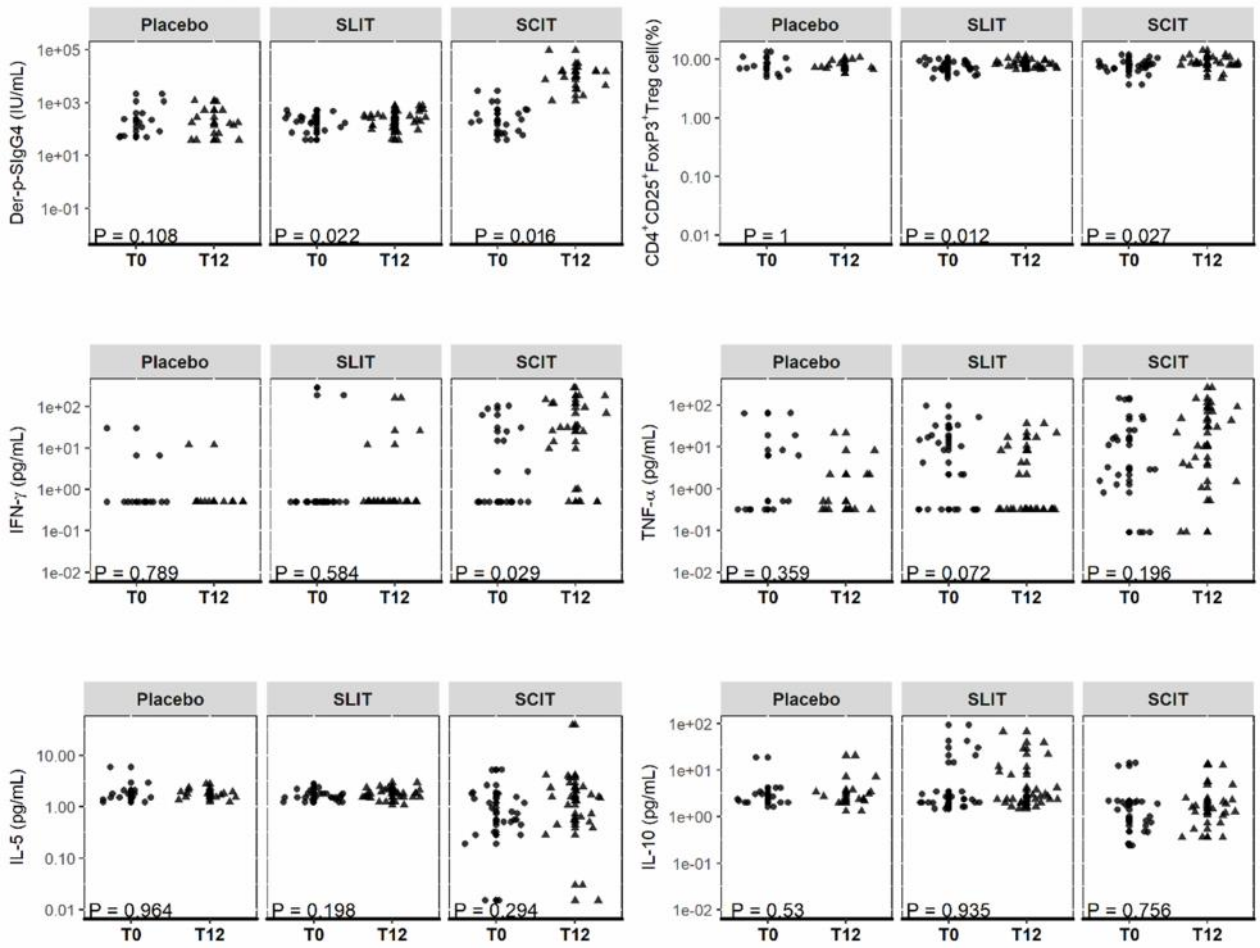
Region	Number	%Gated	X-Mean	X-HPCV	Y-Mean
I1	31	1.05	1.2	1.7	4.1
I2	9	0.31	8.3	0.2	12.8
I3	2809	95.35	1.0	1.6	1.3
I4	97	3.29	4.0	0.1	1.5

f



Region	Number	%Gated	X-Mean	X-HPCV	Y-Mean
L1	29	1.02	1.7	0.2	5.3
L2	193	6.80	13.2	0.2	10.8
L3	2331	82.16	1.1	0.5	1.2
L4	284	10.01	6.1	0.4	1.5

Suppl. Fig. 1. $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs gating strategy. Example result obtained for a single patient before AIT. PBMC was stained with combination of surface antigens (anti-CD4 and anti-CD25) and with anti-FoxP3. CD4 positive cells from PBMC were gated (b, dot plot SS versus CD4-FITC, gate B) and analyzed for CD25 and FoxP3 expression (d, dot plot FoxP3-PE versus CD4-FITC; f, dot plot FoxP3 versus CD25-PC). Cells in L2 gate were considered Tregs. c & e, negative control dot plot.



Suppl. Fig. 2. The analysis of biomarkers before and after 12 months of treatment shown by all individual data points. Paired values for Wilcoxon's signed-rank and Mann–Whitney U-test. *p* value < 0.05 is statistically significant. T0, at baseline; T12, at 12 months after treatment; $CD4^+CD25^+FoxP3^+$ Treg (%), the percentage of $CD4^+CD25^+FoxP3^+$ Treg cells in $CD4^+$ T cells.

Patient Diary Card

DIARY CARD PROVIDED ON: ____|____|____

STARTING REGISTER DATE: ____|____|____

DIARY CARD COLLECTED ON: ____|____|____

The present card has been designed for a daily and accurate control of your allergic symptoms and medication. Please, read carefully the following recommendations and record everyday the data requested. The card must be shown to the investigator when asked for (see date on the front page).

SYMPTOMS

Please, write down daily in the corresponding chart, your rhinitis and asthma symptoms according to their severity as indicated below:

0 = No symptoms (no sign/symptom evident).
1 = Mild symptoms (sing/symptom clearly present, but minimal awareness; easily tolerated).
2 = Moderate symptoms (definite awareness of sing/symptom that is bothersome but tolerable).
3= Severe symptoms (sing/symptom that is hard to tolerate; causes interference with activities of daily living and/or sleeping).

MEDICATION

In case of rescue medication intake, please indicate when you have taken the drug as well as its dosage (2 tables, 1 pill, 2 puffs, etc…) following the investigator recommendation.

Please, remember to complete the charts with a "**0**" in case of **symptoms and/or medication absence**. Do not leave it empty.

	1st week							2nd week						
LUNG	M	T	W	T	F	S	S	M	T	W	T	F	S	S
Cough														
Wheeze														
Chest tightness/shortness of breath (dyspnoea)														
Exercise induced symptoms														
NOSE														
Runny nose														
Blocked nose														
Sneezing														
Itchy nose														
MEDICATION														
Loratadine														
Nasal Budesonide														
Prednisolone														
Salbutamol														
Inhaled Budesonide														

	3rd week							4th week						
LUNG	M	T	W	T	F	S	S	M	T	W	T	F	S	S
Cough														
Wheeze														
Chest tightness/shortness of breath (dyspnoea)														
Exercise induced symptoms														
NOSE														
Runny nose														
Blocked nose														
Sneezing														
Itchy nose														
MEDICATION														
Loratadine														
Nasal Budesonide														
Prednisolone														
Salbutamol														
Inhaled Budesonide														

	5th week							6th week						
LUNG	M	T	W	T	F	S	S	M	T	W	T	F	S	S
Cough														
Wheeze														
Chest tightness/shortness of breath (dyspnoea)														
Exercise induced symptoms														
NOSE														
Runny nose														
Blocked nose														
Sneezing														
Itchy nose														
MEDICATION														
Loratadine														
Nasal Budesonide														
Prednisolone														
Salbutamol														
Inhaled Budesonide														

	7th week							8th week						
LUNG	M	T	W	T	F	S	S	M	T	W	T	F	S	S
Cough														
Wheeze														
Chest tightness/shortness of breath (dyspnoea)														
Exercise induced symptoms														
NOSE														
Runny nose														
Blocked nose														
Sneezing														
Itchy nose														
MEDICATION														
Loratadine														
Nasal Budesonide														
Prednisolone														
Salbutamol														
Inhaled Budesonide														

Supplemental Table 1. Doses and duration of immunotherapy

	SCIT	SLIT	Placebo
Induction phase	Vial 1 (100 SQ-U/mL) : 0.2, 0.4, 0.8 mL Vial 2 (1000 SQ-U/mL) : 0.2, 0.4, 0.8 mL Vial 3 (10 000 SQ-U/mL) : 0.2, 0.4, 0.6, 0.8 mL Vial 4 (100 000 SQ-U/mL) 0.1, 0.2, 0.4, 0.6, 0.8, 1 mL	Vial 0 (1.6 STU/mL) : 1–5 drops Vial 1 (8 STU/mL) : 1–5 drops Vial 2 (40 STU/mL) : 1–5 drops Vial 3 (200 STU/mL) : 1–5 drops Vial 4 (1000 STU/mL) : 1–5 drops	None active ingredients. Package and dosage form were same as SLIT and SCIT.
Duration	Given weekly for 16 weeks	Given daily for 30 days	
Maintenance dose	Vial 4 (100 000 SQ-U/mL) : 1 mL (every 6 weeks)	Vial 4 (1000 STU/mL) : 5 drops (3 times/week)	
Daily maximum dose	Der p 1, 9.8 μg (100 000 SQ-U)	Der p 1/Der f 1, 0.8/0.8 μg (200 STU)	
Cumulative dose (1 year)	Der p 1, 81.2 μg	Der p 1/Der f 1, 118.2 /118.2 μg	

SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; SQ-U, standard quality unit; STU, standard therapeutic unit.