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

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Abstract

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 versus 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. PBMC 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 was 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 was 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 immune-modulatory mechanisms at the cellular level.

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 anti-histamines and intranasal corticosteroids [4, 5]. The only potentially curative treatment available for allergic rhinitis is allergen-specific immunotherapy (AIT), which has been administered for over a century [6, 7]. AIT desensitizes patients to specific allergens through administration of increasing doses of allergen extracts. This regimen is thought to gradually tolerize 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, sublingual AIT (SLIT) is a new treatment option. Its efficacy and safety have 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. However, differences between the two treatments have been reported for certain parameters [14].

Similarities in immunological changes associated with both SCIT [16] and SLIT [17] have been proposed, including an increase in IL-10 production and a decrease in 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 versus SLIT-induced immune-modulatory mechanisms of antibody and T cell responses throughout and after treatment are limited. Especially, immunological studies comparing SCIT versus SLIT, 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 readouts are lacking.

In the present paper, we are utilizing samples collected during a previously published study [19]. The initial publication of this study compared the effects of SCIT and SLIT on allergen-specific antibody responses, including IgE titers, IgG4/IgE-blocking assays, facilitated antigen presentation (FAP) and effector cell activation assessed by basophil activation test (BAT). For this study, 40 Timothy grass (TG)-allergic patients were recruited and randomized into 15+15+10 in SCIT, SLIT and control, respectively. Blood donations were obtained at various time points throughout the treatment over 15 months. 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 to a maximum level of 0.5 (50% of allergen-specific IgE binding is inhibited) compared to the SLIT-treated group that reached a maximum IgE blocking factor of 0.27 after 15 months. Using the facilitated antigen presentation (FAP) assay, inhibition of IgE-facilitated antigen

presentation was determined. Similarly to the IgE blocking factor, no inhibition was observed in the control group at any time point, the SCIT group reached a maximum inhibition of 22.5% after 3 months and the SLIT group reached a maximum inhibition of 17.2% after 15 months [19]. Finally, basophil activations assays (BAT) revealed a significant reduction in basophil activation after SCIT (decreased by a factor of -1.4) and SLIT (-0.71) after 15 months. No reduction was observed in controls.

Here we wanted to investigate if differences between SCIT and SLIT might be present and detectable at the T cell level. Accordingly, in a continuation of the study described above, we examined the effects of SCIT and SLIT on allergen specific T cell responses. Antigen-specific cytokine production was assessed longitudinally in T cells from patients who were treated either by subcutaneous or sublingual AIT or received no treatment at all. To gain insights into the potential mechanisms involved at the T cell level, we investigated how the two treatments influenced TG-specific T cell responses over time using previously validated peptide pools derived from Phl p allergens and recently identified additional TG T cell antigens.

Materials and methods

Patient cohort

Forty grass allergic patients were randomized 15+15+10 in SCIT, SLIT, and untreated controls, respectively, and followed for 15 months 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 months 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*' starting at 20 SQ-U, reaching maintenance dose of 100 000 SQ-U at week 15, after which maintenance dose was given every 2 months throughout the remainder of the study.

SLIT treatment was administered orally with ALK Grazax 75 000 SQ-T sublingual tablets.

Blood collection and PBMC isolation

PBMCs were isolated from freshly drawn (<3 hours) heparinized blood by gradient centrifugation on lymphoprep (Nycomed, Norway), washed twice and resuspended in RPMI 1640 medium supplemented with 5% v/v AB-serum and antibiotics[20]. Cell numbers were adjusted to $10\text{--}20 \times 10^6/\text{ml}$ by addition of freezing medium with DMSO and FCS at final concentrations of 10 and 20 percent v/v, respectively. The PBMC 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. Peptide pools of 19 or 20 immuno-dominant 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 peptides 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. This pool was 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 immuno-dominant peptides from additional T cell-reactive TG antigens described previously[21]. The third pool (MNA) consisted of 20 peptides also derived from the additional TG antigens. It is associated with strong IL-5 production in allergic donors (957 spot forming cells (SFC)), which was then significantly reduced by more than 2 fold in donors who had received SCIT treatment (425 SFC) [22]. The MNA pool and the P19 pool share 9 peptides therefore MNA-reactivity data is not shown in the analysis of Figure 3.

PBMC expansion in vitro

PBMC were thawed and cultured in RPMI 1640 (Ω Scientific, Tarzana, CA) supplemented with 5% human AB serum (Cellgro, Herndon, VA) at a density of 2×10^6 cells/ml in 24-well plates (BD Biosciences, San Jose, CA) and stimulated separately with the 3 peptide pools derived from either the known Phl p allergens or previously described additional TG antigens[22] (5 µg/ml). For in vitro expansion, cells were kept at 37°C, 5% CO₂ and IL-2 (10U/ml, eBioscience, San Diego, CA) was added every 3 days after initial antigenic peptide stimulation. On day 14, cells were harvested and assessed for cytokine production in response to re-stimulation with peptide pool (5 µg/ml) by ELISPOT.

ELISPOT

All ELISPOT assays were performed in a blinded fashion and the key to treatment modality was revealed after full analysis of cellular cytokine responses. ELISPOT assays were performed as described previously [22, 23]. Briefly, flat-bottom 96-well nitrocellulose plates (Millipore, Bedford, MA) were prepared according to the manufacturer's instructions and coated with either a cocktail of 5 µg/ml anti-human IL-5 and anti-human IFN-γ or 5 µg/ml anti-human IL-10 (Mabtech, Cincinnati, OH). The next day, plates were washed, blocked and cells were plated at a concentration of 1×10^5 cells/well and incubated with peptide pool (2.5 µg/ml). After 24 hours, cells were removed and plates were incubated with 2 µg/ml biotinylated anti-human IL-5 Ab and anti-human IFNγ-horseradish peroxidase or biotinylated anti-human IL-10 at 37 °C. After 2 hours, plates were washed and avidin-alkaline phosphatase-complex was added (Vector Laboratories, Burlingame, CA) for 1 h at RT. Alkaline phosphatase spots were developed with Vector Blue Substrate Kit (Vector Labs, Burlingame, CA). Horseradish peroxidase spots were developed with 3-amino-9-ethylcarbazole solution (Sigma-Aldrich, St. Louis, MO). Based on previous experience [21–

23], internal criteria for peptide pool positivity were 100 SFCs/10⁶ PBMCs, $p < 0.05$, and a stimulation index (SI) ≥ 2 comparing stimulated cultures with unstimulated control cultures.

Intracellular cytokine staining

PBMCs were re-stimulated after 14 days of *in vitro* culture with 5 µg/ml peptide pool in complete RPMI medium at 37°C in 5% CO₂. Unstimulated PBMC 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 6h 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, 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 was analyzed using FlowJo software (Tree Star).

Statistical analysis

Statistical analyses were performed using 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 done using Mann-Whitney test (non-parametric, unpaired) and longitudinal comparisons within groups were performed using 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 peptides 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.

As expected in an allergic cohort, the Th2 response was found to be most dominant overall. Significant IL-5 production (> 100 SFC/10⁶ PBMC) was observed in 21/24 patients for P20, 20/24 patients for P19 and in 21/24 patients for MNA (Figure 1A). Overall, 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 ($p < 0.005$) (Figure 1A).

Significant levels of IFNγ production were detected in 22/24 patients for P20 (median response 838 SFC/10⁶ PBMC), 23/24 patients for P19 (median 1,070 SFC/10⁶ PBMC) and 22/24 patients for the MNA pool (median 1,007 SFC/10⁶ PBMC) (Figure 1B). No significant differences in response magnitude between the different peptide pools were

observed. Similarly, significant IL-10 responses were seen in 19/24 donors for P20, 20/24 for P19 and 19/24 for MNA (Figure 1C). Overall, 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 (Figure 1C).

Two months after the first visit, patients were randomized into 3 groups; SLIT (n=9), SCIT (n=8) and untreated (n=7) and treatments were initiated. To study the effect of SCIT and SLIT treatment on T cell responses, PBMC isolated from blood donations from these 3 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). Two fold changes are indicated by dashed lines, corresponding to likely relevant changes in the response taking into account assay variability. The untreated control group showed no fluctuation in median IL-5 responses >2 fold at any time point (Figure 2, statistical analysis follows in Figure 3).

Despite some donor-to-donor variability, a greater than two-fold 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. This decrease in IL-5 responses in SCIT treated patients was most pronounced in the winter months after 1 year of treatment in early 2012 (median SFC values changing from 1918 to SFC for P20; 1293 to 425 for P19; 1608 to 89 for MNA) and 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. By contrast, the SLIT group showed no strong decreases in IL-5 throughout 2 years of treatment (Figure 2) but an increase is observed in response to MNA pool stimulation during and following the second pollen season (800 median SFC increased to 1327 SFC after 15 months and 1453 SFC after 24 months). When comparing the kinetics of the changes in IL-5 and IL-10 production to changes in observed on the antibody level measured in the previous study by Aasbjerg et al. (IgE blocking, facilitated antigen presentation), it appears that changes at the antibody level can be observed much earlier (within 3 months of treatment) compared changes in cytokine production that become detectable after 10–12 months of treatment (repository figure 1).

Interestingly the IL-5 reduction observed in the SCIT group during the winter months of the first and second treatment years was reversed back to base line during the summer months. This trend may indicate a potential effect of the pollen exposure during allergen season, which is a variable that could not be controlled for in this study design. Therefore, 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 (Figure 3). Furthermore, since reactivity patterns in response to all 3 pools were very similar, data from P20 and P19 were combined and presented in a single bar (coded by symbol). Reactivity against MNA pool is excluded from this analysis because it overlaps with P19 by 9 residues. In this data representation – as above - a significant decrease in IL-5 in the SCIT group beginning at 1 month after treatment was observed. IL-5 responses in the SCIT group were significantly lower at all three time points compared to the other groups (Figure 3A). The

decrease within the SCIT group became progressively more pronounced over time. In the SLIT group, there was also a significant decrease in IL-5 after 12 months of treatment relative to the earlier time points but not significant compared to the untreated group. IL-5 production in the control group remained unchanged (Figure 3A).

In addition to IL-5, we also measured IFN γ and IL-10 (Figure 3B,C) (complete data over the entire time course of the study is presented in repository figure 2). A decrease in response was also detected for IFN γ in the SCIT group after 12 months of therapy compared to 10 months and compared to 12 months responses observed in the control group (Figure 3B)..

Interestingly, unique changes in IL-10 response were detected over time. In the treated groups (both SLIT and SCIT), IL-10 production was visibly increased after 10 months of treatment (Figure 3C). P20-specific IL-10 median SFC values were increased from 297 (before treatment) to 723 (after 10 months) in SCIT and 493 SFC to 1097 SFC in SLIT. For P19, values were increased from 333 SFC to 523 SFC in the SCIT group and 317 SFC to 1277 SFC in the SLIT group. This increase was particularly pronounced in the SLIT group where IL-10 was significantly increased compared to both earlier (1 months into treatment) and later (12 months into treatment) time points ($p < 0.0001$) within the same group (Figure 3C).

To determine if counting 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 figure 3). The low number of data points results in a small loss of statistical significance but overall trends i.e. the reduction in IL-5 in the SCIT group and the increase in IL-10 in both the SLIT and SCIT group are still observed (repository figure 3).

Next, we wanted to determine if CD4 T cells are associated with the transient 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]. Since this study aimed at identifying immune-modulatory mechanisms in CD4 T cells, we assessed IL-10 production by intracellular cytokine staining (ICS) experiments to determine if the fold change in IL-10 is observed in CD4 cells. 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. Due to limited sample availability, it was only possible to analyze three SLIT treated and two SCIT treated patients. The ICS assay confirmed the overall increase in IL-10 after 10 months of AIT observed in the ELISPOT (Figure 3C). To determine if the IL-10 production is associated with CD4 T lymphocytes, CD3⁺CD4⁺ cell subpopulation were gated on and the relative production was assessed. This analysis revealed that the IL-10 production is observed CD3/CD4 positive cells (Figure 4A). To assess if the transient IL-10 increase observed by ELISPOT was also observed in the ICS, the fold change of IL-10 production in CD3⁺CD4⁺ cells in all donors was analyzed. Three of five donors showed a > 2 fold increase (Figure 4B), while one donor showed a 1.4 fold increase, and only one donor with low IL-10 production throughout showed a slight

decrease. Overall, these data support the notion that the increase in IL-10 production observed originated from CD4⁺ T cells.

Finally, we wanted to determine whether the IL-10 producing cells were Th1 or Th2 like. IL-10 has been described as both a Th1 and Th2 cytokine [25], and could thus be a by-product of activated Th2 cells or secreted by Th1 like cells, thus likely playing a regulatory role. We assessed whether the IL-10⁺ T cells simultaneously produced either IL-5 (Th2-like) or IFN γ (Th1-like). A representative example of the ICS data and the gating strategy is shown in Figure 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, with an overlap of 25.6% for IL-5 production and 26% for 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%) (Figure 5B). Interestingly, IL-10+IFN γ + cells are already present before treatment was begun, albeit at a lower frequency (median of 7.9% before treatment and 22.0% ten months into treatment). Thus, our data suggest that IL-10 producing T cells are frequently co-producing IFN γ and while these cells already exist before treatment, their frequency is further expanded by SCIT and SLIT treatment.

Discussion

We report that SCIT and SLIT regimens were both associated with sequential alterations of the allergen specific T cell responses. In our donor cohort, which was previously tested for humoral immune modulations[19], samples collected over 15–24 months throughout the course of AIT were assessed for modulation of allergen-specific T cell responses based on cytokine production after allergen-derived peptide stimulation.

Our data show an overall reduction of Th2 responses observed in SCIT-treated individuals between 8 and 13 months of treatment. This reduction is reversed to baseline levels during the onset of the pollen season (15 months of treatment). A limited number of samples collected from SCIT and SLIT patients after 24 months of treatment (out of season) showed a reduced Th2 response in SCIT- but not in SLIT-treated patients suggesting that the antagonistic effect of the grass pollen season masks decreases in IL-5 responses induced by SCIT observed after the first year. However, this observation is limited to a very small sample number and will therefore have to be re-evaluated in future studies. The observed effect of the pollen season on T cell reactivity confirms previously published data of the same cohort where significant changes were observed during grass pollen season for specific IgE levels and basophil sensitivity compared to baseline levels [19]. Consistent with these data, Durham et al. [26] previously reported that the observed treatment effect measured as conjunctivitis symptoms and medication score is highly dependent on pollen exposure. Overall these data emphasize how grass pollen season is a crucial factor strongly influencing allergic immune responses as well as clinical symptoms in treated and un-treated patients, and has to be taken into account for data interpretation.

In light of this conclusion, we assessed cytokine production in allergy-specific T cells obtained from either SCIT or SLIT patients focusing on the out of season time points. In line with what our group and others have shown previously [22, 27], 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 months and 10 months of treatment but not significantly compared to untreated controls. Interestingly, in a similar study by Suarez-Fueyo et al. [28] a significant decrease in Th2 responses after SLIT treatment was reported, however this decrease was detected after 21 months and was not observed in earlier time points. Other studies assessing Th2 responses after 6–12 months of SLIT did not detect any changes [29, 30]. These data suggest that Th2 response modulation may occur at a later time point during SLIT treatment compared to SCIT [31, 32]. While the reasons for this difference in kinetics and intensity of modulation are not clear, this observation underscores that slight differences in immune-modulatory mechanisms might be underlying the efficacy of SCIT and SLIT treatment, likely due to differences in dosage or the route by which the treatment is administered.

In contrast to the treatment-induced reduction in IL-5 production, the increase in IL-10 production was comparable for SCIT and SLIT with regard to the kinetics. In both cohorts significant, transient increases of IL-10 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 seem to exist between mechanisms that contribute to the effect of SLIT versus SCIT.

A key question on mechanisms of AIT is whether it induces new competing T cell specificities or alters existing ones. When comparing to extract pre and post AIT, it is not possible at all to distinguish between these two possibilities. Using pools of peptides instead 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 does show changes in the immune response to these peptide pools suggesting that there is an alteration of existing responses. In line with this observation, Wambre et al. [33] 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 [34], 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 data fits well with the reported plasticity of Th cells in humans [35].

To assess whether the IL-10 production detected by ELISPOT after 10 months of AIT can be linked back to CD4 T cells we performed intracellular cytokine staining (ICS) assays. These assays revealed that CD4 T cells did show a considerable increase in IL-10 in samples collected 10 months into AIT compared to samples collected before treatment was commenced. This is perhaps not surprising, given that the assay was performed with

peptides, and included a 14-day culture aimed at enriching for antigen-specific T cells. It is very possible that in an in vivo situation, there are other cells that make significant amounts of IL-10, which could not be detected by our ICS.

Further characterization of the IL-10 producing CD4 T cells revealed that, in most cases these cells also produced some levels of IFN γ but almost no IL-5, suggesting a closer relation to cells of the Th1 lineage than to cells of the Th2 cell lineage. Other studies have reported similar findings, referring to an IL-10/IFN γ double positive Tr1-like phenotype [16, 27, 33]. 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 suggesting that AIT does not induce but rather expand this subset. Due to the great limitation of sample availability, we were unable to assess Fox p 3 expression to evaluate if an increase of regulatory T cells can be detected as has been shown by other groups [36].

It should be highlighted that samples used in this manuscript were obtained after the original study was concluded. The work was performed with a limited number and amount of sample that was made available to us. In addition, the study design and clinical protocols were already in place. Also, statistical analyses were performed post-hoc without an initial hypothesis 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 a comprehensive assessment of clinical changes to explicitly test if 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 highlights significant differences in the kinetics and magnitude of immune-modulatory changes induced by SCIT and SLIT. In addition, our data suggest that the onset of pollen season may influence immunological read-outs, as the reduction in Th2 cytokine production observed in SCIT-treated patients (Figure 2) is reversed with the onset of the pollen season.

The role of regulatory T cells in AIT has been extensively debated because they are not consistently detected during or after treatment. Our data demonstrates that route of treatment administration can significantly influence AIT-induced cellular changes, providing a potential explanation for the lack of consistency in these findings. Furthermore, the same epitopes can be recognized by Th2-like cells and IL-10 producing cells, suggesting that dominant epitopes can be used as an immune-modulatory reagent more rigorously defined than whole allergen extracts. To our knowledge, this is the first time the immunological effect of SCIT and SLIT were directly compared on both the humoral and cellular level 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIT	Allergen-specific immunotherapy
SCIT	subcutaneous immunotherapy
SLIT	sublingual immunotherapy
TG	Timothy grass
IL	interleukin
Th	T helper

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Figure 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 done using Mann-Whitney test. *-p<0.05, **-p<0.005. Error bars indicate interquartile range.

Figure 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.

Figure 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).

Figure 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 red bar. N=5

Figure 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

Table 1

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