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ORIGINAL PAPER

Sublingual immunotherapy with *Dermatophagoides* monomeric allergoid down-regulates allergen-specific immunoglobulin E and increases both interferon-γ- and interleukin-10-production

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Summary

Background The clinical efficacy and safety of sublingual immunotherapy (SLIT) for aeroallergens has been demonstrated in several trials, whereas the immunological changes induced by this treatment, which may account for the clinical improvement, are still unclear.
Objective To investigate the effects of a successful SLIT on the *in vitro* allergen-driven T cell response and cytokine secretion as well as on the serum levels of chemokines and of IgE, IgG1 and IgG4 antibodies (Abs).
Materials and methods Twenty-five *Dermatophagoides pteronyssinus* (Dp)-sensitive patients with perennial rhinitic and/or rhinitic and asthmatic symptoms were randomized into two groups (13 untreated (UT) and 12 SLIT-treated) for a 1 year and half study. The proliferative response of peripheral blood mononuclear cell (PBMC) to purified Der p1 allergen, their cytokines (IFN-γ, IL-4, IL-10 and TGF-β) production and serum levels of chemokines associated with T helper type 1 (Th1) (CXCL10) or T helper type 2 (Th2) (CCL22) responses and of Dp-specific IgE, IgG1 and IgG4 Abs were evaluated before and after 6 months of treatment.
Results SLIT induced a significant reduction of symptom medication scores after 6, 12 and 18 months of treatment in comparison with UT patients. SLIT-treated patients showed a significant decrease in serum levels of DP-specific IgE Abs, whereas total IgE, and specific IgG1 and IgG4 Abs remained unchanged. The proliferative response of allergen-specific T cells to Der p1 *in vitro* after 6 months of treatment was reduced, while no effect was observed on T cell proliferation to recall antigen (streptokinase). Moreover, Der p1-driven IFN-γ and IL-10 were significantly increased in culture supernatants of PBMC from 6 month-treated patients in comparison with those detected at the beginning of therapy.
Conclusions These data suggest that the allergen-driven enhancement of IL-10- and IFN-γ-producing T cells precedes and associates with SLIT-induced down-regulation of specific IgE, thus providing a rationale to explain the clinical benefit of SLIT in allergic patients.

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Introduction

Allergen-specific immunotherapy is recognized as a highly effective practice in the treatment of patients with severe allergic rhinitis (AR) and/or asthma, and is recommended by WHO as an integrated part of the allergy management strategy [1–3]. Several studies have shown that immunotherapy, based on the subcutaneous administration of increasing doses of allergen (SIT), achieves a hyposensitization and reduces both early and late responses occurring during the natural exposure to allergen itself. SIT is accompanied by increases in the titre of

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allergen-specific IgG antibodies (Abs), particularly the IgG4 isotype [4], which blocks IgE-dependent release of mediators from basophils and mast cells. This leads to a reduced T cell response to the specific allergen [5] and to a long-lasting impairment of serum IgE Abs [6]. It has also been demonstrated that SIT modifies peripheral and mucosal T helper type 2 (Th2)- responses into a prevalent T helper type 1 (Th1) cytokine profile of allergen-specific $CD4^+$ T cells [7–9]. Moreover, the increased proportion of IL-10-producing T cells in response to allergen stimulation after SIT has emerged as a highly consistent finding, which can explain several humoral and cellular alterations observed in treated patients [10, 11]. An important question concerns whether the increased Th1 responses or IL-10 (or both) play some roles in the induction of tolerance to allergen seen during SIT.

Sublingual immunotherapy (SLIT) was introduced during the 1980s as an alternative route of immunotherapy, conferring more safety than SIT. Its efficacy in reducing symptoms and medication usage, as well as in decreasing bronchial/nasal reactivity towards the offending allergen in a considerable number of trials performed in adults and children has been clearly demonstrated [12]. In 1998, WHO, and in 2001, the allergic rhinitis and its impacts on asthma (ARIA) document concluded that SLIT is a valid alternative to the injective SIT and that its use in clinical practice in adults as well in children is justified [9, 13]. In a very recent Cochrane meta-analysis, the efficacy of SLIT has been definitively shown [14].

Because of the few observations on the mechanisms exerted by SLIT, no clear evidence has been provided of whether it is capable of affecting the allergen-specific immune response. A relevant effect of SLIT on serum IgE or IgG isotypes was observed only in a minority of studies [15–17], whereas there is only one report on the reduction of T cell response to allergen [18]. In humans, SLIT has been shown to impair inflammatory phenomena (cellular infiltration and adhesion molecule expression on epithelia) in target organs [16]. Only one report has recently shown that SLIT was able to increase allergen-driven IFN-γ production [19], while other authors did not find any humoral or cellular changes [20]. By contrast, several evidence in animal models of asthma indicate that the oral route of immunotherapy is tolerogenic and can redirect the Th1/Th2 differentiation, in part by eliciting dendritic cells of oral mucosa producing high levels of IL-12 [21]. The *Dermatophagoides* monomeric allergoid used in this study for SLIT was submitted to a 2-year double-blind placebo-controlled trial, demonstrating clinical efficacy and a significant decrease of allergen-driven inflammatory response [22].

In this study, some specific immune parameters were assessed in a panel of *Dermatophagoides pteronyssinus* (Dp)-sensitive patients before and during SLIT, and were compared with those from a matched group of untreated (UT) Dp-sensitive patients. SLIT induced an improvement of symptoms accompanied by a decrease in serum-specific IgE, but not of IgG1 and IgG4, Abs within 12–18 months of therapy. More importantly, SLIT increased IL-10 and IFN-γ production upon allergen stimulation of peripheral blood mononuclear cell (PBMC) before the clinical improvement. As both cytokines are able to impair Th2 responses, this finding provides a biological basis for the clinical effects of SLIT.

Materials and methods

Reagents

Dp extract in glycero-saline (glycerol 50%, sodium chloride 3.4 mg/mL, sodium bicarbonate 1.375 mg/mL, phenol 4 mg/mL) for skin prick tests (SPT), and purified Der p 1, allergen (obtained by mAb affinity chromatography) for *in vitro* tests were purchased from Lofarma S.p.A. (Milan, Italy).

All selected patients were prescribed a commercial SLIT treatment with a monomeric allergoid of *Dermatophagoides* (50/50 mix of Dp and *D. farinae*) (LAIS®, Lofarma SpA), obtained by carbamilation with potassium cyanate at alkaline pH, a reaction that leads to a substantial substitution of ε-amino groups of lysine residues and consequently a strong decrease in the capacity to react with IgE Abs [23]. The product, standardized for allergenic potency by RAST/EAST-inhibition in comparison with an in-house reference preparation and titrated in allergenic units (AU), was formulated in orosoluble tablets and administered sublingually.

The medium used was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% pyruvate, 2×10^{-5} M 2-mercaptoethanol (2-ME) (all from Gibco Laboratories, Grand Island, NY, USA) and 5% autologous serum. Unconjugated and flourescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridin chlorophyll protein (PerCP)-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-IL-10, anti-IL-10R, anti-IFN-γ and anti-IL-4 monoclonal antibodies (mAbs) were purchased from Becton Dickinson Biosciences (Mountain View, CA, USA).

All unconjugated and conjugated, isotype-matched, control Abs were purchased from Southern Biotechnology Associated Inc. (Birmingham, AL, USA). Streptokinase (SK) antigen was purchased by Behring (Behring, L'Aquila, Italy). Phorbol-12-myristate-13-acetate (PMA), ionomycin, brefeldin A, phytohaemagglutinin (PHA) and saponin were from Sigma Chemical Co. (St Louis, MO, USA). IL-2 was a kind gift from Eurocetus (Milan, Italy).

Patients

A total number of 25 (14 men and 11 women) age-matched Dp-sensitive patients having perennial rhinitis

and/or rhinitis plus mild asthma were recruited from November to December 2002. The criteria for inclusion in the study were as follows: age below 45 years; history of perennial allergic symptoms for at least 3 years; positive SPT for Dp; RAST positivity (more than class 3); and no chronic usage of drugs or prior SIT. Six out of the 20 selected patients showed positive SPTs for seasonal allergens: five for grass pollens and one for compositae pollens.

Balancing of experimental groups

The selected patients were randomized into two groups (consisting of 13 UT patients and 12 treated with SLIT) taking into account age, sex, type and importance of symptoms. Six of them also had seasonal symptoms and were included in UT- [3] or SLIT-treated [3] group. Descriptions of the patients are summarized in Table 1.

Immunotherapy programmes

SLIT was carried out by self-administration and was continued from January 2003 to December 2004. The patients were carefully instructed by the prescribing physician on the modality of use of the SLIT and the dosage schedule. Moreover, the manufacturer provided clear written instructions. The buildup phase of about 8 weeks involved the administration, every other day, of increasing doses (25, 50, 100, 200, 300, 600, 1000 AU) until the maintenance dose of 1000 AU was reached. This maintenance dose was then administered once a week. The cumulative dose of allergen extract received by each patient was about 60 000 AU/year. The tablets had to be taken in the morning, dissolved in the mouth for 1–2 min and then swallowed.

All patients were prescribed an appropriate drug therapy to control their symptoms. Regular visits for clinical evaluation and safety assessment were scheduled. Blood samples were taken before starting treatment, at 6, 12 and 18 months of therapy. The study was conducted according to Good Clinical Practices rules. The patients gave informed consent before inclusion.

Table 1. Description of patients

	SLIT group	Control group
Number of patients	12	13
Mean age	28	30
Age range	16–43	20–36
Gender (M/F)	7/4	5/4
Single sensitization	8/11 (72%)	6/9 (67%)
Rhinitis alone	6/11 (55%)	5/9 (56%)
Rhinitis + mild asthma	5/11 (45%)	4/9 (44%)
Follow-up period (months)	18	18
Duration of disease (years)	4.3 ± 3.8	4.0 ± 3.1
Drop out	1	4

SLIT, sublingual immunotherapy.

Clinical evaluation

Both treated and UT patients maintained a weekly diary of allergic symptoms during natural exposure periods. Specific symptoms scores (SSS) recorded were nasal blockage, nasal itching, sneezing, rhinorrhoea, eye irritation and watering, wheezing, cough and asthma. Symptom medication scores (SMS) were calculated from patient diaries, as described previously [24]. Arbitrary scores were attributed to the drugs used (0.5 points for each dose of nasal corticosteroids and 2 points for each dose of antihistamine). Patients were instructed to use local steroids only (plus antihistamines if they did not improve symptoms) and to report each administration or variation of the initial drug therapy in the diary. The values reported in Table 3 are the average of daily SMS in the indicated period. Patients were also instructed to stop drugs at least 7 days before blood sampling. At each time point (time 0, 6, 12 and 18 months) of the study, patient self-evaluation was carried out: each one was asked for his/her overall evaluation of the treatment with categories of symptoms gravity (from 0 to 15).

Detection of immonuglobulin E and immunoglobulin G antibodies and chemokines in the serum

Sera were collected in January, June, December 2003 and June 2004. Total and anti-Dp IgE Abs were detected in the sera by the commercial IgE CAPSYSTEM® (Pharmacia, Uppsala, Sweden). Allergen-specific IgG1 and IgG4 Abs were also measured by the common ELISA procedure [25] using monoclonal anti-IgG1 and anti-IgG4 (Unipath Limited, Bedford, UK) as first Ab, and anti-murine-IgG raised in goat and peroxidase-conjugated (Sigma-Aldrich Srl, Milan, Italy) as second Ab. The absorbance was read at 492 nm, and results were expressed as the ratio between OD of the sample and OD of the control negative sera as described previously. Detection of CXCL10 and CCL22 in the serum was performed using commercial kits (R & D Systems, Minneapolis, MN, USA).

Flow cytometric analysis

Flow cytometry analysis of PBMC was performed as detailed elsewhere [26]. Cells were analysed on a BDLSRII cytofluorimeter, using the Diva software (BD Biosciences, Mountain View, CA, USA). The area of positivity was determined using an isotype-matched control mAb. Ten thousand events for each sample were acquired.

Allergen-specific proliferative response

Allergen-specific T cell response was assessed on blood samples taken at the beginning and in 6th month of therapy. PBMC (10^6/mL), suspended in medium additioned of 5% of autologous serum, were cultured in triplicate on 96 U-bottomed plates (Nunc, Kamstrup, Denmark) for 5 days in the presence of increasing doses of Der p 1 (0.4, 2 and 10 μg/mL) and of unrelated recall antigen (SK, at 400, 2000 and 10 000 IU/mL). In some experiments, PBMC were stimulated with Der p 1 in the absence or presence of neutralizing anti-IL10 (10 μg/mL) or anti-IL-10R (10 μg/mL), or an isotype-matched, mAbs (10 μg/mL). After a 16 h pulse with 0.5 μCi (^{3}H)-thymidine/well (^{3}H-TdR; Amersham International, Bucks, UK), cultures were harvested and (^{3}H)-TdR was measured [27].

Quantitation of cytokine production

To assess cytokine production, 10^6 PBMC were activated with different doses of allergen or antigen and 5-day cell-free supernatants were frozen at −70 °C until use. The quantitative determination of IL-4, IL10, TGF-β and IFN-γ were performed using a commercial ELISA (Quantikine R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Flow cytometric analysis of intracellular cytokines

Allergen-specific short-term T cell lines were generated from PBMCs (1×10^6/mL) of two SLIT-treated donors with Der p 1 (10 μg/mL). On day 7, allergen-activated T cells were expanded with rIL-2 as described [27].

Flow cytometric analysis at the single cell level of intracellular IL-4, IL-10 and IFN-γ synthesis of the T cell lines was performed, as detailed elsewhere [26]. Briefly, 1×10^6 cells were stimulated with PMA (10 ng/mL) plus ionomycin (1 μM) for 6 h, the last four in the presence of brefeldin A (5 μg/mL). After stimulation, cells were washed twice with Phosphate buffered solution (PBS) pH 7.2, fixed for 15 min with formaldehyde (2% in PBS pH 7.2), washed twice with 0.5% bovine serum albumin (BSA) in PBS pH 7.2, permeabilized with PBS pH 7.2, containing 0.5% BSA and 0.5% saponin, and then incubated for 15 min at room temperature with the specific mAbs. Cells were then washed and analysed on a BDLSRII cytofluorimeter, using the Diva software (BD Biosciences). The area of positivity was determined using an isotype-matched control mAb. 10^4 events for each sample were acquired.

Statistical analysis

Statistical analysis was performed using the paired and unpaired Student's *t*-test, as appropriate. A P-value of less than 0.05 was considered significant.

Results

Clinical efficacy of sublingual immunotherapy for Dermatophagoides pteronyssinus

Twenty out of 25 enrolled patients (80%) completed the study in the year 2004. The 'dropouts' were four among the UT patients and one in the SLIT-treated group, who stopped SLIT owing to pregnancy. The dropouts among UT patients occurred because of low compliance in providing blood samples.

Clinical results of the 1-year and half follow-up were based on the patient self-evaluation scores obtained at the beginning and after 6, 12 and 18 months of treatment. The SSS of both SLIT-treated and UT patients at 6, 12 and 18 months were significantly reduced in comparison with those shown at time 0 (Table 2). Of note, in SLIT-treated, but not in UT, patients a significant reduction in SMS was also observed (Table 3).

Effects of sublingual immunotherapy on serum levels of allergen-specific immunoglobulin E, immunoglobulin G1 and immunoglobulin G4 antibodies

Dp-specific IgE Abs decreased in serum samples from SLIT-treated patients in comparison with those detected before the treatment, whereas total IgE were not affected. As shown in Fig. 1, the reduction of Dp-specific IgE was significant after 12 (in 10 out of 11 patients) and 18 (in all 11 patients) months ($P < 0.05$ and $P < 0.005$, respectively) of therapy. By contrast no changes of both total and Dp-specific IgE were observed in UT patients analysed at 0, 6, 12 and 18 months of the study. The amounts of Dp-

Table 2. Specific symptoms scores

	SLIT group	Control group
Time 0	10.0 ± 1.8	10.1 ± 1.6
6 months	6.2 ± 1.2	7.1 ± 2.4*
12 months	5.7 ± 1.1*	6.0 ± 1.7*
18 months	5.3 ± 0.8*	5.6 ± 1.0*

Clinical scores (± SE) were evaluated as described in Materials and methods.
*$P < 0.05$ (vs. time 0 in each group)
SLIT, sublingual immunotherapy.

Table 3. Daily Symptom medication scores

	SLIT group	Control group
6 months	2.6 ± 0.8	3.3 ± 0.9
12 months	1.6 ± 0.5[a]	2.4 ± 0.6[b]
18 months	1.1 ± 0.4[c]	2.7 ± 0.8[d]

Daily symptom medication scores (± SE) were evaluated as described in Materials and methods. a vs. b, and c vs. d: $P < 0.05$
SLIT, sublingual immunotherapy.

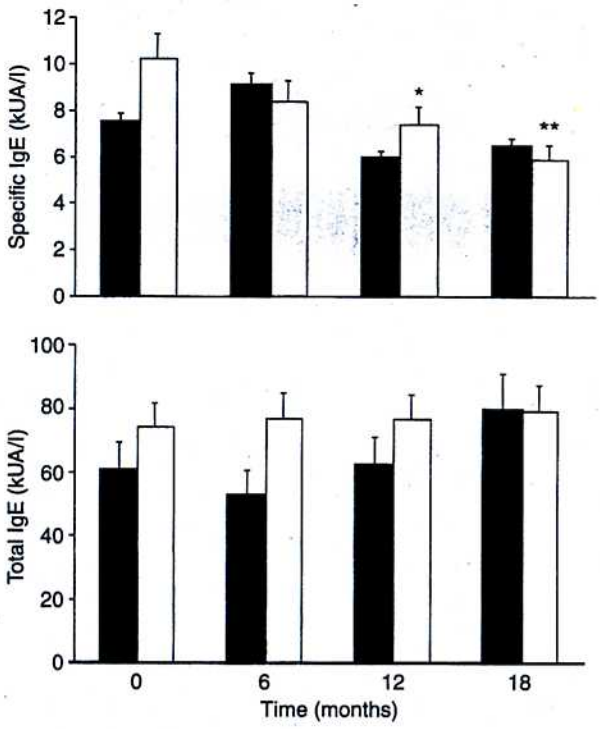

Fig. 1. Der p 1-specific and total IgE in the serum of untreated and sublingual immunotherapy (SLIT)-treated patients. Serum samples taken from nine untreated (black columns) and 11 SLIT-treated (white columns) patients at different times (0, 6, 12, 18 months) from the beginning of treatment were assayed for Der p 1-specific (upper panel) and total (lower panel) IgE antibodies (Abs) by commercial kits as described in Materials and methods. The mean values (± SE) are reported. $^{*}P < 0.05$; $^{**}P < 0.005$.

specific IgG1 and IgG4 were low in both groups and were not changed after the treatment (data not shown).

Effects of sublingual immunotherapy on the allergen-driven proliferative response and on T helper type 1/T helper type 2 balance

The proliferative response to increasing doses (0.4, 2 and 10 μg/mL) of Der p1 of PBMC from patients in the 6th months of therapy was lower than that found before treatment (Fig. 2). No change in the proliferative response to increasing doses of SK was found in PBMC from both UT- and SLIT-treated patients, analysed in the beginning and after 6 months of treatment (Fig. 2).

When, we compared the Th1- and Th2-related cytokines secreted in culture supernatants of PBMC, the levels of IFN-γ were significantly ($P < 0.05$) increased upon stimulation with high doses of allergen in PBMC of patients in the 6th month of SLIT in comparison with those detected in supernatants at the beginning of treatment (Figs 3a and 3b). By contrast, the amounts of IFN-γ in culture supernatants of PBMC stimulated with recall antigen (SK) from the same patients at 0 or 6 months of therapy were virtually unchanged (data not shown), as were those from UT patients stimulated with both antigens at the same time-points (Fig. 3a and data not shown).

Of note, the levels of IFN-γ in comparable samples (culture supernatants from the same patient, stimulated with the same doses of allergen performed at 0 and at 6 months) were increased (more than doubled) in 45% of culture supernatants from SLIT-treated patients and only in 14% of those from UT patients ($P < 0.01$).

In addition, the amounts of IL-4 in culture supernatants of Der p 1- or SK-stimulated PBMC from UT- and SLIT-treated patients performed before and after 6 months of treatment were unchanged (data not shown).

The serum levels of CXCL10 (an IFN-γ-driven chemokine) after 12 and 18, but not after 6, months of treatment were significantly higher ($P < 0.05$) than those found in serum samples of patients at the beginning of therapy (Fig. 3c), thus confirming, at least indirectly, the *in vitro* IFN-γ increase by allergen-specific T cells during therapy. Of note, serum CXCL10 levels remained unchanged in UT analysed at 0, 6, 12 and 18 months. Moreover, the amounts of CCL22 (a Th2-related chemokine) in the sera of treated and UT patients at the same time-points were similar to those of the same patients at the beginning of the study (Fig. 3c).

Effects of sublingual immunotherapy on regulatory mechanisms of immune responses

The effects of SLIT on regulatory mechanisms were initially studied in freshly isolated PBMC by evaluating the proportions of $CD4^+$ T cells highly expressing CD25, referred to as T-regulatory (Treg) cells. The proportions of circulating $CD4^+CD25$ high T cells in SLIT-treated patients (monitored at 6 months) were similar to those found at the beginning of therapy (data not shown).

Then, we considered the effects of SLIT on the production of cytokines with regulatory activity such as TGF-β1 and IL-10. The mean values (± SE) of TGF-β1 in culture supernatants of allergen-driven PBMC from UT- and SLIT-treated patients after 6 months of treatment were not significantly different (5900 ± 623 vs. 5117 ± 456 pg/mL; $P = 0.3$) from those of the same patients analysed at the beginning of the treatment.

By contrast, the mean values of IL-10 were significantly ($P < 0.05$) increased in culture supernatants of allergen-stimulated PBMC from patients after 6 months of SLIT in comparison with those obtained at the beginning of therapy, but only when low doses of allergen were used (Figs 4a and 4b). Of note, the amounts of IL-10 remained unchanged in culture supernatants of SK-stimulated PBMC at 0 or 6 months of therapy, as well as those from UT patients stimulated with both antigens at the same time points (Fig. 4a and data not shown).

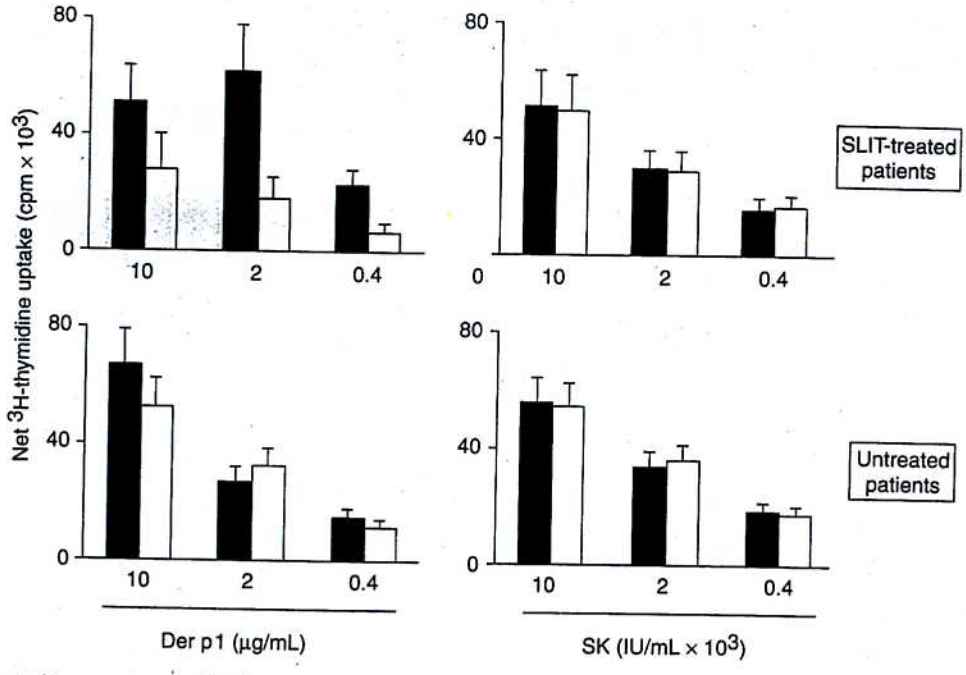

Fig. 2. Effect of sublingual immunotherapy (SLIT) on the proliferative response to allergen. Der p1- and streptokinase (SK)-driven proliferative response of peripheral blood mononuclear cell (PBMC) from nine untreated and 11 SLIT-treated patients, examined before the beginning of immunotherapy (black columns) and at the 6th month of treatment (white columns). 10^6 PBMC were cocultured in triplicate for 5 days with increasing doses of Der p 1 or SK, and the proliferative response was assessed by the measurement of ^{3}H-thymidine uptake. The mean values of cpm (± SE) are reported.

Of note, the levels of IL-10 in comparable samples (culture supernatants from the same patient, stimulated with the same doses of allergen performed at 0 and at 6 months) were increased (more than twice) in 55% of culture supernatants from SLIT-treated patients and only in 11% of those from UT patients ($P < 0.001$).

In order to evaluate whether IL-10 could influence the proliferation and IFN-γ production to allergen, PBMC from two SLIT-treated patients were cultured with Der p 1 in the presence or in the absence of neutralizing anti-IL-10 and anti-IL-10R mAbs. No effect on allergen-driven proliferation and of IFN-γ production with the two mAbs was observed (data not shown).

In addition, we took the opportunity to re-evaluate two patients after 22 and 24 months of therapy. Allergen-stimulated PBMC from these two patients showed increased IL-10 and IFN-γ levels comparable with those of 6-month samples and higher than those detected before therapy (data not shown), thus suggesting that the up-regulation of IFN-γ and IL-10 observed after 6 months of SLIT was maintained during the entire treatment period. Moreover, intracellular cytokines were evaluated in allergen-specific short-term T cell lines generated from the two patients. Whereas the proportions of IL-4-producing T cells were unchanged after therapy (Fig. 4c), thus confirming the previous observation, the proportions of allergen-specific $CD4^+$ T cells producing IFN-γ (21.5% vs. 7.9% and 20.4% vs. 6.9%) or IL-10 (5.1% vs. 1.1% and 3.7% vs. 1.6%) were highly increased in comparison with those found in T cell lines generated before SLIT (Fig. 4c). In addition, whereas the proportions of IFN-γ- and IL-10-producing $CD4^+$ T cells were largely in favour of those secreting IFN-γ, interestingly, more than half of those producing IL-10 co-expressed cytoplasmic IFN-γ (2.9% and 2.0% of total $CD4^+$ T cells, respectively, vs. 0.2% and 0.4% of the same patients before therapy) (Fig. 4c).

When the amounts of IFN-γ and IL-10 produced by allergen-stimulated PBMC of SLIT-treated (6 months) patients were compared, no correlation ($R = -0.02$) was found (as in as those obtained from PBMC of the same patients before therapy or of UT patients, data not shown).

When we verified whether the immunological changes correlate with clinical benefit, indeed, no relation was found (at the single patient level) between the SSS decrease and the *in vitro* increase of IFN-γ ($r = 0.036$, P = NS), or IL-10 ($r = 0.098$, P = NS), as well as the up-regulation of soluble CXCL10 levels ($r = 0.121$, P = NS) (data not shown), after 6 months of SLIT. In addition, a slight correlation between the SSS or SMS decrease and the reduction of specific IgE Ab ($r = 0.511$, $P < 0.1$; $r = 0.587$, $P < 0.1$) was observed. Lastly, in SLIT-treated patients the IL-10 or the IFN-γ increases were not related ($r = 0.026$, P = NS; and $r = -0.081$, P = NS) to the IgE decrease (data not shown).

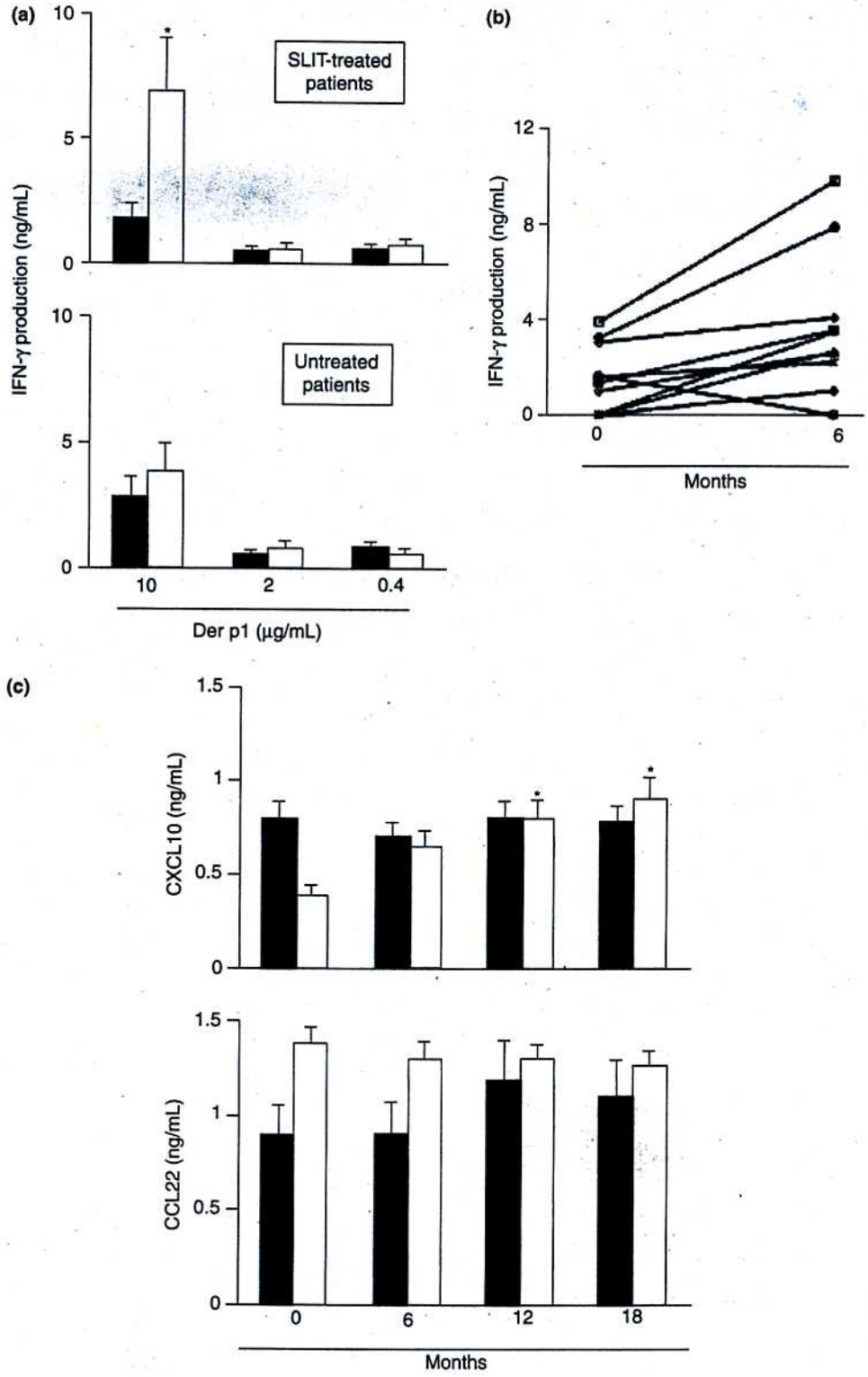

Fig. 3. Effects of sublingual immunotherapy (SLIT) on IFN-γ production by allergen-stimulated peripheral blood mononuclear cell (PBMC), and on CXCL10 and CCL22 serum levels. (a) IFN-γ levels were evaluated in culture supernatants of PBMC of nine untreated (UT) and 11 SLIT-treated patients stimulated for 5 days with increasing doses of Der p 1 as described in Materials and methods. Supernatants were obtained at the beginning (black column) and after the 6th month of treatment (white columns). Mean values (± SE) are reported. (b) IFN-γ levels detected in culture supernatants of PBMC from each SLIT-treated patient, stimulated for 5 days with 10 μg/mL of Der p 1, at 0 and after 6 months of therapy. (c) Serum samples taken from nine UT (black columns) and 11 SLIT-treated (white columns) patients at different times (0, 6, 12 and 18 months) from the beginning of treatment were assayed for the content of CXCL10 and CCL22 by commercial kits as described in Materials and methods. Mean values (± SE) are reported. $^*P < 0.05$.

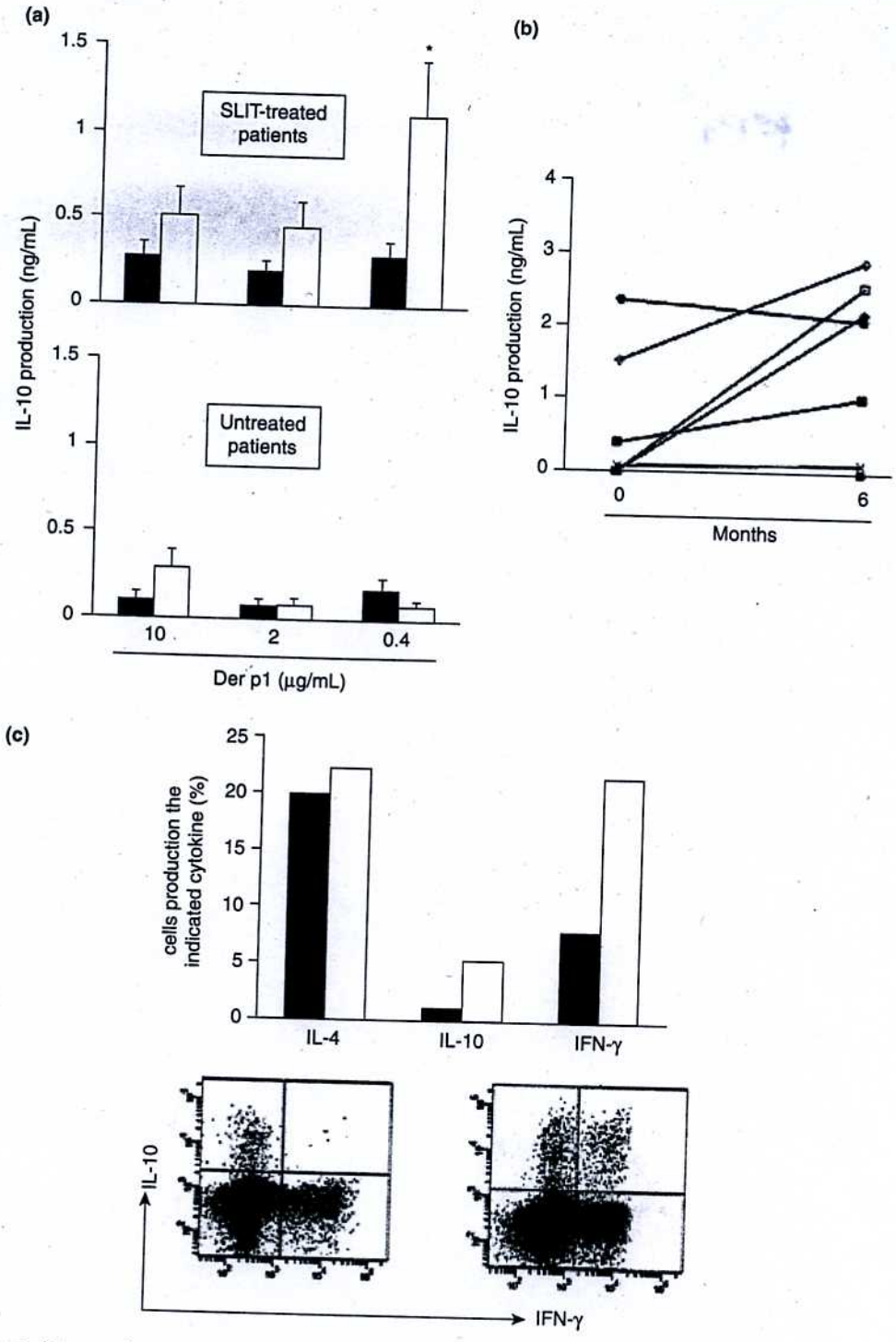

Fig. 4. Effects of sublingual immunotherapy (SLIT) on IL-10 production by allergen-stimulated peripheral blood mononuclear cell (PBMC). (a) IL-10 levels were evaluated in culture supernatants of PBMC of nine untreated (UT) and 11 SLIT-treated patients stimulated for 5 days with increasing doses of Der p 1 as described in Materials and methods. Supernatants were obtained at the beginning (black column) and after the 6th month of treatment (white columns). Mean values (± SE) are reported. (B) IL-10 levels detected in culture supernatants of PBMC from each SLIT-treated patient, stimulated for 5 days with 0.4 μg/mL of Der p 1, at 0 and after 6 months of therapy. (C) Flow cytometric evaluation of intracellular levels of IL-4, IL-10 and IFN-γ on polyclonally stimulated Der p 1-specific CD4+ T cell lines obtained from the same SLIT-treated patient at 0 (black columns) and after 24 months of therapy (white columns). Plots represent contemporaneous evaluation of IL-10 and IFN-γ in Der p 1-specific CD4+ T cell lines at 0 (left panel) and after 24 months of therapy (right panel). One representative experiment is depicted. $^{*}P < 0.05$.

Discussion

This study was addressed to evaluate the effect of SLIT for Dp on immunological response to allergen in randomized Dp-sensitive patients having perennial rhinitis and/or asthma. Even though a high dropout rate (20%) was observed along the study, however, this does not alter the representativeness of the surviving groups, as in the baseline assay before treatment the mean values of Dp-specific IgE, IgG1 and IgG4, as well as of proliferative response to allergen in the surviving groups, are comparable with those of dropout patients.

When examined after 1 year and a half of SLIT, the treated group of patients showed a clearcut reduction of nasal and bronchial symptoms in comparison with those displayed at the beginning of therapy, as well as a clearcut reduction of SMS in comparison with those displayed by UT patients.

In agreement with previously published results on groups of patients treated with different non-injective therapies [20, 28], SLIT-treated patients did not show any increase of allergen-specific IgG1 and IgG4 Abs, which, by contrast, are consistently up-regulated during subcutaneous SIT [4, 28]. More importantly, even if Dp-specific IgE Abs remained unchanged in UT subjects, SLIT induced a progressive reduction (significant after 12 months of therapy) of allergen-specific IgE Abs serum levels, thus giving a biological rationale to the improvement of symptoms [12].

The effects of SLIT, as well as of other non-injective therapies, on Ig isotypes have been extensively reported, but the changes in allergen-specific IgE or IgG do not seem to be constant and reproducible [15, 17, 20, 28]. However, more clearcut data have been obtained in animal models indicating that the repeated inhalation of ovalbumin or ragweed antigens reduced IgE response in rats with poor or no modification of IgG response [29]. In agreement with its activity on humoral responses, SLIT was able to reduce slightly the proliferative response of peripheral blood T lymphocytes to increasing doses of the major allergen of house dust mite (Der p 1) in comparison with that found in UT patients. Whereas the majority of studies on SIT described a decreased T cell responsiveness to allergen, until now contradictory results have been reported in SLIT-treated patients [18–20]. On the whole, the data reported in this study suggest that SLIT affects both systemic humoral and cellular allergen-specific responses.

Several hypotheses have been proposed to explain the immunoregulatory effects of SIT by attributing it to the induction of a T cell tolerance [30], which may reflect generation of allergen-specific suppressor T cells, clonal anergy or extrathymic deletion of allergen-reactive memory T cells [31, 32]. More recently, the Th2 to Th1 deviation [7–9, 30], the up-regulation of $CD4^+CD25^+$ regulatory T cells [10] as well as the increased production of IL-10 by memory T cells have been proposed to be involved in SIT [10, 11]. Another study reported that T cells generated by SIT were $CD4^+CD25^+$ T cells producing both IL-10 and TGF-β [33]. Of note, some authors suggest that IL-10 produced by adaptive Treg cells acts mainly by shifting the allergen-specific Ab production from the dangerous IgE to the protective IgG4 isotype rather than inducing a suppression of Th2 immune response [34].

At present, few data are available on the T cell mechanisms operating in non-injective therapy. It has been reported that in local nasal immunotherapy the cytokine impairment reflects more the decrease of T cell proliferative response than a Th2–Th1 switch [28]. Moreover, in SLIT-treated patients some papers reported no change of types 1 or 2 cytokines [18, 20], and in others a significant increase of allergen (Dp)-driven IFN-γ production was found [19]. The proportions of IL-10-producing T cells, analysed in two of these studies, remained unchanged [20].

Our results indicate that the proportions of circulating $CD4^+CD25^+$ Treg cells remained unchanged after 6 months of SLIT, in agreement with a recent study performed in SIT-treated patients [35]. As there is no clearcut evidence that $CD4^+CD25^+$ Treg cells are confined at the mucosal or lymphnodal levels, it seems unlikely that they can play some role in T cell tolerance during SLIT. On the other hand, our previous results suggest that $CD4^+CD25^+$ Treg cells are less active on Th2 than on Th1 responses, and that peripheral Treg cells, sorted by CD25 expression, are also contaminated by large and variable amounts of activated allergen-specific effector T cells [36].

Furthermore, no change of allergen-driven TGF-β production was detected before and after 6 months of therapy in culture supernatants from UT- and SLIT-treated patients, thus also suggesting that TGF-β, or TGF-β-producing cells, are not involved in T cell tolerance of SLIT-treated patients, as also observed for SIT-treated patients [37].

When T cell derived cytokines were analysed, no change of allergen-induced IL-4 was detected in culture supernatants after 6 months of therapy. More importantly, PBMC from SLIT-treated (6 months of therapy) patients produced significantly higher amounts of IL-10 and of IFN-γ in comparison with those observed at the beginning of therapy, whereas no changes were found in corresponding supernatants from UT patients, the duration of this increase being at least for the entire treatment period. Noteworthy, it has been shown that allergen-specific T cells, expanded during SLIT, produce higher levels of IFN-γ or IL-10 or both, and are, indeed, potentially able to suppress IgE response. Whether these T cell subsets increase in all SLIT-treated patients, as well as the implications of expanded IFN-γ/IL-10 double-positive T cells, are at present unknown. Regulatory IFN-γ/IL-10 double-positive T cells (called Th1-like T1 cells) have been described in mice undergoing chronic stimulation with

antigens [38] or in protection against certain pathogens such as leishmania, borrelia and mycobacteria [39]. Even though the contemporaneous presence of IL-10 and IFN-γ in PBMC in SIT-treated patients has been reported [37, 40, 41], this is the first time that the co-expression of both cytokines by allergen-specific $CD4^+$ T cells has been described in patients undergoing immunotherapy. Nevertheless, it is also relevant to state that allergen-specific T cell response during SLIT is characterised by high variability in cytokine production, suggesting different mechanisms acting at different times during therapy and/or interacting each other in different ways.

These findings suggest that, even though the analysis is limited to circulating and not to tissue or lymphnodal memory T cells (where the changes are likely more evident), both immunodeviation and immunoregulation mechanisms are elicited by SLIT. Indeed, IL-10 and IFN-γ are able to impair *in vitro* allergen-specific Th2 responses as well as to down-regulate IgE-producing cells (IFN-γ) directly, or to switch towards IgG4 subclass (IL-10), thus giving a biological rationale for the well-documented improvement of symptoms with SLIT [14]. Some data indicate that immunodeviation may play a critical role in SLIT-treated patients more than immunoregulation. Firstly, we reported the increased levels of CXCL10 in the serum of SLIT-treated patients after 12–18 months of therapy. It has been shown that CXCL10 is an IFN-γ-induced chemokine that stimulates IFN-γ production in an autocrine potentiating loop [42], and that *in vivo*, enhanced levels of IP-10/CXCL10 have been detected in several inflammatory Th1-oriented diseases [43]. Furthermore, increased IL-10 production seems to have a questionable role *in vivo* as there were undetectable levels of allergen-specific IgG4 Ab in SLIT-treated patients, a subclass whose switch factor is IL-10 [34], or *in vitro* where no effects on proliferative response and IFN-γ production under IL-10 neutralizing conditions were observed. Finally, the absence of an inverse correlation between the two cytokines in supernatants of allergen-stimulated PBMC from SLIT-treated patients excludes that IL-10, at least *in vitro*, inhibits IFN-γ production of allergen-specific T cells. This agrees with the finding that IFN-γ production by grass pollen-stimulated PBMC from SIT-treated patients is inhibited *in vitro* by high doses of IL-10, generally exceeding those generally found in culture supernatants [37].

An explanation of the absence of any inverse correlation between the production of the two cytokines at all allergen doses used at present remains only speculative. One possibility is that it is due to the expansion of allergen-specific T cells producing both IL-10 and IFN-γ. The predominant expansion of $IFN\text{-}\gamma^+IL\text{-}10^-$ T cells when the highest dose of allergen was used is in agreement with previous studies reporting that high doses of allergens selectively induce a $CD4^+$-mediated type 1 cytokine response in patients undergoing SIT [44, 45] as well as to recall antigens and their peptides [32, 46, 47]. Of note, it has also been clearly shown that the preferential IL-10 or IFN-γ response to allergens strictly depends on the type of stimulating epitopes regardless of the allergic status of the donor [48], and it cannot be excluded that some epitopes favour in nature the stimulation of a T cell subset producing both cytokines.

A further relevant query concerns why SIT and SLIT share some immunological mechanisms. Indeed, SLIT is not simply a local immunotherapy leading to local modifications, but is also a systemic therapy as shown in a pharmacokinetics study with radiolabelled Par j 1 allergen locally administered in healthy volunteers, which appeared in the plasma 15–20 min and peaks after 2–3 h [49].

A central question remains the possible correlation between the clinical improvement and some *in vitro* or *ex vivo* changes. It is unlikely that the clinical efficacy of SLIT, which is due to a number of known and unknown local and systemic mechanisms, may be strictly related to one single parameter. Indeed, no relation was found (at the single patient level) between the clinical benefit and changes of IFN-γ, IL-10 production, serum CXCL10 or IgE levels, thus resembling a vast bibliography on SIT and SLIT [1, 2, 9, 12]. However, it is noteworthy that all SLIT-treated patients with clinical benefit showed a significant increase of IFN-γ or of IL-10 or of both cytokines, whereas only two out of nine UT patients showed this trend.

In conclusion, based on the data available currently, we do not know whether a predominant Th1-skewing or an immunoregulatory mechanism plays the major role in the modification of allergen-specific Th2 response leading to reduction of IgE and clinical. The presence of T cells producing both IFN-γ and IL-10 in SLIT-treated patients may be relevant but requires more in depth studies on their origin and functions. It cannot be excluded that both mechanisms are contemporaneously operating in SLIT, and under some circumstances they may synergise [50], the predominance of one or the other depending on several factors: the rate of sensitivity and of the atopic status of the subject, the type of allergen and its major epitopes, the rate of absorption, the local dose reached at tissue level, the contemporaneous activation of DC by other pathogens, etc. These double potential mechanisms could provide a convincing biological explanation for the clinical efficacy of SLIT and may reconcile contradictory results of the literature on this issue.

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References

1 Creticos PS. Immunotherapy with allergens. *JAMA* 1992; 268:2834–9.

2 Malling H-J, Weeke BE. AACI position paper: immunotherapy. *Allergy* 1993; 48:9–35.

3 Bousquet J, Lockey R, Malling HJ World Health Organization position paper: allergen immunotherapy – therapeutical vaccines for allergic diseases. *J Allergy Clin Immunol* 1998; 102 (4 Part 1):558–62.

4 Aalberse RL, Van der Gaag R, Van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4 restricted response. *J Immunol* 1983; 130: 722–6.

5 Chapman MD, Platts-mills TAE, Gabriel M *et al.* Antibody response following prolonged hyposensitization with *Dermatophagoides pteronyssinus* extract. *Int Arch Allergy Appl Immunol* 1980; 61:431–40.

6 Gleich GJ, Jacob GL, Yunginger JW, Henderson LL. Measurement of the absolute levels of IgE antibodies in patients with ragweed hay fever. Effect of immunotherapy on seasonal changes and relationship to IgG antibodies. *J Allergy Clin Immunol* 1977; 60:188–98.

7 Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in $CD4^+$ T cells from allergic individuals. *J Exp Med* 1993; 178:2123–30.

8 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.

9 Norman PS. Immunotherapy: 1999–2004. *J Allergy Clin Immunol* 2004; 113:1013–23.

10 Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102:98–106.

11 Akdis M, Verhagen J, Taylor A *et al.* Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004; 199:1567–77.

12 Canonica GW, Passalacqua G. Noninjection routes for immunotherapy. *J Allergy Clin Immunol* 2003; 111:437–48.

13 Bousquet J, Van Cauwenberge P. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108 (Suppl. 5):S240–5.

14 Wilson DR, Lima MT, Durham SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. *Allergy* 2005; 60:4–12.

15 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.

16 Passalacqua G, Albano M, Riccio AM *et al.* Clinical and immunological effects of a rush sublingual immunotherapy to parietaria species: a double blind placebo controlled triall. *J Allergy Clin Immunol* 1999; 104:964–8.

17 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.

18 Fanta C, Bohle B, Hirt W *et al.* Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. *Int Arch Allergy Immunol* 1999; 120:218–24.

19 Arikan C, Bahceciler NN, Deniz G *et al.* Bacillus Calmette-Guerin-induced interleukin-12 did not additionally improve clinical and immunological parameters in asthmatic children treated with sublingual immunotherapy. *Clin Exp Allergy* 2004; 34:398–405.

20 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.

21 Macatonia SE, Hosken NA. Dendritic cells produce IL-12 and direct the development of Th1 cells from naïve $CD4^+$ T cells. *J Immunol* 1994; 154:5071–9.

22 Passalacqua G, Albano M, Fregonese L *et al.* Randomised controlled trial of local allergoid immunotherapy on allergic inflammation in mite-induced rhinoconjunctivitis. *Lancet* 1998; 351:629–32.

23 Mistrello G, Brenna O, Roncarolo D, Zanoni D, Gentili M, Falagiani P. Monomeric chemically modified allergens: immunologica and physicochemical characterization. *Allergy* 1996; 51:8–15.

24 La Rosa M, Ranno C, Andrè C, Carat F, Tosca MA, Canonica GW. Double-blind placebo-controlled evaluation of sublingual-swallow immunotherapy with standardizaed *Parietaria Judaica* extract in children with allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 1999; 104:425–32.

25 Ariano R, Panzani RC, Chiapella M, Augeri G, Falagiani P. Local immunotherapy of seasonal allergic rhinitis in children due to *Parietaria officinalis* pollen: a preliminary report. *Pediatr Asthma Allergy Immunol* 1993; 7:227–37.

26 Annunziato F, Cosmi L, Liotta F *et al.* Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J Exp Med* 2002; 196:379–87.

27 Parronchi P, Macchia D, Piccinni MP *et al.* Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine secretion. *Proc Natl Acad Sci USA* 1991; 88:4538–42.

28 Giannarini L, Maggi E. Decrease of allergen-specific T cell response induced by local nasal immunotherapy. *Clin Exp Allergy* 1998; 28:404–12.

29 Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR. Inhibition of T cell and antibody response to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* 1993; 178: 1783–8.

30 Van Neerven RJJ, Ebner C, Yssel H, Kapsenberg ML, Lamb JR. T-cell responses to allergens: epitope-specificity and clinical relevance. *Immunol Today* 1996; 17:526–32.

31 Fasler S, Aversa G, Terr A, Thestrup-Pedersen K, de Vries JE, Yssel H. Peptide-induced anergy in allergen-specific human Th2 cells results in lack of cytokine production and B cell help for IgE synthesis. Reversal by IL-2, not IL-4 or IL-13. *J Immunol* 1995; 155:4199–206.

32 Secrist H, Dekruyff RH, Umetsu DT. Interleukin 4 production by CD4+ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J Exp Med* 1995; 181:1081–9.

33 Jutel M, Akdis M, Budak F *et al.* IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003; 33:1205–14.

34 Nouri-Aria KT, Wackholz PA, Francis JM *et al.* Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004; 172: 3252–9.

35 Smith TR, Alexander C, Kay AB, Larche M, Robinson DS. Cat allergen peptide immunotherapy reduces CD4(+) T cell responses to cat allergen but does not alter suppression by CD4(+) CD25(+) T cells: a double-blind placebo-controlled study. *Allergy* 2004; 59:1097–101.

36 Cosmi L, Liotta F, Angeli R *et al.* Th2 cells are less susceptible than Th1 cells to the suppressive activity of $CD25^+$ regulatory thymocytes because of their responsiveness to different cytokines. *Blood* 2004; 103:3117–21.

37 Francis JN, Till SJ, Durham SR. Induction of IL-$10^+CD4^+CD25^+$ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003; 111:1255–61.

38 Stock P, Akbari O, Berry G, Freeman GJ, Dekruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol* 2004; 5:1149–56.

39 Trinchieri G. Regulatory role of T cells producing both interferon gamma and interleukin 10 in persistent infection. *J Exp Med* 2001; 194:F53–7.

40 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.

41 Verhoef A, Alexander C, Kay AB, Larche M. T cell epitope immunotherapy induces a $CD4^+$ T cell population with regulatory activity. *PLoS Med* 2005; 2:253–61.

42 Gangur V, Simons ER, Hyglass K. Human IP-10 selectively promotes dominance of polyclonally activated and environmental antigen-driven IFN-g over IL-4 responses. *FASEB J* 1998; 12:705–13.

43 Luster AD. IP-10. In: Oppenheim JJ, Feldman M., eds. *Cytokine reference, a compendium of cytokines and other mediators of host defense.* London: Academic Press, 2000; 1103–9.

44 Gardner LM, Spyroglou L, O'Hehir RE, Rolland JM. Increased allergen concentration enhances IFN-γ production by allergic donor T cells expressing a peripheral tissue trafficking phenotype. *Allergy* 2004; 59:1308–17.

45 Gardner LM, O'Hehir RE, Rolland JM. High dose allergen stimulation of T cells from house dust mite-allergic subjects induces expansion of IFN-γ+ T Cells, apoptosis of CD4+IL-4+ T cells and T cell anergy. *Int Arch Allergy Immunol* 2004; 133:1–13.

46 Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J Exp Med* 1995; 182:1591–6.

47 Rogers PR, Croft M. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* 1999; 163:1205–13.

48 Reefer AJ, Carneiro RM, Custis NJ *et al.* A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. *J Immunol* 2004; 172:2763–72.

49 Bagnasco M, Mariani G, Passalacqua G *et al.* Absortion and distribution kinetics of the major Parietaria allergen administered by noninjectable routes to healthy human beings. *J Allergy Clin Immunol* 1997; 100:121–9.

50 Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 2005; 5:271–83.