

Two year follow-up of immunological response in mite-allergic children treated with sublingual immunotherapy. Comparison with subcutaneous administration

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Although the efficacy of allergen-specific sublingual immunotherapy (SLIT) is now accepted, the underlying mechanisms remain elusive. Such mechanisms are better documented in the case of subcutaneous immunotherapy (SCIT). In order to understand the T-lymphocyte response in patients receiving SLIT, we compared children with respiratory disease monosensitized to *Dermatophagoides pteronyssinus* receiving SLIT or SCIT over a 2-yr period. Peripheral blood was obtained before beginning immunotherapy, and after 3 months, 1 yr and 2 yr. Total IgE, specific IgE and IgG4 to *D. pteronyssinus* were determined in serum. T-cell markers (CD3, CD4, CD8, CD25) and intracellular cytokine production (TNF- α , IL-2, IL-4 and IFN- γ) were determined in peripheral blood mononuclear cells (PBMC) by flow cytometry. No differences between SCIT and SLIT were detected in the clinical variables or in the subjective evaluation. Although an increase in specific IgE and IgG4 was only detected in SCIT, a significant decrease in the specific IgE/IgG4 ratio was found in both groups. SCIT and SLIT experienced an increase in the CD4/CD8 ratio over time, but an increase in the CD4⁺CD25⁺ and a decrease in the CD8⁺CD25⁺ subsets were only found with SCIT. A slight shift from a Th2 to a Th1 pattern, measured by the IFN- γ /IL-4 ratio, was only detected in the CD4 T cells with SCIT. A decrease in both groups was found in TNF- α and IL-2 production over time. Children with respiratory allergic diseases receiving SCIT or SLIT had a different immunologic response in peripheral blood during treatment, though the clinical improvement was similar. Whether SLIT induces a mucosal protective response should be studied.

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Allergen-specific immunotherapy (IT) administered subcutaneously (SCIT) has been used to treat allergic diseases since around 1910. However, because of the possibility of severe systemic reactions, other routes (nasal, oral, bronchial and sublingual) have been tried, with the sublingual route generating most interest (1).

Sublingual immunotherapy (SLIT) is specifically useful in children, because it can be easily administered at home. Both SCIT and SLIT are considered an effective therapy for respiratory allergic disease (1–4), with the clinical effect lasting at least 3 yr after treatment has been stopped (5). It is particularly important in

children, for whom it can prevent new sensitizations (6) and progression from rhinitis to asthma (7).

Although immunological changes are usually better documented with SCIT, there are no clear-cut qualitative differences between SCIT and SLIT, suggesting that immune mechanisms involved could be quite similar (8). More research comparing SCIT and SLIT could contribute to a better understanding of both mechanisms. Many studies have been carried out to assess the mechanisms underlying allergen-specific IT, focussing on increases in levels of IgG₁ and IgG₄ (9, 10). This led to the hypothesis of the induction of blocking antibodies, capable of competing with IgE for binding to the allergen, preventing basophil and mast cell degranulation. However, a good correlation has not always been found between an increase in these immunoglobulins and the clinical benefit obtained (11, 12). Later, the capacity of IT to change from a Th2 to a Th1 pattern was detected (13–15), with decreased levels of IL-4 and IL-5 and increased levels of IFN- γ . In addition to this effect, an increase in TGF- β and IL-10 was also found (16). Recent studies suggest the involvement of T regulatory cells (T_{Reg}) in the production of TGF- β and IL-10 (17).

Based on our current understanding of the immune mechanisms underlying allergen-specific immune responses, optimizing vaccines to control the response of T lymphocytes during exposure to the allergen or vaccination could be critical. Accordingly, we undertook this explorative study to examine the immunological changes in peripheral blood over a 2-yr follow-up period in a group of children with respiratory allergic disease and monosensitized to *Dermatophagoides pteronyssinus* (D.pter) receiving SLIT, when compared with a group receiving SCIT with the same extract. A better knowledge of the T-lymphocyte response to SCIT and SLIT could contribute to the development of a second generation of more efficient vaccines.

Methods

Patients

The study included 23 children referred to the Paediatric Allergy Unit of Carlos Haya Hospital, Malaga, Spain, in November and December 2001 for evaluation of respiratory allergic diseases. They were all monosensitized to house dust mite, with skin test positive to D.pter and serum

specific IgE to D.pter. The respiratory allergic disease was diagnosed according to the ARIA (18) and GINA (19) consensus. The skin prick test was done with D.pter extract (100 BU/ml) provided by ALK-Abello (Madrid, Spain) and a wheal > 3 mm was considered positive. Parents decided whether their children were to receive just symptomatic treatment or symptomatic treatment plus IT, with the latter being those who were finally included in the study. The children were then randomized into two treatment groups, either SCIT (n = 12) or SLIT (n = 11). Before starting and after finishing the 2 yr treatment, the patients gave a subjective evaluation using a semiquantitative scale (Much better, Better, Equal, Worse, Much worse) and a visual analogical scale (VAS).

In no patient was IT contraindicated, according to WHO criteria (2), and none had been previously treated with IT or was sensitized to other inhalant allergens.

The study was approved by the institutional review boards, and informed consent was obtained from the parents of all the children.

Treatment

All the patients were treated for 2 yr with a D.pter 100% extract, provided by ALK-Abello. SCIT was administered using a depot allergen extract adsorbed in aluminium hydroxide (Pangramin[®] Depot-UM) and SLIT using a glycerinated allergenic extract (SLIT[®] *Tratamiento sublingual*). All the extracts were biologically standardized and measured in mass units, i.e., the major allergens, Der p1 and Der p2, were quantified in $\mu\text{g/ml}$, with a ratio between the two of 2:1. Table 1 shows the rate of administration of SCIT and SLIT according to the manufacturers' recommendations. The maximum dose administered in SCIT and SLIT was 4 and 2 $\mu\text{g/ml}$, respectively, of the two major mite allergens, Der p1 and Der p2, in accordance with the standardization procedure followed (19, 20).

Sample collection

Six millilitres of heparinized venous blood was obtained at different times: before beginning IT, at 3 months (once the maximum dose had been reached in both types of IT), at 1 yr, and at the end of the study (2 yr). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Nycomed, Oslo, Norway) and serum samples were also obtained and stored at -20°C until assayed.

Table 1. Immunotherapy characteristics

		SLIT	SCIT
Build up phase	Dose scheduled	Vial 0: 1–10 drops Vial 1: 1–5 drops Vial 2: 1–5 drops Vial 3: 1–5 drops Vial 4: 1–5 drops Daily dose 30 days	Vial 1: 0.1, 0.2, 0.4, 0.8 cc Vial 2: 0.1, 0.2, 0.4, 0.8 cc Vial 3: 0.1, 0.2, 0.4, 0.6, 0.8 cc One dose a week
Maintenance phase	Duration Dose scheduled Maximum Dose Administrated Der p1/Der p2 Cumulated Dose per month Der p1/Der p2 Duration	5 drops vial 4 (Three times a week) 0.8/0.4 µg 10.8/5.4 µg Up 2 yr	13 wk 0.8 cc vial 3 (One dose a month) 3.2/1.6 µg 3.2/1.6 µg Up 2 yr

Total IgE, specific IgE and specific IgG4 determination

Total and specific IgE to D.pter was determined by CAP-FEIA System (Pharmacia, Uppsala, Sweden) following the manufacturer's recommendations. Results were obtained from a standard curve and expressed as kU/l. The cut-off point to be included in the study was a value ≥ 0.71 kU/l (class 2).

The determination of specific IgG₄ to D.pter in serum was done by ELISA, as described (20). Briefly, ELISA plates (Costar, 3590) were coated with 40 µg/ml of D.pter (Alk-Abello) for 1–2 h at 37°C (50 µl/well). After blocking with PBS 1% BSA (100 µl/well), 50 µl of serum sample was added at 1/10, 1/30 and 1/90 dilutions for 1 h at room temperature. After washing with 0.1% Tween-20 in PBS, plates were incubated for 30 min with 50 µl of anti-IgG₄ monoclonal antibody (G4T9) conjugated to peroxidase at 1/5000 dilution. After washing, o-phenylenediamine dihydrochloride (OPD) peroxidase was added and the enzymatic reaction stopped by adding 50 µl of H₂SO₄. The absorbance was read at 492 nm. Results were obtained from a standard curve obtained using *Phleum pratense* extract and expressed in UA/ml.

PBMC phenotype immunofluorescence analysis

The measurement of lymphocyte subsets and cell activation markers was carried out by incubating PBMC for 15 min at room temperature in the dark with the following monoclonal antibodies (moAbs): CD3-PerCP, CD4 (α-phycoerythrine; PE, allophycocyanin; APC), CD8 (fluorescein isothiocyanate; FITC, APC) and CD25-PE (all provided by Becton-Dickinson, San Jose, CA, USA).

Cytokine production was determined in cells stimulated for 4 h at 37°C with phorbol 12-myristate 13-acetate (Sigma, St Louis, MO, USA) and ionomycin (Sigma) at a final concentration of 25 ng/ml and 1 µg/ml of cell suspen-

sion, respectively, in which the intracellular protein transport of cytokines was disrupted with monensin (Sigma) at 10 µg/ml of cell suspension. After subset cell surface staining for 15 min, cells were fixed for 15 min using Fix and Per cell permeabilization kit (Caltag Laboratories, Burlingame, CA, USA) and permeabilized with the same kit and stained intracellularly with moAb to cytokines (TNF-α-PE, IL-2-FITC, IL-4-PE and IFN-γ-PE; all provided by Becton-Dickinson) for 30 min at room temperature. After washing, cells were analysed on a FacsCalibur flow-cytometer using Cell Quest software (Becton-Dickinson). Negative isotype controls were used to verify the staining specificity of the antibodies used.

Statistical analysis

The mean, median, minimum and maximum values, standard deviation, interquartile range and 95% confidence interval for the mean value were calculated for the quantitative variables. In those results that adjusted to a normal distribution, ANOVA was done between serial determinations and in significant cases, the Student *t*-test for paired data were used to detect statistically significant differences (***p* < 0.005 and **p* < 0.05). In those results without a normal distribution, Friedman test was done between serial determinations and in significant cases the Wilcoxon test for related data was done for paired sample (***p* < 0.005 and **p* < 0.05). The statistical analysis was performed using the spss program, version 11.5.

Results

Characteristics of the patient groups and treatment

Table 2 shows the clinical characteristics of the patients. No significant differences were found between the two groups of patients, SCIT and

Table 2. Characteristics of the group of patients studied: overall group, those receiving subcutaneous immunotherapy (SCIT) and those receiving sublingual immunotherapy (SLIT)

	Total	SCIT	SLIT
Sex, n(%)			
Female	7 (30)	4 (33)	3 (27)
Male	16 (70)	8 (67)	8 (73)
Diagnostic, n(%)			
Rhinitis (R)	6 (26)	3 (25)	3 (27)
Asthma (A)	2 (9)	1 (8)	1 (9)
R + A	15 (65)	8 (67)	7 (64)
Age (yr) (mean $\pm$ S.D.; Interval)	11.9 $\pm$ 2.3; 8–17	12.3 $\pm$ 2.8; 8–17	11.5 $\pm$ 1.5; 9–14
Time evolution (yr) (mean $\pm$ S.D.; Interval)	7.3 $\pm$ 3.8; 1–12	6.5 $\pm$ 4.2; 1–12	8.1 $\pm$ 3.4; 2–12

n, absolute numbers.

SLIT, in any of the clinical variables analysed. Two patients dropped out of the study, one in the SCIT group because of an acute attack of atopic dermatitis after 1 yr treatment, and one in the SLIT group after 1 yr, for unknown reasons. Both groups showed good tolerance and no systemic reactions were detected.

The total number of doses administered was 400 in the SCIT group and 3300 in the SLIT group. The cumulative dose in the maintenance phase was 70/35 μ g of Der p1/Der p2 for SCIT, and 216/108 μ g of Der p1/Der p2 for SLIT.

Before starting and after finishing the 2 yr treatment, the patients gave a subjective evaluation using a semiquantitative scale (Much better, Better, Equal, Worse, Much worse) and a VAS. In the SCIT group, 90% of the patients reported feeling better or much better, while 83% in the SLIT group also reported feeling better or much better. The VAS results were 8.1 ± 1.4 (SCIT) and 8.5 ± 1.3 (SLIT).

Serum antibody responses

We quantified total IgE and specific IgE and IgG₄ to D.pter at different times (baseline, and 3 months, 1 yr and 2 yr after beginning IT) in both SCIT and SLIT. At baseline, no significant differences were observed between SCIT and SLIT in any of the total and specific immunoglobulins analysed. In SCIT, no differences were detected in the total IgE levels, although significant increases were found in specific IgE and IgG₄. There was a gradual increase in specific IgE and IgG₄ from the baseline level to the sample obtained at 2 yr, although the increase in specific IgE appeared later (after 1 yr) than the increase in specific IgG₄ (after 3 months). No differences were found in the SLIT group for any of the determinations (Fig. 1a). On the other hand, the IgE/IgG₄ ratio specific to D.pter showed a significant decrease in both types of IT (SCIT,

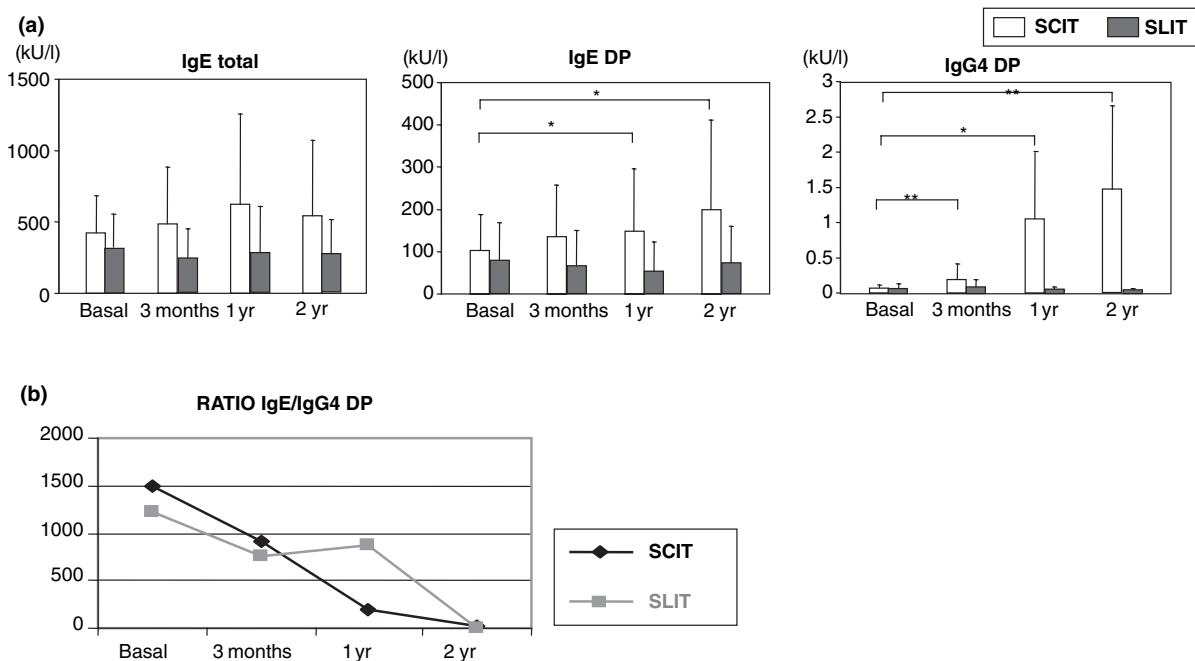


Fig. 1. (a) Comparison of total IgE, specific IgE and specific IgG₄ to *Dermatophagoides pteronyssinus* (DP) at different time intervals (before immunotherapy, and three months, 1 yr and 2 yr after beginning treatment) in patients with subcutaneous (SCIT) and sublingual immunotherapy (SLIT). The results are expressed in kU/l as mean $\pm$ s.d. concentration (* p < 0.05; ** p < 0.005). (b) IgE/IgG₄ ratio at different time intervals, in patients with subcutaneous (SCIT) and sublingual immunotherapy (SLIT).

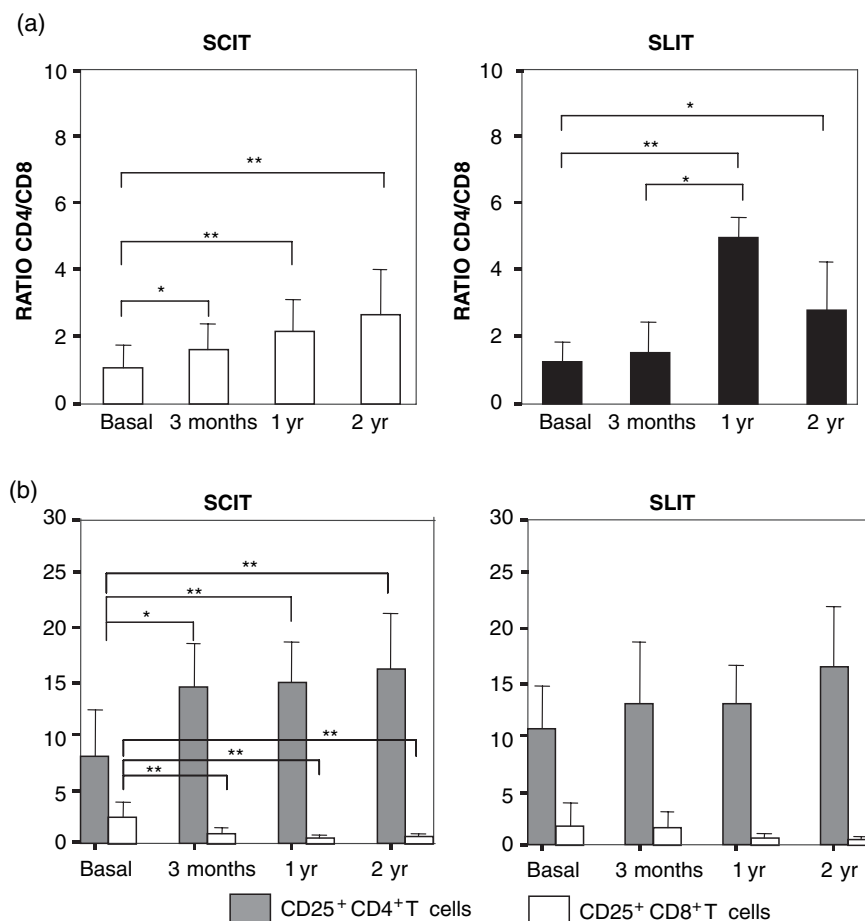


Fig. 2. Comparison of T cell subpopulations in peripheral blood at different time intervals (before immunotherapy, and three months, 1 yr and 2 yr after beginning treatment) in patients with subcutaneous (SCIT) and sublingual immunotherapy (SLIT). (a) CD4⁺/CD8⁺ ratio in SCIT (left) and SLIT (right). (b) Expression of CD25 in CD4⁺ and CD8⁺ T cells in SCIT (left) and SLIT (right) The results are expressed in percentage of cells as mean $\pm$ s.d. (* $p < 0.05$; ** $p < 0.005$).

$p = 0.002$; SLIT, $p = 0.016$). Changes were observed earlier in SCIT, from the first month, while with SLIT we detected no significant changes until 2 yr of treatment (Fig. 1b).

T-cell response in peripheral blood

We analysed the CD4/CD8 T lymphocyte ratio at different times in SCIT and SLIT. Comparison between the two groups at baseline showed no significant differences. We detected a significant increase in both groups over time (SCIT: $p < 0.001$; SLIT: $p = 0.004$). However, the increase appeared earlier with SCIT (at 3 months, $p = 0.041$) than with SLIT (at 1 yr, $p = 0.004$) (Fig. 2, top). The increase in the ratio was mainly due to a significant increase in the CD3⁺CD4⁺ subpopulations in both SCIT ($p = 0.01$) and SLIT ($p = 0.048$) (data not shown). The bottom part of Fig. 2 shows the expression of the CD25 marker in the CD4 and CD8 subpopulations. Significant changes were detected in SCIT, with an increase in the CD4⁺CD25⁺ subpopulation ($p < 0.001$) and a decrease in the CD8⁺CD25⁺ subpopulation

($p = 0.005$) between 3 months and the end of the study. A similar trend was seen in SLIT, though it was not significant. The measurements carried out in this study did not enable us to determine whether these CD4⁺CD25⁺ cells were T_{reg} or activated cells.

Cytokine production in PBMC

Using intracellular cytokine staining and flow cytometry we analysed changes at the single cell level and compared the effect induced by SCIT and SLIT. We checked again that there were no differences between both groups at baseline. First, we analysed the production of TNF- α and IL-2 in both CD4⁺ and CD8⁺ T cells (Fig. 3). In the CD4⁺ T cells, we detected an important decrease in all the children in the production of TNF- α (SCIT: $p = 0.002$; SLIT: $p = 0.019$) and IL-2 (SCIT: $p = 0.001$; SLIT: $p < 0.001$). These changes appeared with effect from the first post-IT sample obtained and did not return to baseline levels at 2 yr. Analysis of the CD8⁺ T cells showed significant changes with SCIT (TNF- α : $p = 0.009$; IL-2: $p = 0.001$) and SLIT (IL-2: $p < 0.001$).

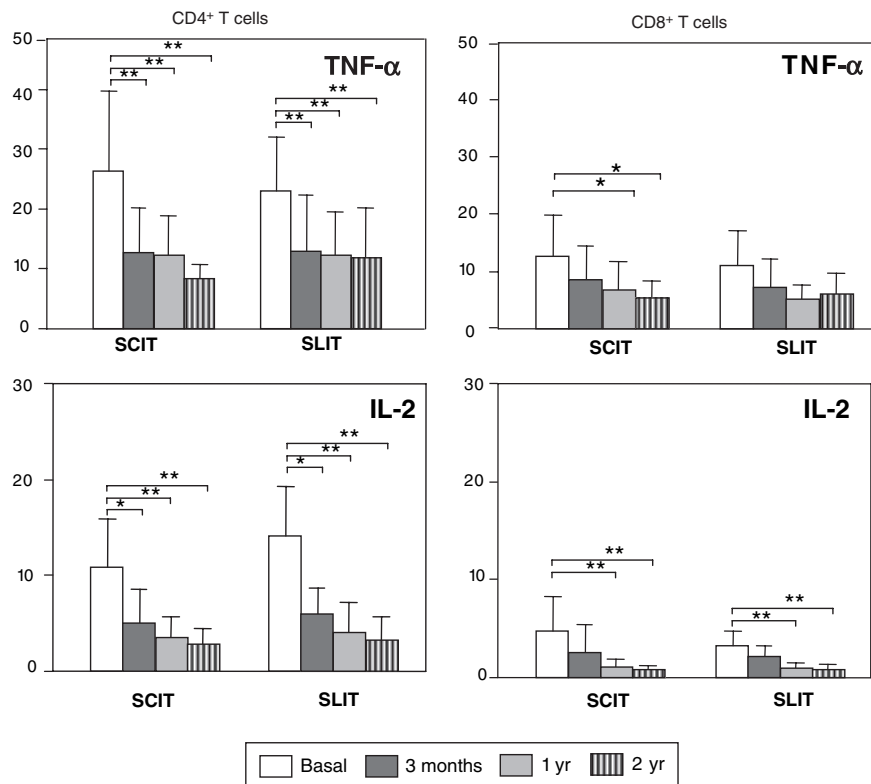


Fig. 3. Comparison of the different cytokine expression (IL-10, IL-2 and TNF- α) in the CD4⁺ and CD8⁺ T cells at different time intervals (before immunotherapy, and three months, 1 yr and 2 yr after beginning treatment) in patients with subcutaneous (SCIT) and sublingual immunotherapy (SLIT). The results are expressed in percentage of cells as mean $\pm$ s.d. (*p < 0.05; **p < 0.005).

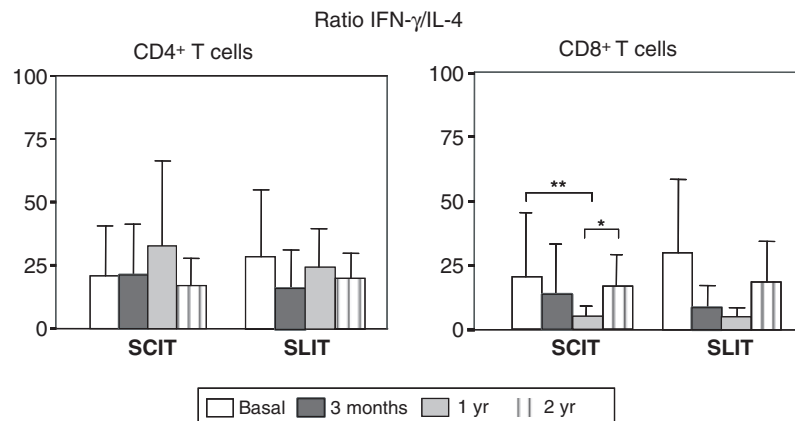


Fig. 4. Comparison of the IFN- γ /IL-4 ratio in circulating CD4⁺ and CD8⁺ T cells at different time intervals (before immunotherapy, and three months, 1 yr and 2 yr after beginning treatment) in patients with subcutaneous (SCIT) and sublingual immunotherapy (SLIT). The results are expressed as ratio of percentage of cells as mean $\pm$ s.d. (*p < 0.05; **p < 0.005).

We also analysed the percentage of cells producing IL-4 and IFN- γ , expressing the results as the IFN- γ /IL-4 ratio in both CD4 and CD8 T cells (Fig. 4). With SCIT we detected a increase in the IFN- γ /IL-4 ratio in the CD4 T cells from baseline to year one, with levels tending to normalize at 2 yr. These changes were nearly significant (p = 0.060). A parallel significant decrease in this ratio occurred in the CD8⁺ T cells (p = 0.039). With SLIT, however, no tendency for the IFN- γ /IL-4

ratio to increase was detected in the CD4⁺ T cells. Changes in the CD8⁺ subsets had a similar tendency to the changes in SCIT.

Discussion

Specific IT is a well-documented treatment for allergic diseases and is highly effective in carefully selected patients with IgE mediated diseases. It has been shown to be effective for

venom anaphylaxis and for rhinoconjunctivitis and asthma caused by inhalant allergens (3). The non-injectable route for IT was initially introduced as a solution to problems related with the classical subcutaneous route, with SLIT being the more widely accepted form (4). Numerous controlled trials have shown the efficacy of SLIT in asthma and rhinitis in adults and children (18, 21–22). However, several important points remain to be elucidated, especially the underlying mechanisms of SLIT. We monitored the immunological changes in peripheral blood, both humoral and T lymphocyte response, in a group of children sensitized to D.pter with respiratory disease receiving SLIT, and compared the changes with those occurring in children receiving SCIT.

The design of this study might involve a certain bias that requires analysis. (i) Ours was not a double-blind placebo-controlled study for various reasons. The study involved a 2-yr follow up in children, a period that was too long to be accepted by the ethical committee of our hospital and, even more so, by the parents if we consider that what we wanted to analyse was the *in vitro* immunological changes induced by SLIT. However, the aim of this study was not to evaluate the effectiveness of IT, which has already been done by others in a double-blind manner using the same doses as we used (23–25), but rather to evaluate *in vitro* changes in patients receiving SLIT compared to those receiving SCIT. (ii) Regarding the evaluation of the VAS, although a placebo effect may exist in our study, this effect should be similar in both groups and not affect the *in vitro* results. Moreover, this scale does not show effectivity but rather the perception of the patients about their illness over time. (iii) Another possible bias is that both SCIT and SLIT children were initially different in the *in vitro* determinations. However, comparisons between both groups at baseline showed no differences, indicating that both groups were homogenous.

In SCIT, as previously described (26), we found a gradual increase in specific IgE and IgG₄ antibodies to D.pter, although this increase began significantly sooner for IgG₄ (3 months) than for IgE (1 yr), with no significant changes in total IgE. However, we detected no changes in total IgE, or specific IgE and IgG₄ to D.pter in the group of patients receiving SLIT. The peripheral blood humoral response in patients receiving SLIT is controversial. Some studies have reported changes in allergen-specific antibodies (26, 27), whereas others have found a limited impact (23, 28, 29). A recent

meta-analysis concluded that a consistent increase in allergen-specific IgG₄ levels existed (21), but other studies, including ours, have not found any change (14, 30). However, when we analysed the specific IgE/IgG₄ ratio a decrease was indeed observed in both types of IT, SCIT and SLIT. These changes have also been detected by other authors (31–33), with some exceptions (34), and were found to be correlated with the clinical efficacy (32, 35).

Concerning T lymphocytes, a number of recent studies have shown that they may play a central role in allergic respiratory diseases (36, 37). IT acts preferentially on T lymphocytes, suggesting an important role in the treatment of these patients (38–40). Several lines of evidence indicate that specific IT may act by modifying the patterns of cytokines produced by T helper cells, with a shift from a Th2 to a Th1 response (13, 14, 41). However, no monitoring of the different T-cell subpopulations in patients receiving SLIT has been done.

In our study, we found similar increases in the CD4/CD8 ratio in both SCIT and SLIT, though detected earlier in the former. These results show a role for T lymphocytes in the IT response in both types of IT. Previous reports have demonstrated the modulation of IL4 and IFN γ (13, 14, 41). Using intracellular cytokine staining and flow cytometry, in SCIT we found only a small rise in the IFN γ /IL4 ratio among peripheral blood CD4 T cells (nearly significant) accompanied by a decrease in CD8 T cells; these changes appeared with effect from the first determination and tended to return to baseline levels at the end of IT. These results are in agreement with those previously published (13–15), indicating a shift from a Th2 to a Th0 or Th1 cytokine pattern induced by injectable IT. However, this tendency was not detected in SLIT, either in CD4 or CD8 T cells, in any of the samples analysed. We also analysed the expression of the CD25 marker in the two T-cell subpopulations, finding differences only in SCIT, with an increase in CD4 T cells and a parallel decrease in CD8 T cells. Although it is tempting to speculate that this increase in CD4⁺CD25⁺ T cells is due to an increase in T_{reg} cells, the design of this study did not enable us to demonstrate this, although the decrease in the IFN γ /IL4 ratio specifically in CD8 only in SCIT could reinforce this hypothesis. Indeed, the presence of T_{reg} decreases the number of CD8 T cells producing IFN γ (42).

Finally, as other cytokines are known to play a key role in the initiation and maintenance of the inflammatory response (43), we analysed the

production of TNF- α and IL-2. As others, we too found a decreased production of TNF- α and IL-2 in peripheral blood (44). These results may indicate a reduction of the inflammatory response, as these cytokines are known to be involved in allergic inflammation (2, 20).

Summarizing, this study shows that children with respiratory allergic diseases receiving SCIT or SLIT had a different immunological response in peripheral blood during therapy, though the clinical improvement, measured by subjective evaluation, was similar. The main difference detected in humoral responses was that, despite a final decrease in both therapies, in SCIT significant changes were detected in IgE/IgG₄ with effect from the first month while in SLIT they only were observed at 2 yr. With respect to the cellular response, changes were observed mainly in SCIT. The route and dose of antigen administered may determine these differences. As the oral mucosa is an immunocompetent site and SLIT may benefit from the physiological mechanisms of oral tolerance, the possibility of a mucosal protective immune response should be analysed in depth.

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References

- CANONICA GW, PASSALACQUA G. Noninjection routes for immunotherapy. *J Allergy Clin Immunol* 2003; 111: 437–48.
- BOUSQUET J, LOCKEY RF, MALLING HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998; 102: 558–62.
- LOCKEY RF. "ARIA": global guidelines and new forms of allergen immunotherapy. *J Allergy Clin Immunol* 2001; 108: 497–9.
- PASSALACQUA G, GUERRA L, PASQUALI M, LOMBARDI C, CANONICA GW. Efficacy and safety of sublingual immunotherapy. *Ann Allergy Asthma Immunol* 2004; 93: 3–12.
- DURHAM SR, WALKER SM, VARGA EM, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999; 341: 468–75.
- PAGNO GB, BARBERIO G, DE LUCA F, MORABITO L, PARMIANI S. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. *Clin Exp Allergy* 2001; 31: 1392–7.
- MOLLER C, DREBORG S, FERDOUSI HA, et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* 2002; 109: 251–6.
- MOINGEON P, BATARD T, FADEL R, FRATI F, SIEBER J, VAN OVERTVELT L. Immune mechanisms of allergen-specific sublingual immunotherapy. *Allergy* 2006; 61: 151–65.
- GEHLHAR K, SCHLAACK M, BECKER W, BUFE A. Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clin Exp Allergy* 1999; 29: 497–506.
- VAN NEERVEN RJ, WIKBORG T, LUND G, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4⁺ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999; 163: 2944–52.
- EWAN PW, DEIGHTON J, WILSON AB, LACHMANN PJ. Venom-specific IgG antibodies in bee and wasp allergy; lack of correlation with protection from stings. *Clin Exp Allergy* 1993; 23: 647–60.
- DJURUP R, MALLING HJ. High IgG4 antibody level is associated with failure of immunotherapy with inhalant allergens. *Clin Allergy* 1987; 17: 459–68.
- VARNEY VA, HAMID QA, GAGA M, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993; 92: 644–51.
- JUTEL M, PICHLER WJ, SKRBIC D, URWYLER A, DAHINDEN C, MULLER UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995; 154: 4187–94.
- EBNER C, SIEMANN U, BOHLE B, et al. Immunological changes during specific immunotherapy of grass pollen allergy; reduces lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. *Clin Exp Allergy* 1997; 27: 1007–15.
- JUTEL M, AKDIS M, BUDAK F, et al. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003; 33: 1205–14.
- AKBARI O, STOCK P, DEKRUY RH, UMETSU DT. Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol* 2003; 15: 627–33.
- BOUSQUET J, VAN CAUWENBERGE P. (eds). Allergic rhinitis and its impact on asthma (ARIA). Position paper endorsed by WHO. *J Allergy Clin Immunol* 2001; 108 (Suppl. 5): 147–334.
- VON MUTIUS E. Presentation of new GINA guidelines for paediatrics. The Global Initiative on Asthma. *Clin Exp Allergy* 2000; 30 (Suppl. 1): 6–10.
- AKDIS CA, AKDIS M, BLESKEN T, et al. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* 1996; 98: 1676–83.
- WILSON DR, LIMA MT, DURHAM SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. *Allergy* 2005; 60: 4–12.
- BOUSQUET J, SCHEINMANN P, GUINNEPAIN MT, et al. Sublingual-swallow immunotherapy (SLIT) in patients with asthma due to house-dust mites: a double-blind, placebo-controlled study. *Allergy* 1999; 54: 249–60.
- PAGNO GB, MORABITO L, BARBERIO G, PARMIANI S. Clinical and immunologic effects of long-term sublingual immunotherapy in asthmatic children sensitized to

- mites: a double-blind, placebo-controlled study. *Allergy* 2000; 55: 842–9.
24. MARCUCCI F, SENSI L, FRATI F, et al. Effects on inflammation parameters of a double-blind, placebo controlled one-year course of SLIT in children mono-sensitized to mites. *Allergy* 2003; 58: 657–62.
25. TABAR AI, ECHECHIPIA S, GARCIA BE, et al. Double-blind comparative study of cluster and conventional immunotherapy schedules with *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 2005; 116: 109–18.
26. CLAVEL R, BOUSQUET J, ANDRE C. Clinical efficacy of sublingual-swallow immunotherapy: a double-blind, placebo-controlled trial of a standardized five-grass-pollen extract in rhinitis. *Allergy* 1998; 53: 493–8.
27. VOURDAS D, SYRIGOU E, POTAMIANOU P, et al. Double-blind, placebo-controlled evaluation of sublingual immunotherapy with standardized olive pollen extract in pediatric patients with allergic rhinoconjunctivitis and mild asthma due to olive pollen sensitization. *Allergy* 1998; 53: 662–72.
28. HIRSCH T, SAHN M, LEUPOLD W. Double-blind placebo-controlled study of sublingual immunotherapy with house dust mite extract (D.pt.) in children. *Pediatr Allergy Immunol* 1997; 8: 21–7.
29. KAY AB, DURHAM SR. T-lymphocytes, allergy and asthma. *Clin Exp Allergy* 1991; 21: 17–21.
30. GUEZ S, VATRINET C, FADEL R, ANDRE C. House-dust-mite sublingual-swallow immunotherapy (SLIT) in perennial rhinitis: a double-blind, placebo-controlled study. *Allergy* 2000; 55: 369–75.
31. LA ROSA M, RANNO C, ANDRE C, CARAT F, TOSCA MA, CANONICA GW. Double-blind placebo-controlled evaluation of sublingual-swallow immunotherapy with standardized *Parietaria judaica* extract in children with allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 1999; 104: 425–32.
32. TROISE C, VOLTOLINI S, CANESSA A, PECORA S, NEGRINI AC. Sublingual immunotherapy in *Parietaria* pollen-induced rhinitis: a double-blind study. *J Investig Allergol Clin Immunol* 1995; 5: 25–30.
33. BAHCECILER NN, ARIKAN C, TAYLOR A, AKDIS M, BLASER K, BARLAN IB, AKDIS CA. Impact of sublingual immunotherapy on specific antibody levels in asthmatic children allergic to house dust mites. *Int Arch Allergy Immunol* 2005; 136: 287–94.
34. ROLINCK-WERNINGHAUS C, KOPP M, LIEBKE C, LANGE J, WAHN U, NIGGEMANN B. Lack of detectable alterations in immune responses during sublingual immunotherapy in children with seasonal allergic rhinoconjunctivitis to grass pollen. *Int Arch Allergy Immunol* 2005; 136: 134–41.
35. LIMA MT, WILSON D, PITKIN L, et al. Grass pollen sublingual immunotherapy for seasonal rhinoconjunctivitis: a randomized controlled trial. *Clin Exp Allergy* 2002; 32: 507–14.
36. CHANG JH, CHAN H, QUIRCE S, et al. In vitro T-lymphocyte response and house dust mite-induced bronchoconstriction. *J Allergy Clin Immunol* 1996; 98: 922–31.
37. VAN BEVER HP, BRIDTS CH, MOENS MM, et al. Lymphocyte transformation test with house dust mite (*Dermatophagoides pteronyssinus*) in normal children, asthmatic children and asthmatic children receiving hyposensitization. *Clin Exp Allergy* 1993; 23: 661–8.
38. BONNO M, FUJISAWA T, IGUCHI K, et al. Mite-specific induction of interleukin-2 receptor on T lymphocytes from children with mite-sensitive asthma: modified immune response with immunotherapy. *J Allergy Clin Immunol* 1996; 97: 680–8.
39. HSIEH KH. Altered interleukin-2 (IL-2) production and responsiveness after hyposensitization to house dust. *J Allergy Clin Immunol* 1985; 76: 188–94.
40. VISSERS JL, VAN ESCH BC, HOFMAN GA, KAPSENBERG ML, WELLER FR, VAN OOSTERHOUT AJ. Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model. *J Allergy Clin Immunol* 2004; 113: 1204–10.
41. GARDNER LM, THIEN FC, DOUGLASS JA, ROLLAND JM, O'HEHIR RE. Induction of T 'regulatory' cells by standardized house dust mite immunotherapy: an increase in CD4+ CD25+ interleukin-10+ T cells expressing peripheral tissue trafficking markers. *Clin Exp Allergy* 2004; 34: 1209–19.
42. GORBACHEV AV, FAIRCHILD RL. CD4+ T cells regulate CD8+ T cell-mediated cutaneous immune responses by restricting effector T cell development through a Fas ligand-dependent mechanism. *J Immunol* 2004; 172: 2286–95.
43. ZUANY-AMORIM C, MANLIUS C, DALUM I, et al. Induction of TNF- ∞ Autoantibody Production by AutoVac TNF106: A Novel Therapeutic Approach for the Treatment of Allergic Diseases. *Int Arch Allergy Immunol* 2004; 133: 154–63.
44. DE AMICI M, PUGGIONI F, CASALI L, ALESINA R. Variations in serum levels of interleukin (IL)-1 β , IL-2, IL-6, and tumor necrosis factor- α during specific immunotherapy. *Ann Allergy Asthma Immunol* 2001; 86: 311–3.