

Distinct modulation of allergic T cell responses by subcutaneous vs. sublingual allergen-specific immunotherapy

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Clinical & Experimental Allergy

Summary

Background Allergen-specific immunotherapy is the only curative treatment for type I allergy. It can be administered subcutaneously (SCIT) or sublingually (SLIT). The clinical efficacy of these two treatment modalities appears to be similar, but potential differences in the immunological mechanisms involved have not been fully explored.

Objective To compare changes in the allergen-specific T cell response induced by subcutaneous vs. sublingual administration of allergen-specific immunotherapy (AIT).

Methods Grass pollen-allergic patients were randomized into groups receiving either SCIT injections or SLIT tablets or neither. PBMCs were tested for Timothy grass (TG)-specific cytokine production by ELISPOT after *in vitro* expansion with TG-peptide pools. Phenotypic characterization of cytokine-producing cells was performed by FACS.

Results In the SCIT group, decreased IL-5 production was observed starting 10 months after treatment commenced. At 24 months, T cell responses showed IL-5 levels significantly below the before-treatment baseline. No significant reduction of IL-5 was observed in the SLIT or untreated group. However, a significant transient increase in IL-10 production after 10 months of treatment compared to baseline was detected in both treatment groups. FACS analysis revealed that IL-10 production was associated with CD4⁺ T cells that also produced IFN γ and therefore may be associated with an IL-10-secreting type 1 cell phenotype.

Conclusion and clinical relevance The most dominant immunological changes on a cellular level were a decrease in IL-5 in the SCIT group and a significant, transient increase of IL-10 observed after 10 months of treatment in both treated groups. The distinct routes of AIT administration may induce different immunomodulatory mechanisms at the cellular level.

Keywords SLIT, SCIT, T cell, timothy grass allergy

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Cite this as: V. Schulten, V. Trippl, K. Aasbjerg, V. Backer, G. Lund, P. A. Würtzen, A. Sette and B. Peters.

Clinical & Experimental Allergy, 2016 (46) 439–448.

Introduction

Allergic rhinitis affects up to 40% of the general population in North America [1, 2] and is associated with significant symptoms for the patients and high costs for society [3]. Allergic rhinitis symptoms are commonly treated with prescription drugs such as oral antihistamines and intranasal corticosteroids [4, 5]. The only potentially curative treatment available for allergic rhinitis is allergen-specific immunotherapy (AIT), introduced over a century ago [6, 7]. AIT desensitizes patients to specific allergens through the administration

of increasing doses of allergen extracts. This regimen is thought to gradually tolerate the immune system [8]. While subcutaneous injection (SCIT) is the most common route of administration, sublingual administration of drops or tablets (SLIT) is becoming increasingly popular [9, 10].

Numerous studies have demonstrated SCIT's efficacy in terms of long-lasting control of allergic symptoms [11]. Relative to SCIT, efficacy and safety of SLIT tablets have also been demonstrated in large double-blind placebo-controlled registration trials [12, 13] and consequently received FDA approval. As reviewed by

Nelson [14, 15], results from meta-analyses suggest that both treatments are effective, though differences between the two have been reported for certain parameters [14].

Similarities in immunological changes associated with SCIT [16] and SLIT [17] have been reported, including increased IL-10 production and decreased Th2 responses [18]. However, differences in the dosing and route of administration of SCIT and SLIT might suggest that differences in the underlying mechanisms of action may exist. Data comparing SCIT- vs. SLIT-induced immunomodulatory mechanisms of antibody and T cell responses throughout and after treatment are limited. Studies in which patients with similar sensitizations are recruited and recalled at the same time points, treated with the same allergen species and assessed by well-defined allergen-specific immunological read-outs are very rare, and conclusive data are lacking.

In this study, we utilized samples collected during a previously published study [19] that compared the effects of SCIT and SLIT on allergen-specific antibody responses, including IgE titres, IgG4/IgE-blocking assays, facilitated antigen presentation (FAP) and effector cell activation assessed by basophil activation test (BAT). In that study, 40 Timothy grass (TG)-allergic patients were recruited and randomly assigned to SCIT, SLIT and control groups ($n = 15$, 15 and 10, respectively). Blood donations were obtained at various time points throughout the 15 month treatment period. Analysis of TG-specific IgE-blocking factor (representing IgE inhibition) revealed that both SLIT- and SCIT-induced significant increases in IgE blocking compared to untreated controls. However, SCIT-treated patients exhibited a more rapid increase of IgE blocking compared to the SLIT-treated group. Inhibition of IgE-facilitated antigen presentation was observed in the SCIT group (max inhibition of 22.5% after 3 months) and the SLIT group (max. inhibition of 17.2% after 15 months) but no inhibition was observed in the controls [19]. Finally, basophil activation tests (BAT) revealed a significant reduction in basophil activation following SCIT (decreased by a factor of -1.4) and SLIT (-0.71) after 15 months. No reduction was observed in controls.

In a continuation of the study described above, we examined the effects of SCIT and SLIT on allergen-specific T cell responses. Cytokine production after antigen stimulation was assessed longitudinally in T cells from patients who were treated either by subcutaneous or by sublingual AIT or received no treatment. The TG-specific T cell responses were investigated over time using previously validated peptide pools derived from Phl p allergens and recently identified additional Phl p T cell targets.

Materials and methods

Patient cohort

Forty grass-allergic patients were randomized 15 + 15 + 10 in SCIT, SLIT, and untreated control groups, respectively, and followed for 15 months of treatment. Clinical data and inclusion/exclusion criteria are reported elsewhere [19]. Demographic and serological information is presented in Table 1. The current study on the cellular immunology included 24 patients (7 untreated controls, 8 SCIT, and 9 SLIT) from whom sufficient cell numbers were available at -2 , 1, 3, 7, 10, 12, and 15 month time points relative to treatment start in January 2011. For a small subset of SCIT and SLIT donors, cells were available after 24 months of treatment and responses were assessed (SCIT $n = 2$, SLIT $n = 5$).

SCIT and SLIT treatment

Treatment was administered as described before [19]. Briefly, SCIT patients received gradual up-dosing weekly injections of ALK Alutard 225 'P. pratense'

Table 1. Demographic and clinical patient information

Donor ID	Gender	Age	Treatment modality	Phl p IgE (kU/L)*	Phl p SPT**
GABL001	M	27	SLIT	21.68	9
GABL007	F	39	SLIT	3.97	12
GABL008	M	32	SLIT	65.93	10
GABL009	M	40	SLIT	15.39	14
GABL028	M	24	SLIT	0.43	9
GABL029	M	35	SLIT	18.22	13
GABL030	M	47	SLIT	28.87	19
GABL040	M	41	SLIT	5.46	10
GABL042	M	28	SLIT	3.04	9
GABL023	F	22	SCIT	64.60	8
GABL036	M	24	SCIT	114.25	10
GABL037	M	31	SCIT	31.73	12
GABL045	F	44	SCIT	9.04	13
GABL057	M	39	SCIT	1.56	10
GABL059	M	59	SCIT	6.48	13
GABL060	M	45	SCIT	47.82	11
GABL078	M	20	SCIT	7.48	12
GABL039	M	20	CONTROL	41.39	9
GABL043	F	22	CONTROL	49.64	12
GABL049	F	42	CONTROL	51.59	15
GABL050	F	64	CONTROL	10.58	14
GABL051	M	27	CONTROL	8.45	8
GABL061	M	43	CONTROL	1.64	8
GABL062	F	45	CONTROL	1.52	14

*Timothy grass extract (Phl p)-specific IgE as measured by RAST.

**Timothy grass extract (Phl p)-specific skin prick test (SPT) diameter (mm) measured as largest measure of the two perpendicular SPT diameters.

starting at 20 SQ-U, reaching a maintenance dose of 100 000 SQ-U at week 15, after which this maintenance dose was given every 2 months throughout the remainder of the study. SLIT treatment was administered daily with ALK Grazax 75 000 SQ-T sublingual tablets taken orally.

Blood collection and PBMC isolation

PBMCs were isolated from freshly drawn (< 3 h) heparinized blood by gradient centrifugation as described elsewhere [20]. Cell numbers were adjusted to $10\text{--}20 \times 10^6/\text{mL}$ by the addition of freezing medium with DMSO and FCS at final concentrations of 10 and 20 per cent v/v, respectively. The PBMCs were subsequently cryopreserved in freezing media containing 10% DMSO.

Peptide synthesis

Peptides were purchased from A and A (San Diego, CA) as purified material on a large (10 mg) scale. Individual peptides were resuspended in DMSO at a final concentration of 40 mg/mL. Pools of 19 or 20 immunodominant peptides from known Phl p allergens or additional TG T cell antigens that were defined in previous studies [21–23] were used.

Peptide pools

Three peptide pools, each comprised of previously identified T cell epitopes, were used. The first pool (P20) consisted of 20 peptides derived from Phl p 1, 2, 3, 4, 5 and 13, which were found to be associated with the great majority of IL-5 production (> 75%) in a previous study [23]. The second pool (P19) was comprised of 19 immunodominant peptides from additional T cell-reactive TG antigens described in a subsequent study [21]. The third pool (MNA) consisted of 20 peptides also derived from the additional TG antigens. It is associated with strong IL-5 production (957 spot-forming cells (SFC)) in allergic donors, but significantly reduced (> twofold) production in donors who had received SCIT treatment (425 SFC) [22]. As MNA pool and the P19 pool share 9 peptides, MNA-reactivity data are not shown in the analysis summarized in Fig. 3.

PBMC expansion in vitro

PBMCs were thawed and cultured in RPMI 1640 (Ω Scientific, Tarzana, CA, USA) supplemented with 5% human AB serum (Cellgro, Herndon, VA, USA) at a density of 2×10^6 cells/mL in 24-well plates (BD Biosciences, San Jose, CA, USA) and stimulated separately

with the 3 peptide pools derived from either the known Phl p allergens or previously described additional TG antigens [22] (5 $\mu\text{g}/\text{mL}$). For *in vitro* expansion, cells were kept at 37°C in 5% CO₂ and IL-2 (10 U/mL, eBioscience, San Diego, CA, USA) was added every 3 days after initial antigenic peptide stimulation. On day 14, cells were harvested and assessed for cytokine production in response to restimulation with peptide pool (5 $\mu\text{g}/\text{mL}$) by ELISPOT.

ELISPOT

All ELISPOT assays were performed in a blinded fashion, and treatment modality was revealed after full analysis of cellular cytokine responses. ELISPOT assays were performed as described previously [22, 23]. Briefly, plates were coated with either 5 $\mu\text{g}/\text{mL}$ anti-human IL-5 and anti-human IFN- γ or 5 $\mu\text{g}/\text{mL}$ anti-human IL-10 (Mabtech, Cincinnati, OH). The next day, 1×10^5 cells/well were plated and incubated with peptide pool (2.5 $\mu\text{g}/\text{mL}$). After 24 h, plates were developed with 2 $\mu\text{g}/\text{mL}$ biotinylated anti-human IL-5 Ab and anti-human IFN- γ -horseradish peroxidase or biotinylated anti-human IL-10 at 37°C. After 2 h, plates were washed and avidin-alkaline phosphatase complex was added (Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. Alkaline phosphatase spots were developed with Vector Blue Substrate Kit (Vector Labs, Burlingame, CA, USA). Horseradish peroxidase spots were developed with 3-amino-9-ethylcarbazole solution (Sigma-Aldrich, St. Louis, MO, USA). Based on previous experience [21–23], internal criteria for peptide pool positivity were 100 SFCs/ 10^6 PBMCs, $P \leq 0.05$, and a stimulation index (SI) ≥ 2 comparing stimulated cultures with unstimulated control cultures.

Intracellular cytokine staining

PBMCs were restimulated after 14 days of *in vitro* culture with 5 $\mu\text{g}/\text{mL}$ peptide pool in complete RPMI medium at 37°C in 5% CO₂. Unstimulated PBMCs were used to assess background cytokine production, and PMA/ionomycin was used as positive control. Golgiplug (BD Bioscience) was added after the first 2 initial hours of stimulation. After a total of 6 h incubation time, cells were harvested and stained for surface antigens CD4, CD3, CD8, CD14, CD19 and CRTh2 and Fixable Viability Dye (all from eBioscience).

After washing, cells were fixed with 4% paraformaldehyde followed by permeabilization using permeabilization buffer (0.5% saponin, 0.1% sodium azide and 1% BSA in PBS) and then stained for IL-10, IFN- γ and IL-5. Samples were acquired on a BD LSR II flow cytometer. Data were analysed using FlowJo software (Tree Star).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software package, version 6.0f (GRAPHPAD Software, Inc., La Jolla, CA, USA). *P*-values < 0.05 were considered significant. Comparisons across groups were carried out using the Mann–Whitney *U*-test (nonparametric, unpaired) and longitudinal comparisons within groups were performed using the Wilcoxon rank test (non-parametric, paired). No corrections were made for multiple hypothesis testing.

Results

In total, 24 donors were recruited (~ 30% females, average age 36). All donors had detectable Phl p-specific IgE (average of 25 kU/L) and positive skin prick test (average wheal of flare 11.4). Baseline T cell responses to TG-peptide stimulation were determined in 24 allergic patients before allergen-specific immunotherapy was initiated. Peripheral blood mononuclear cells (PBMCs) from TG-allergic patients were cultured *in vitro* with the three peptide pools constituting the most dominant IL-5-inducing Phl p peptides. Peptides in these pools were derived either from the known Phl p allergens (peptide pool P20) [23] or recently described additional T cell antigens (peptide pools P19 and MNA) [21, 22]. After 14 days of culture, cells were restimulated and cytokine production was measured by ELISPOT. Overall, in the allergic cohort tested, the Th2 response was most dominant. Significant IL-5 production (> 100 SFC/10⁶ PBMC) was observed in 21 of 24 patients for P20, 20 of 24 patients for P19 and in 21 of 24 patients for MNA (Fig. 1a). IL-5 production was highest in response to P20 (median 2,102 SFC/10⁶ PBMC), followed by P19 (median 1,632 SFC/10⁶ PBMC). The MNA pool triggered significantly lower IL-5 responses (median 818 SFC/10⁶ PBMC) compared to both P20 (*P* < 0.05) and P19 (*P* < 0.005) (Fig. 1a).

Significant levels of IFN γ production were detected in 22 of 24 patients for P20 (median response 838 SFC/10⁶ PBMC), 23 of 24 patients for P19 (median 1070 SFC/10⁶ PBMC) and 22 of 24 patients for the MNA pool (median 1007 SFC/10⁶ PBMC) (Fig. 1b). No significant differences in response magnitude between the different peptide pools were observed. Similarly, significant IL-10 responses were seen in 19 of 24 donors for P20, 20 of 24 for P19 and 19 of 24 for MNA (Fig. 1c). However, IL-10 production was much lower compared to IL-5 and IFN γ and no variation in response to the different peptide pools was observed (Fig. 1c).

Two months after the first visit, patients were randomized into SLIT (*n* = 9), SCIT (*n* = 8) and untreated (*n* = 7), and treatments were initiated. PBMC isolated from blood donations from these three groups of donors obtained throughout 15 months of treatment were assessed in ELISPOT assays as described above. For a small subset of SCIT and SLIT donors, cells were available after 24 months of treatment and responses were assessed (SCIT *n* = 2, SLIT *n* = 5). Figure 2 depicts fold changes in IL-5 responses, relative to the response before treatment, after stimulation with P20 (top panel), P19 (middle panel) or the MNA pool (lower panel) in the three groups (SLIT, SCIT and controls). Twofold changes are indicated by dashed lines, corresponding to likely relevant changes in the response, taking into account assay variability. Median IL-5 responses of untreated controls did not change > twofold at any time point (Fig. 2, statistical analysis follows in Fig. 3).

A greater than twofold decrease in IL-5 production in response to the three different peptide pools was observed in the SCIT group during months outside the pollen season. These decreases in IL-5 responses in SCIT-treated patients were most pronounced in early winter months of 2012 after 1 year of treatment (median SFC values changing from 1918 to 352 SFC for P20; 1293 to 425 for P19; 1608 to 89 for MNA) and

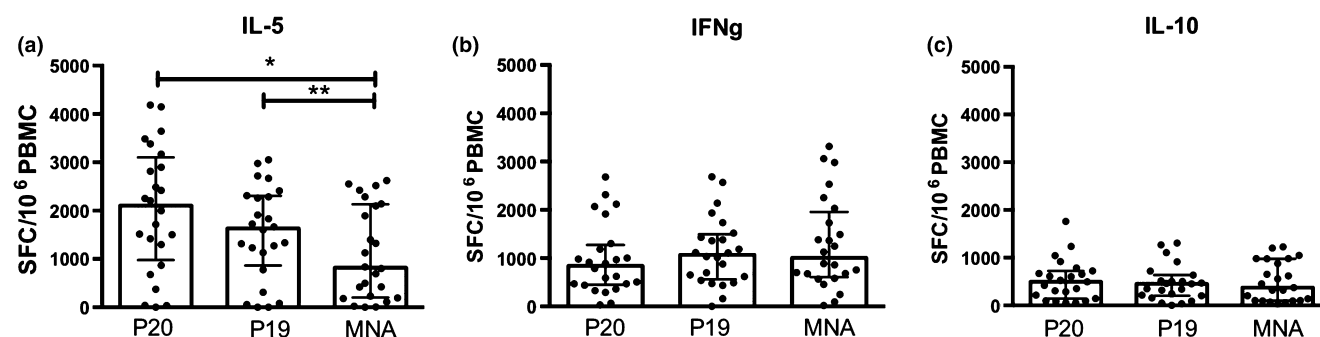


Fig. 1. Response magnitude in PBMC from TG-allergic patients 2 months before allergen-specific immunotherapy. A bar chart showing (a) IL-5, (b) IFN γ and (c) IL-10 production (SFC) of allergic patients in response to stimulation with P20, P19 and MNA; *N* = 24. Statistical analysis was performed using Mann–Whitney *U*-test. **P* < 0.05, ***P* < 0.005. Error bars indicate interquartile range.

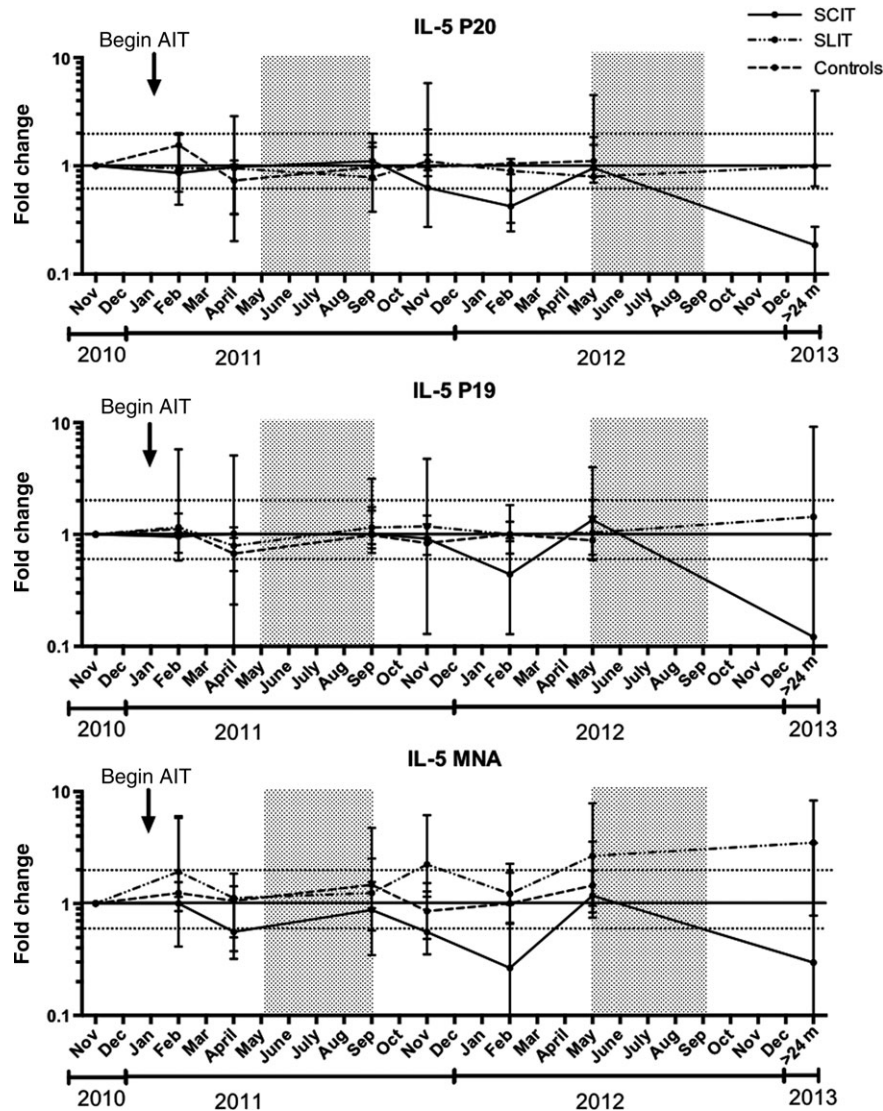


Fig. 2. Long-term changes in TG-specific IL-5 responses in PBMC from allergic subjects and patients undergoing SCIT or SLIT. IL-5 production is expressed here as fold change relative to the response before treatment commenced. The local grass pollen season is indicated as shaded area. Error bars indicate interquartile range.

after 24 months. During the second year of treatment, between the February 2012 and May 2012 time points, a rise back to baseline in IL-5 was detected. In contrast, the SLIT group showed no strong decreases in IL-5 throughout the 2 years of treatment (Fig. 2). An increased response to MNA pool stimulation during and following the second pollen season was, however, noted (800 median SFC increased to 1327 SFC after 15 months and 1453 SFC after 24 months). Comparing the kinetics of changes in IL-5 and IL-10 production to antibody changes (IgE blocking, facilitated antigen presentation, see Aashjerg *et al.*) suggests that the antibody response may be altered within 3 months of commencing treatment, while changes in cytokine

production only become detectable after 10–12 months (repository Fig. 1).

To avoid a potential effect of pollen exposure during allergen season, we focused our analysis on the out-of-season time points to better assess the impact of therapy on T cell responses in the absence of potential additional allergen exposure (Fig. 3). Furthermore, as reactivity patterns in response to all three pools were very similar, data from P20 and P19 were combined. Reactivity against MNA pool is excluded from this analysis because it overlaps with P19 by 9 peptides. IL-5 responses in the SCIT group were significantly lower at all three time points compared to the other groups (Fig. 3a) and the decreased response within the SCIT group became progressively

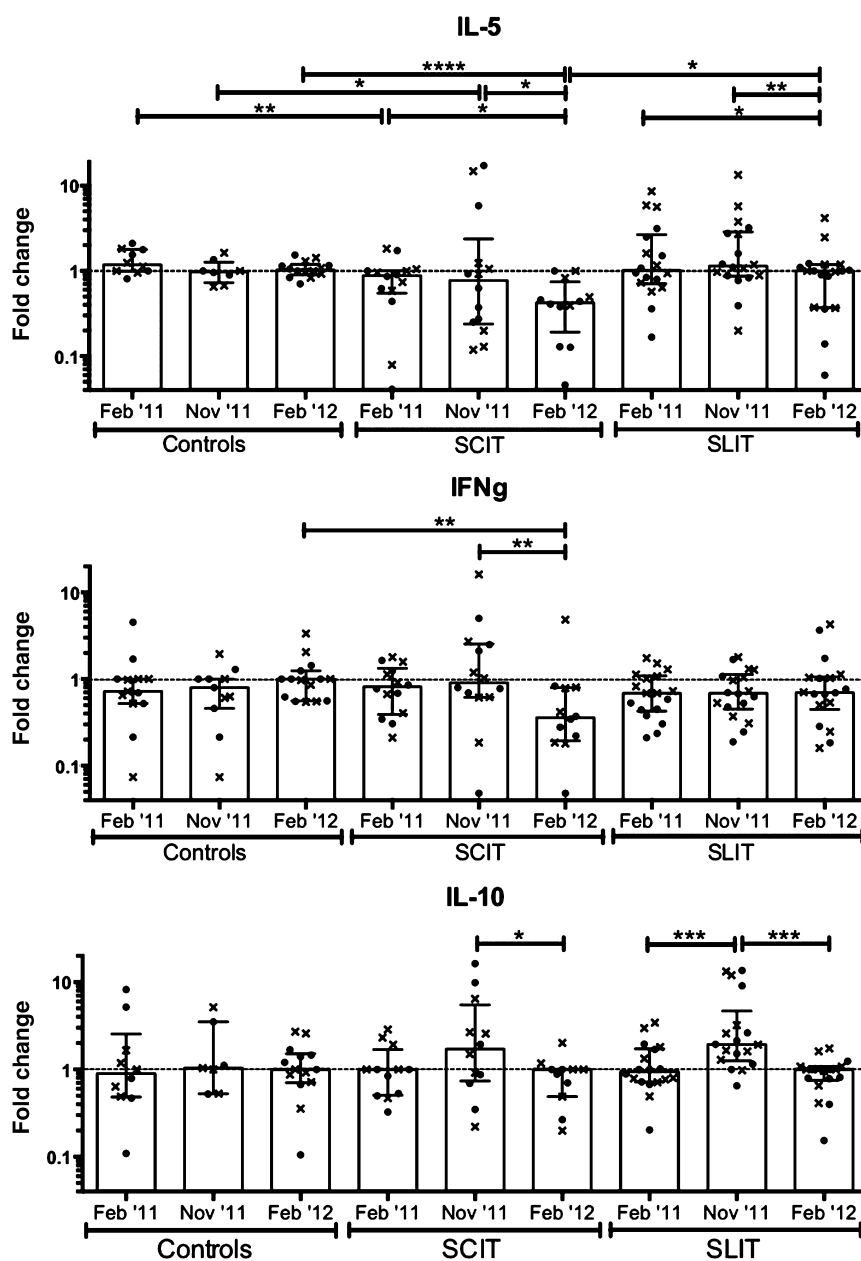


Fig. 3. Fold changes in longitudinal TG-specific responses at selected out-of-season time points throughout SCIT and SLIT and in allergic controls compared to responses before treatment. Bars are indicating the median. Dots and crosses represent individual donor- pool responses (P20- dot, P19- cross).

more pronounced over time. The SLIT group also showed a significant decrease in IL-5 responses after 12 months of treatment relative to the earlier time points but the difference from responses in untreated donors was not significant. IL-5 production in the control group remained unchanged (Fig. 3a).

In addition to IL-5, we also measured IFN γ and IL-10 responses (Fig. 3b,c) (complete data over the entire time course of the study is presented in repository Fig. 2). In the SCIT group, IFN γ responses were decreased after 12 months of therapy, compared to those at 10 months

and those of the control group at 12 months (Fig. 3b). Interestingly, unique changes in IL-10 production were detected over time (Fig. 3c). After 10 months, P20-specific IL-10 median SFC values were increased from 297 to 723 in SCIT and from 493 SFC to 1097 SFC in SLIT. P19 values were increased from 333 SFC to 523 SFC in the SCIT group and from 317 SFC to 1277 SFC in the SLIT group. This increase in the SLIT group was significant when compared to both earlier (1 month into treatment) and later (12 months into treatment) time points ($P < 0.0001$) within the same group (Fig. 3c).

To determine whether evaluating each donor-pool combination as a separate data point would introduce a bias for the cases where all pools induce similar cytokine production in an individual, we plotted the sum of all 3 pool responses per donor (repository Fig. 3). The low number of data points results in some loss of statistical significance. However, the overall trends towards reduction in IL-5 in the SCIT group and increases in IL-10 in both the SLIT and SCIT groups were still observed (repository Fig. 3).

Next, we wanted to determine whether CD4 T cells are associated with the transiently increased IL-10 production observed 10 months into both SCIT and SLIT treatment. IL-10 is an important anti-inflammatory cytokine that is produced by several different immune cells including T cells, dendritic cells and macrophages [24]. Intracellular cytokine-staining (ICS) assays were performed after peptide pool stimulation of cells from before treatment and 10 months into AIT, the time point at which increased IL-10 production was observed. The ICS assay confirmed the overall increase in IL-10 after 10 months of AIT observed in the ELISPOT (Fig. 3c). Gating on the CD3⁺ CD4⁺ cell subpopulation revealed that the IL-10 production is associated with CD4⁺ T cells (Fig. 4a). Three of five donors showed a > twofold increase (Fig. 4b), one donor showed a 1.4-fold increase and one donor (with low IL-10 production throughout) showed a slight decrease, confirming that the transient IL-10 increase observed by ELISPOT was also observed in the ICS.

Finally, we wanted to determine whether the IL-10-producing cells coproduced IFN γ (Th1-like) or IL-5 (Th2-like). A representative example of the ICS data and the gating strategy is shown in Fig. 5a. The percentage overlap of IL-10/IL-5 and IL-10/IFN γ double producers was calculated from the total IL-10 positive

population in donors where significant IL-10 production was observed (4 out of 5 donors). In 3 out of 4 patients, IL-10 production was more associated with a Th1-like phenotype (simultaneous IFN γ production) (range 8–79%). One donor showed no clear polarization (25.6% for IL-10/IL-5 co-production and 26% for IL-10/IFN γ). No donor showed a Th2-like polarization, and overlap of IL-5 and IL-10 production was low overall (range 1–25.6%) (Fig. 5b). Interestingly, IL-10⁺ IFN γ ⁺ cells were already present before treatment began, albeit at a lower frequency (median of 7.9% before treatment and 22.0% 10 months into treatment). Thus, our data suggest that IL-10-producing T cells are frequently coproducing IFN γ and while these cells already exist before treatment, their frequency is further expanded by SCIT and SLIT treatment.

Discussion

Using patient samples collected over 15–24 months of AIT and sensitive measurements of cytokine production after peptide stimulation, we have shown that both SCIT and SLIT regimens are associated with sequential alterations of allergen-specific T cell responses. In SCIT-treated individuals, an overall reduction of Th2 responses was observed between 8 and 13 months of treatment. This reduction was reversed to baseline levels during the onset of the pollen season (15 months of treatment). The observed effect of the pollen season on T cell reactivity is in line with previously published data from the same cohort where significant changes were observed during grass pollen season for specific IgE levels and basophil sensitivity compared to baseline levels [19]. Overall, these data emphasize the importance of allergen exposure during grass pollen season, which has to be taken into account for data interpretation.

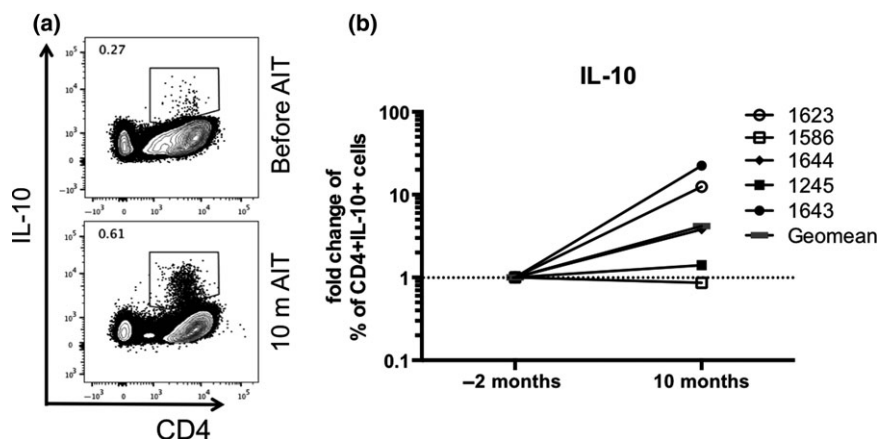


Fig. 4. Identification of IL-10-producing cells. (a) FACS plots depicting IL-10 production from CD4⁺ T cells before and 10 months into AIT from one representative donor. (b) Fold change of IL-10 production in SCIT (open symbols) and SLIT (black symbols) donors before and 10 months into AIT. The geomean is indicated as a bar. $N = 5$.

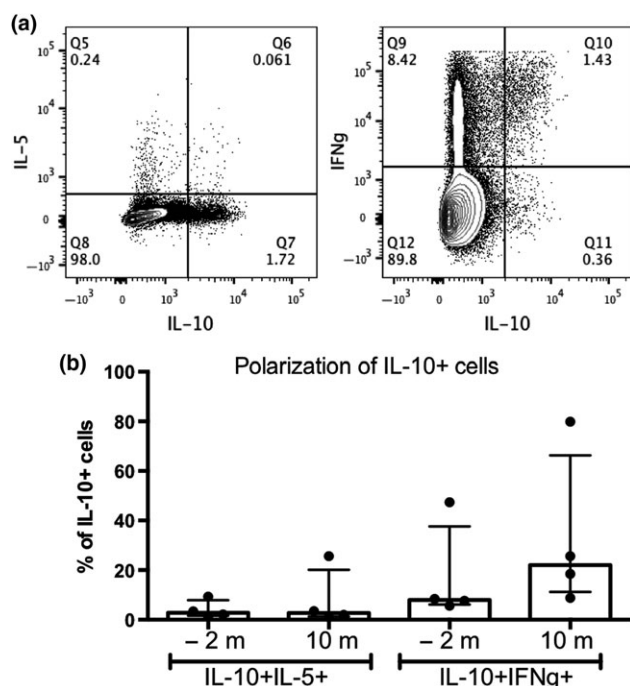


Fig. 5. Analysis of polarization of IL-10-producing T cells. (a) FACS plots depicting IL-5 (left panel) and IFN γ (right panel) production of IL-10-producing T cells 10 months into AIT from one representative donor. (b) A bar chart showing the median percentage of IL-10-producing T cells that also produce IL-5 or IFN γ before treatment (-2 m) and 10 months into treatment (10 m). Error bars represent interquartile range. *N* = 4.

To avoid the effect of any possible pollen exposure, we assessed cytokine production from allergen-specific T cells by focusing on the out-of-season time points. In line with what our group and others have shown previously [22, 25], we observed a significant decrease in IL-5 production in donors who had received SCIT compared to untreated controls. In the SLIT-treated group, this effect was observed to a much lesser extent. IL-5 levels were significantly decreased after 12 months compared to 1 month and 10 months of treatment but not significantly different from those of untreated controls. Interestingly, in a similar study by Suarez-Fueyo *et al.* [26] a significant decrease in Th2 responses after SLIT treatment was reported. However this decrease was detected after 21 months and was not observed at earlier time points. Other studies assessing Th2 responses after 6–12 months of SLIT did not detect any changes [27, 28]. These data suggest that Th2 response modulation may occur later during SLIT treatment compared to SCIT [29, 30]. While the reasons for this difference are not clear, this observation highlights that slight differences in immunomodulatory mechanisms might underlie differential efficacy of SCIT and SLIT treatments, likely due to differences in dosage and/or the route by which the treatment is administered.

In contrast to the treatment-induced reduction in IL-5 production, the kinetics of increased IL-10 production were comparable for SCIT and SLIT. In both cohorts, significant and transient increases of IL-10 production were detected in response to antigen stimulation after 10 months of treatment, which subsided again after 12 months. This rise in IL-10 production was more pronounced in the SLIT group, suggesting that this mode of treatment has a larger effect on increasing IL-10 production than reducing IL-5 production compared to SCIT treatments. These data further emphasize that potential differences in mechanisms seem to exist that contribute to the immunological changes induced by SLIT vs. SCIT.

A key question on mechanisms of AIT is whether it induces new competing T cell specificities or alters existing ones. Comparing extract-specific T cell responses pre- and post-AIT, it is not possible to distinguish between these two possibilities. Instead, using pools of peptides that were originally identified based on their ability to trigger IL-5 production in allergic donors before treatment is a first step towards addressing this question. Our data do show changes in the immune response to these peptide pools, suggesting an alteration of existing responses. In line with this observation, Wambre *et al.* [31] have shown that allergic T cell modulation occurs on an epitope-specific level. In patients who had received SCIT for a minimum of 3 years, a modulation from a Th2 to a Th1 response for the dominant epitopes was far more prominent than for non-dominant epitopes. This observation is relevant for potential epitope-based immunotherapy regimens [32], as it suggests that the same dominant epitopes might be, in different immunological contexts, linked to pathogenic (Th2) or regulatory (IL-10 production) responses. This result agrees well with the reported plasticity of Th cells in humans [33].

Characterization of the IL-10-producing CD4 T cells showed that, in most cases, these cells also coproduce IFN γ but almost no IL-5, suggesting a closer relation to cells of the Th1 lineage than Th2. Other studies have reported similar findings, referring to an IL-10/IFN γ double positive Tr1-like phenotype [16, 25, 31]. Our data further show that these IL-10/IFN γ double positive cells are already detectable before treatment but their frequency is increased after 10 months of AIT. This suggests that AIT does not induce, but rather expands this subset. Due to limited sample availability, we were unable to assess FOXP3 expression to evaluate whether an increase of regulatory T cells can be detected, as has been shown by other groups [34].

It should be noted that this is an exploratory study conducted with samples from a subset of patients obtained after the original study by Aashjerg *et al.* was concluded. Statistical analyses were performed *post hoc*

without an initial hypothesis of how and at what time points differences in T cell responses could be expected, and no correction for multiple hypothesis testing was performed. Thus, it will be necessary to perform follow-up studies in larger cohorts including a non-allergic control group and undertake a comprehensive assessment of clinical changes to explicitly test whether our findings can be confirmed, and if they correlate with clinical benefit. Furthermore, as the duration of the study was limited to 15 months of treatment, changes that might occur after this time period were not investigated. In particular, it is possible that a decrease in IL-5 responses in SLIT-treated donors would have been observed in later time points. Additionally, we cannot rule out that IL-10 responses might re-emerge at later time points. Such questions are beyond the scope of this work and will be addressed in future studies.

In conclusion, our study suggests significant differences in the kinetics and magnitude of immunomodulatory changes induced by SCIT and SLIT and that the onset of pollen season may influence immunological read-outs.

Further our data lead us to speculate that the dose and route of treatment administration may significantly influence AIT-induced cellular changes, providing a potential explanation for the lack of consistency in findings related to the role of regulatory T cells in AIT.

Finally, the observation that the same epitopes can be recognized by IL-5- and IL-10-producing cells suggests that dominant epitopes can be used to monitor the effect of AIT on a T cell level. To our knowledge, this is the first time the immunological effect of SCIT and SLIT was directly compared on both the humoral and cellular levels in a longitudinal cohort. Differences in the underlying mechanism by which immunomodulation is achieved may be highly relevant for the definition of biomarkers and clinical read-outs designed to assess SCIT or SLIT efficacy.

Acknowledgements

This project has been funded in whole or in part with Federal funds from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN272200700048C and U19 Grant No. U19 AI100275. Donor recruitment and sample accrual were funded by ALK.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A comparison of the kinetics of immunotherapy-induced changes observed on the T cell vs. the antibody level.

Figure S2. Long-term changes in TG-specific (a) IFN γ responses and (b) IL-10 responses in PBMC from allergic subjects and patients undergoing SCIT or SLIT.

Figure S3. Fold changes in longitudinal TG-specific responses at selected out-of-season time points throughout subcutaneous (SCIT) and sublingual (SLIT) treatment and in allergic controls.