# Effect of sublingual immunotherapy with grass monomeric allergoid on allergen-specific T-cell proliferation and interleukin 10 production

Samuele E. Burastero, MD*; Gianni Mistrello, PhD†; Paolo Falagiani, DVM†; Clara Paolucci, PhD*; Daniela Breda, BSc*; Daniela Roncarolo, PhD†; Stefania Zanotta, PhD†; Giorgio Monasterolo, MD‡; and Renato E. Rossi, MD§

**Background:** Sublingual immunotherapy (SLIT) is safe and efficacious in the treatment of patients with allergic rhinitis. Although favorable clinical effects have been observed with controlled trials as early as a few months since the beginning of treatment, few biological changes induced by SLIT have been demonstrated.

**Objective:** To investigate in grass-allergic patients the effect of a 2-month SLIT regimen, administered with a simplified protocol without up-dosing, on proliferation and production of cytokines characteristic of the regulatory T-cell phenotype (interleukin 10 [IL-10] and transforming growth factor $\beta$ [TGF-$\beta$]) by allergen-specific T cells.

**Methods:** Patients were recruited to the study in January 2006. SLIT was performed by self-administration and was continued for 60 days from February to April 2006. Eleven grass pollen–allergic patients with seasonal rhinitis were treated daily before the pollen season for 2 months with a modified allergen (monomeric allergoid) derived from a 3-grass pollen extract. Allergen-specific proliferation and production of IL-10 and TGF-$\beta$ were measured on peripheral blood mononuclear cells at baseline and treatment end. Tetanus toxoid served as the control antigen.

**Results:** After SLIT, allergen-specific ($P = .002$) but not tetanus toxoid–specific proliferation decreased, whereas IL-10 transcription increased ($P < .001$). TGB-$\beta$ transcription was also increased after treatment, although not statistically significantly ($P = .06$). Changes in proliferation to allergen and in IL-10 transcription were correlated ($r = -0.82$, $P = .003$).

**Conclusions:** A short-term course of SLIT with modified allergen in grass-allergic patients is associated with the reduction of allergen-specific proliferation and with the up-regulation of the IL-10 regulatory cytokine.

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## INTRODUCTION

Allergen-specific immunotherapy is highly effective in the treatment of patients with severe allergic rhinitis, conjunctivitis, and/or asthma. Immunotherapy has been recommended by the World Health Organization as an integrated part of the allergy management strategy.[1] Several controlled studies have shown that subcutaneous immunotherapy (SCIT) achieves hyposensitization and reduces both early and late responses that occur during the natural exposure to allergen. The efficacy of SCIT has been referred to as the increase in allergen-specific "blocking" IgG4 antibodies,[2] which would prevent IgE-dependent release of mediators from basophils and mast cells. This parameter likely reflects the modification of the phenotype of peripheral and mucosal CD4 $T_H2$ responses toward a prevalent $T_H1$ cytokine profile.[3–5] More recently, the increased proportion of interleukin 10 (IL-10)–producing T cells in response to allergen stimulation after SCIT has emerged as a consistent biological end point, which can explain previously reported humoral and cellular alterations observed in treated patients.[6,7] Thus, increased $T_H1$ responses and IL-10 up-regulation in regulatory T cells likely cooperate in the induction of tolerance to allergen seen during SCIT.

Sublingual immunotherapy (SLIT) was introduced during the 1980s as an alternative route of immunotherapy, characterized by a higher safety profile than SCIT. SLIT efficacy in reducing such clinical parameters as symptoms and medication use has been clearly demonstrated with controlled studies in adults and children.[8–10] The Allergic Rhinitis and Its Impact on Asthma document concluded that SLIT is a valid alternative to the injective immunotherapy and that its use in clinical practice in adults and children is justified.[11] In a Cochrane meta-analysis, the efficacy of SLIT has been definitively proven at the Ia evidence level.[12]

The biological mechanisms of SLIT are still a matter of controversy. Indeed, it appears intuitively not obvious to explain how an extremely limited absorption of allergen as

**Affiliations:** * San Raffaele Scientific Institute, Milan, Italy; † Lofarma Allergeni SpA, Milano, Italy; ‡ Laboratorio Analisi Chimico-Cliniche e Microbiologia, Fossano e Savigliano; § Allergy Unit National Health Service, Rete di Allergologia Regione Piemonte, Cuneo, Italy.

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that obtained by the sublingual route[13,14] may significantly affect the systemic response to allergen. However, some evidence in animal models of asthma indicates that the oral route of immunotherapy is indeed tolerogenic and can redirect the $T_H1/T_H2$ differentiation via induction of IL-12 production by mucosal dendritic cells.[15] Furthermore, SLIT has been shown to impair inflammatory phenomena, such as cellular infiltration and expression of adhesion molecule expression on epithelia in target organs,[16,17] and simultaneously to increase systemic allergen-driven interferon-$\gamma$[18,19] and IL-10[20,21] production. However, other authors did not find any significant humoral or cellular changes.[22]

Here, we evaluated allergen-specific T-cell proliferation and the induction of IL-10 and transforming growth factor $\beta$ (TGF-$\beta$) production, as prototypical anti-inflammatory[23–25] and regulatory T-cell cytokines,[26] in grass-allergic patients undergoing SLIT. According to controlled trials, even short preseasonal immunotherapy courses induce clinical improvements in pollen-allergic patients.[27–29] Therefore, a monomeric allergoid, derived from a 3-grass pollen extract, was administered for a 2-month period, preceding the beginning of the symptomatic season. Our results indicate that a short course of SLIT can induce a decrease in allergen-specific T-cell proliferation associated with an increase in cytokines capable of impairing $T_H2$ responses. These findings provide a biological basis for explaining the effect of this form of treatment.

## MATERIALS AND METHODS

### *Patients*

This was an open, observational pilot study to evaluate peripheral blood mononuclear cell (PBMC) proliferation to grass allergen and grass-induced IL-10 and TGF-$\beta$ production in PBMCs from grass-allergic patients undergoing 2 months of SLIT. Patients were recruited to the study in January 2006, and the whole study was performed when the grass pollen count was below 5 grains per cubic meter in the area where patients were living and patients were asymptomatic. Blood was drawn in 2 outpatients visits, before SLIT started (February 2006) and 2 months later (April 2006).

### *Inclusion Criteria*

All patients were sensitized to grass pollen and selected by in vivo and in vitro tests. The skin prick tests were performed with commercial extracts from *Phleum pretense* extract, containing 60 $\mu$g/mL of Phl p 5; *Parietaria judaica* pollen, containing 20 $\mu$g/mL of Par j 1; birch, containing 45 $\mu$g/mL of Bet v 1; olive tree, containing 60 $\mu$g/mL of Ole e 1; mugwort, containing 135 $\mu$g/mL of Art v 1; cat dander, containing 60 $\mu$g/mL of Fel d 1; and mites, containing 40 $\mu$g/mL of Der p 1 and Der f 1 and 20 $\mu$g/mL of Der p 2 and Der f 2 (all produced by Alk-Abellò, Lainate, Milan, Italy). No recombinant allergens were used for in vivo diagnosis. IgE-specific antibodies for recombinant allergens rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl 6, rPhl p 7, rPhl 11, and rPhl 12 were evaluated using the immunoenzymatic CAP system (Phadia Diagnostics, Uppsala, Sweden) following the manufacturer's instructions.

### *Exclusion Criteria*

Exclusion criteria were previous treatment with SCIT or SLIT and permanent treatment with topical or systemic corticosteroids within the last 4 weeks before the start of the study. Eleven individuals (4 women and 7 men; age range, 19–56 years) sensitized to grass pollen were included in the study. They were all informed about the nature of the study and gave informed consent before inclusion. The study was conducted according to Good Clinical Practice rules. All patients had been experiencing rhinoconjunctivitis with (3/11) or without (8/11) mild asthma for at least 2 years. Six patients had a single sensitization to grass, whereas 5 had further sensitizations to cat, pellitory, mites, or olive. Age, sex, sensitization, and serologic characteristics of patients are indicated in Table 1.

Table 1. Grass-Specific IgE Sensitization Profile

| Patient No. | Sex | Age, y | Sensitization | IgE concentration, KU/L | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total IgE | Phl p 1 | Phl p 2 | Phl p 4 | Phl p 5b | Phl p 6 | Phl p 7 | Phl p 11 | Phl p 12 |
| 1 | F | 32 | G, P, C | 261 | 86.4 | 4.63 | 16.2 | 19.2 | 0.96 | <0.35 | <0.35 | <0.35 |
| 2 | F | 30 | G, C | 232 | 7.1 | 1.36 | 17.44 | 5.75 | 6.62 | <0.35 | 1.16 | <0.35 |
| 3 | F | 30 | G, M, B | 271 | 8.31 | 4.4 | 11.9 | 8.20 | 2.84 | <0.35 | 6.00 | <0.35 |
| 4 | F | 33 | G | 73 | 10.8 | <0.35 | <0.35 | 0.95 | <0.35 | <0.35 | 1.57 | <0.35 |
| 5 | M | 56 | G | 161 | 6.26 | 3.14 | 9.00 | 6.8 | 4.00 | <0.35 | 1.82 | <0.35 |
| 6 | M | 23 | G | 158 | 9.91 | 0.56 | 10.86 | 8.79 | <0.35 | <0.35 | <0.35 | 0.76 |
| 7 | M | 37 | G | 453 | 12.4 | 1.13 | 6.61 | 6.34 | 0.76 | <0.35 | 5.97 | <0.35 |
| 8 | F | 46 | G | 12 | 0.68 | <0.35 | 0.35 | 0.35 | <0.35 | <0.35 | <0.35 | <0.35 |
| 9 | M | 19 | G, M, O | 874 | 5.83 | <0.35 | <0.35 | 1.84 | <0.35 | <0.35 | <0.35 | <0.35 |
| 10 | F | 33 | G | 36 | 2.92 | 0.58 | 2.59 | 4.72 | <0.35 | <0.35 | 2.37 | <0.35 |
| 11 | F | 27 | G, O | 112 | 13.5 | <0.35 | <0.35 | 7.30 | <0.35 | <0.35 | 5.56 | <0.35 |

Abbreviations: G, grass; P, pellitory; C, cat; M, mites; O, olive.

*Immunotherapy Treatment*

All selected patients were prescribed a commercial SLIT treatment (LAIS; Lofarma Allergeni S.p.A., Milan, Italy) with a monomeric allergoid of grass pollen mix (*Holcus lanatus,* 33%; *P pratense,* 33%; *Poa pratensis,* 33%) obtained by carbamylation with potassium cyanate at alkaline pH, a reaction that leads to a substantial substitution of e-amino groups of lysine residues and consequently a strong decrease in the capacity to react with IgE antibodies.[30] The product, standardized for allergenic potency by the radioallergosorbent test and reverse enzyme allergosorbent test inhibition compared with an in-house reference preparation and titrated in allergenic units (AU), was formulated in orosoluble tablets and administered sublingually.

SLIT was performed by self-administration and was continued for 60 days from February to April 2006. No build-up phase was performed, and patients received from the first day a dose of 2,000 AU (2 tablets, equivalent to 2.2 $\mu$g of Phl p 1) once a day, which was maintained throughout the study. The cumulative dose of allergen extract received by each patient was therefore approximately 60,000 AU, equivalent to 132 $\mu$g of Phl p 1 major allergen. The tablets had to be taken in the morning, were dissolved in the mouth for 1 to 2 minutes, and then swallowed. Since both phlebotomies were performed before the pollen season, and all patients were monosensitized to grass, rescue medications were not needed.

*Measurement of Grass Allergen–Specific T-Cell Proliferation*

*Cell culture reagents.* Complete medium used for cell cultures was prepared as follows: RPMI 1640 (PBI, Milan, Italy) was supplemented with gentamicin, 5 $\mu$g/mL; glutamine, 2 mmol/L (PBI); and 5% normal human serum (Danish Red Cross Laboratories, Utrecht, Holland). As a native allergen extract, a lyophilized commercial preparation of a grass mixture (*H lanatus,* 33%; *P pratense,* 33%; *P pratensis,* 33%) from Lofarma Allergeni S.p.A. was used, whose total protein concentrations were calculated using a commercial kit (Bio-rad Laboratories, Segrate, Italy). This antigen was used at a 20-$\mu$g/mL total protein concentration. Furthermore, a recombinant Phl p 1 (rPhl p 1) allergen was used, which was prepared by Lofarma Allergeni S.p.A.

*Expression and purification of rPhl p 1.* Total RNA was isolated from *P pratense* pollen (Allergon AB, Ángelholm, Sweden) using the TRIzol reagent (Invitrogen, Milan, Italy) and complementary DNA (cDNA) synthesized with M-MLV reverse transcriptase (Invitrogen). The coding sequence for Phl p 1 protein (GenBank X78813) was amplified using polymerase chain reaction (PCR) with the following primers: PH1 to 5′ECO RI (CCG*GAATTC-CATATG*ATCCCCAAGGTTCCCCCGG) PH1 to 3′BAM HI (CGC*GGATCC*TCACTTGGACGAGTAGCTGG).

A sequence coding for a 6-histidine tag was inserted at the 5′ end by PCR using the primer PH1 to 6H 5′(CCG*G-AATTCCATATG*CATCACCATCACCATCACATCCCCA-AGGTTCCC) instead of primer PH1 to 5′ECORI. *Eco*RI, *Bam*HI, and *Nde*I restriction sites are italicized. The cDNA coding for Phl p 1 was then checked by sequence, inserted into the pET-3c vector, and expressed in *Escherichia coli* BL21 (DE3) pLys (Stratagene, La Jolla, California). The solubilized recombinant proteins were purified by affinity chromatography on Ni-NTA matrices (Qiagen, Milan, Italy) and refolded in column with urea gradient. Endotoxin level, measured by the LAL test (Cape Cod, Falmouth, Massachusetts), was less than 0.06 EU/mL at the rPhl p 1 concentration used in the assay. The procedure for producing this molecule is part of the patent application: "Variants of allergen proteins from *Phleum pratense*" published in the European Patent Office, Bulletin N.15/05, April 13, 2005 (proprietor's name: Consiglio Nazionale delle Ricerche. Date of filing of the application: 11/09/2001. Filing N. 1317484. European Patent Office publication number: 01976216.0).

*Cells.* Cell preparations that contain T lymphocytes were obtained as PBMCs isolated by standard gradient separation (Ficoll Hypaque, Pharmacia, Uppsala, Sweden) from heparinated blood. Cells were cultured in complete medium with allergen or tetanus toxoid for 5 days in 96-well flat-bottom plates in standard 5-day proliferation assays where 200,000 cells per well were plated in 96-well flat bottom plates (Costar; PBI).

Tritiated thymidine (3H-TdR; Dupont, Cologno M., Italy) was added (1 $\mu$Ci per well) in the last 6 to 18 hours of culture. Cells were harvested with a FilterMate Universal Harvester (Perkin Elmer Life Sciences, Boston, Massachusetts) and incorporated thymidine, expressed as counts per minute after scintillation counting was used as a measure of allergen-specific cell proliferation (TopCount NXT Microplate Scintillation and Luminescence Counter; Perkin Elmer Life Sciences). Controls were included (PBMCs without antigen). The stimulation index (SI) for each antigen was calculated as the ratio between the mean 3H-TdR incorporation in test vs control wells. All assays were performed in triplicate, and mean values were used for calculation.

*Measurement of IL-10 and TGF-$\beta$ Transcription*

PBMCs cultured as described herein were plated for cytokine assays in 48-well plates (1 mL per well) for 3 days. This time of culture was chosen as the most suitable to allow detection of these cytokines, on the basis of preliminary experiments where PBMCs were exposed to allergen for different periods and cytokine levels measured as specified herein.

The cell pellets were collected, lysed, and frozen at −80°C in TRIzol (Invitrogen Life Technologies) until the day of RNA extraction. Before reverse transcription, RNA was treated using RNAse-free DNase (Ambion Europe, Huntingdon, England) according to the manufacturer's instructions, 1.6 $\mu$g of oligo$(dT)_{15}$ was added, and the sample was denatured at 70°C for 10 minutes. RNA was reverse transcribed using 10 U of SUPER RT (HT Biotechnology Ltd, Cambridge, England) per microgram of total RNA, 1 SUPER RT buffer, 1-mmol/L each deoxynucleotide triphosphate, and 40

U of RNaseOUT (Invitrogen Life Technologies) at 42°C for 40 minutes. Relative quantitation of specific cDNA species to $\beta$-actin message was conducted on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using TaqMan chemistry in a multiplex PCR and the comparative threshold (CT) method[31] for IL-10, TGF-$\beta$, and $\beta$-actin. Sequences for primers and probes were designed by the Assay-On-Demand Taqman service of Applied Biosystems. Relative quantification of signal was determined by subtracting the CT for the target gene from the CT for $\beta$-actin ($\Delta$CT). Relative expression levels of cytokines for each culture condition were expressed as $2^{\Delta CT}$, and cytokine SIs were calculated by elevating the log base "2" to the $\Delta$CT values of the stimulated minus the $\Delta$CT of the unstimulated culture, following the indications of the manufacturer.

*Intracellular Staining*

PBMCs ($1 \times 10^6$) were cultured for 6 days with rPhl p 1. Cells were harvested and restimulated for 4 hours with phorbol-12-myristate-13-acetate (5 ng/mL) (Sigma) in the presence of a protein transport inhibitor (brefeldin A, 10 $\mu$g/mL) (Sigma), as previously described.[32] Briefly, cells were first reacted with either peridinin chlorophyll-$\alpha$ protein–labeled anti-CD3 monoclonal antibody (Becton Dickinson) or peridinin chlorophyll-$\alpha$ protein–labeled IgG1 isotype control (Becton Dickinson), fixed with paraformaldehyde 1% (Sigma) in PBS and subsequently reacted with either phycoerythrin-conjugated IgG1 isotype control (Pharmingen, BD Biosciences, Erembodegen, Belgium) or phycoerythrin-labeled anti-human IL-10 monoclonal antibody (Pharmingen) in permeabilizing buffer (PBS buffer containing 0.05% saponine; Sigma). Readings were performed with a FACScan cytofluorimeter, equipped with Cellquest software (Becton Dickinson). Cells were preliminary gated for physical parameters to exclude cellular debris (lymphogate). The percentage of CD3 cells positive for IL-10 was determined by a dot-plot representation with quadrant statistics, compared with control cell preparations reacted with the fluorochrome- and isotype-matched control monoclonal antibodies.

*Statistical Analysis*

The Wilcoxon matched-pair signed-rank test and the Spearman rank order correlation test were used to evaluate whether there were differences and correlation among the compared groups of values, respectively. $P < .05$ was considered statistically significant.

## RESULTS

*Decrease in Allergen-Specific Proliferation After 2 Months of SLIT*

Proliferation of PBMCs stimulated in vitro for 5 days with tetanus toxoid, native grass extract, and rPhl p 1 was measured at baseline and 2 months from the beginning of SLIT. An SI (ie, the ratio between proliferation in the presence of antigen vs proliferation of cells in medium only) above 2 was considered as an index of specific proliferation to antigen. At baseline, a signal compatible with specific proliferation to tetanus toxoid was measured in all patients (median value of SI, 17.6; range, 3.0–48.3). This was expected, since they had been all boosted with this vaccination within the previous 5 years. Also proliferation to rPhl p 1 and to the grass extract, which all patients were sensitized to (Table 1), yielded an SI of more than 2 in all patients (29.8 [range, 16.6–50.7] and 31.2 [range, 16.6–70.8], respectively).

Proliferation to the rPhl p 1 and to the raw grass extract was 45.4% and 62.5% diminished after immunotherapy ($P = .002$ and .04, respectively) (Fig 1). The specificity of proliferation was maintained in all patients with both rPhl p 1 and the grass extract (median values of SI, 22.1 [range, 2.1–68.8] and 18.7 [range, 2.5–58.8], respectively).

*Increase in Transcription of IL-10 and TGF-$\beta$ at rPhl p 1–Stimulated Lymphocytes After 2 Months of SLIT*

Transcription of IL-10 and TGF-$\beta$ were measured in 3-day rPhl p 1–stimulated PBMCs before and after immunotherapy by means of quantitative PCR. The median values of the gene-specific signal were 2.6 and 1.4 times increased after immunotherapy compared with baseline for IL-10 ($P < .001$) and TGF-$\beta$ ($P = .06$), respectively (Fig 2).

*Correlation Between Allergen-Dependent Changes of Cytokine Transcription and Proliferation*

Pretreatment vs posttreatment changes in allergen-specific proliferation, expressed as counts per minute, were correlated with changes in IL-10 expression, expressed as cytokine SIs, both in the case of grass-stimulated ($r = -0.82$, $P = .003$) and Phl p 1–stimulated ($r = -0.80$, $P = .004$) cell cultures (Fig 3). No correlations were found between either absolute values or SLIT-associated changes in IL-10 and TGF-$\beta$ expression. Namely, correlation indexes of pretreatment and posttreatment changes in

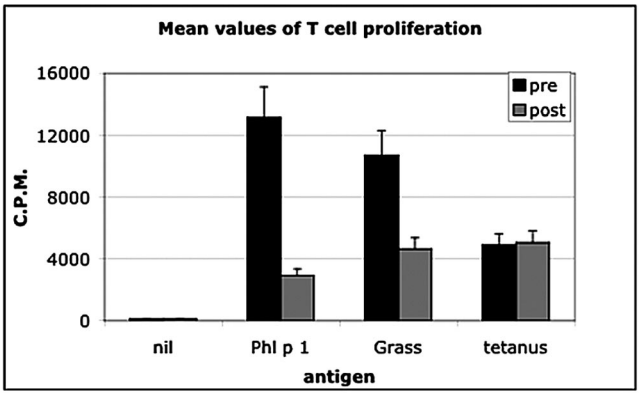


Figure 1. Allergen-dependent T-lymphocyte proliferation in peripheral blood mononuclear cells (PBMCs). Proliferation was measured with a standard 3H-thymidine incorporation assay and expressed as counts per minutes (on the y-axis). Columns represent mean values of T-cell proliferation measured before the beginning of sublingual immunotherapy (SLIT) (pre-SLIT) and after 2 months of SLIT (post-SLIT). Antigens used for T-cell stimulation are indicated on the x-axis. Nil indicates medium only; Phl p 1, rPhl p 1 major allergen; grass, native grass extract; tetanus, tetanus toxoid.

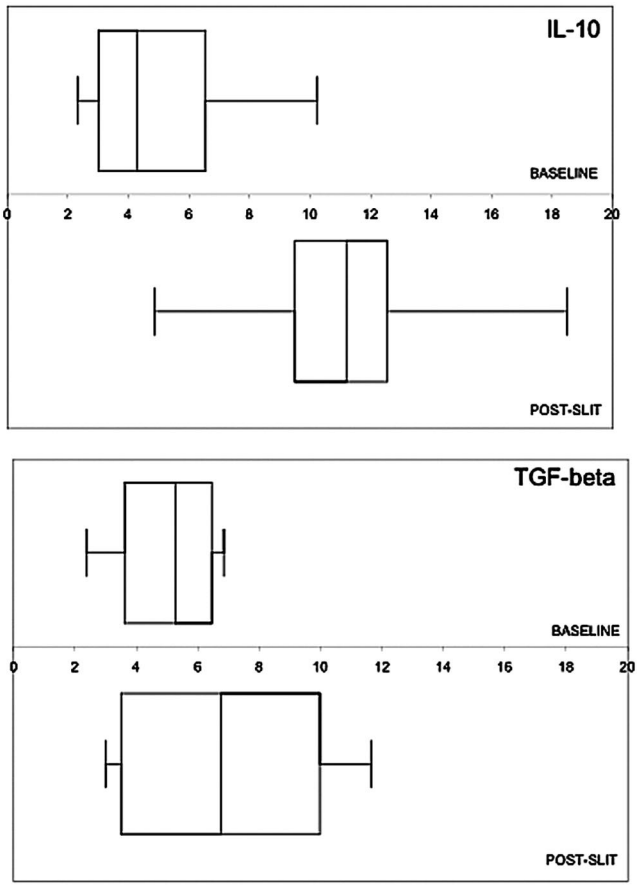


Figure 2. Whisker plot representation of the median, lower and upper quartile, minimum, and maximum values of interleukin 10 (IL-10) (upper panel) and transforming growth factor $\beta$ (TGF-$\beta$) (lower panel) expression level compared with the $\beta$-actin control gene. Cytokine stimulation indexes were obtained by subtracting the relative (to $\beta$-actin) expression levels for the target gene in stimulated vs unstimulated cultures. Whisker plots refer to values measured before sublingual immunotherapy (SLIT) (baseline) and after 2 months of SLIT (post-SLIT).

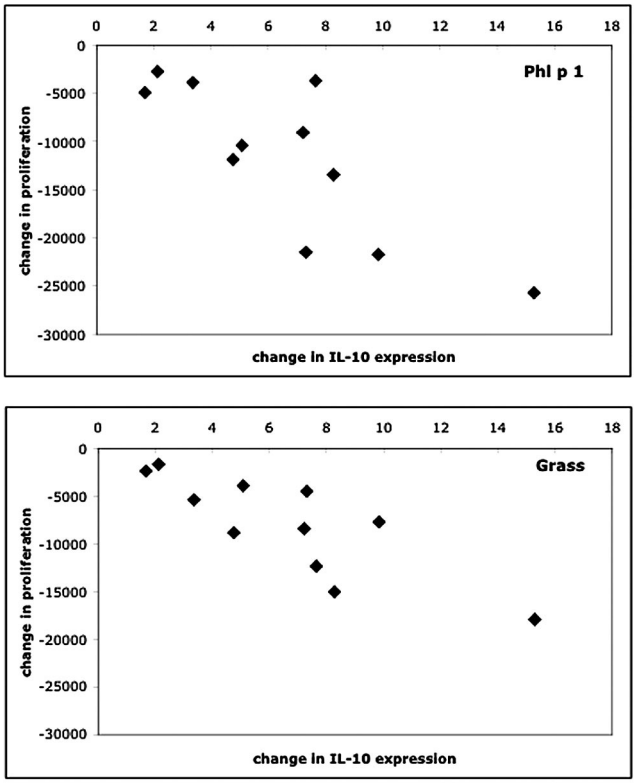


Figure 3. Correlations of the observed changes in allergen-dependent interleukin 10 (IL-10) expression after sublingual immunotherapy (SLIT) (post-SLIT minus pre-SLIT values) with the corresponding allergen-dependent changes in proliferation. Results obtained with comparison of proliferation with Phl p 1 recombinant allergen (top panel, $r = -0.80$, $P = .004$) and with grass extract (bottom panel, $r = -0.82$, $P = .003$) are represented. Proliferation changes are expressed on the y-axis as counts per minute. Interleukin 10 expression changes are expressed as changes in cytokine stimulation indexes.

allergen-specific proliferation vs TGF-$\beta$ expression in grass and rPhl p 1–stimulated cultures were 0.15 ($P = .60$) and 0.10 ($P = .63$), respectively. Correlation indexes of pretreatment and post-treatment changes in IL-10 vs TGF-$\beta$ expression were 0.17 ($P = .47$) and 0.16 ($P = .70$), respectively.

*T-Lymphocyte Origin of IL-10 Produced by rPhl p 1–Stimulated PBMCs*

Double staining of PBMCs with monoclonal antibodies to membrane CD3 and intracellular IL-10 showed at the protein level that, in the 5 patients who could be tested, production of IL-10 took place in allergen-stimulated T cells (Fig 4) and increased after 2 months of SLIT (Table 2).

## DISCUSSION

The main finding of the present study is that the proliferation of allergen-stimulated lymphocytes was decreased and the transcription of IL-10 was augmented by a short course of SLIT with a modified allergen, a pattern consistent with an increased regulatory T-cell activity. Furthermore, TGF-$\beta$ transcription, which can also be attributed to regulatory T cells, were augmented in allergen-stimulated PBMCs after immunotherapy, and this increase was barely statistically nonsignificant ($P = .06$).

Taken together, our data demonstrate that even a short course of SLIT can affect biological parameters associated with the systemic down-regulation of the $T_H2$ allergen-specific immune response in patients with allergic rhinitis and with the tolerance to subsequent allergen exposure.

Notably, although we made a horizontal follow-up in a single group of patients, and no control individuals were included, we performed both phlebotomies before the pollen season. This allowed avoidance of expression changes of these and other cytokines due to environmental exposure to allergen, which we observed in preliminary experiments, which might act as confounding factors. Indeed, in our hands

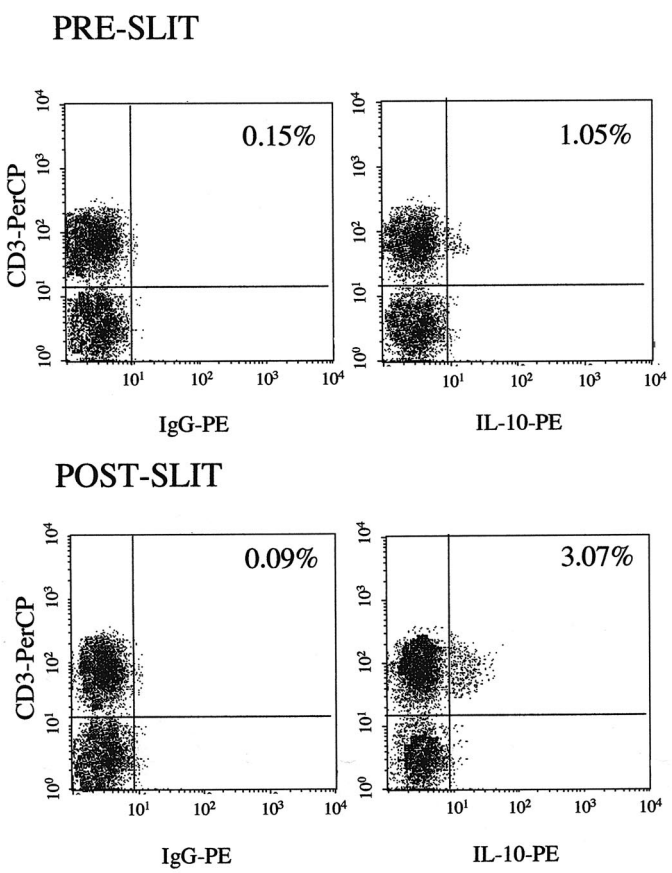


Figure 4. Dot plot cytofluorimetric analysis of interleukin 10 (IL-10) expression by CD3 T lymphocytes from one grass-allergic patient stimulated in vitro with recombinant Phl p 1. The test was performed on samples taken from the same patient before sublingual immunotherapy (SLIT) (pre-SLIT, top panels) and 2 months after the beginning of SLIT (post-SLIT, bottom panels). Samples were simultaneously stained with a peridinin chlorophyll-$\alpha$ protein–conjugated monoclonal antibody to the membrane CD3 (on the y-axis) and with phycoerythrin (PE)–conjugated monoclonal antibody to intracellular IL-10 (IL-10-PE, right panels) or with an isotype matched control (IgG-PE, left panels) (on the x-axis). Ten thousands events were acquired. The percentage of CD3 T cells expressing IL-10 is indicated in the top right quadrant of each panel.

5 birch- or grass-allergic patients, devoid of further sensitizations, when evaluated at the peak of the pollen season, displayed an IL-10 expression ranging from 1.8 to 3.2 times the values observed in the same individuals tested during the winter. This observation is reminiscent of a different model of natural exposure (ie, IL-10 hyperproduction and associated T-cell anergy) in hyperimmune patients who recently had received multiple bee stings.[6]

Similarly to previously published results in patients treated with different noninjective therapies,[22,33] SLIT-treated patients included in the present study showed no increase of allergen-specific IgG4 antibodies and no decrease of allergen-specific IgE (not shown).

Table 2. Percentage of IL-10–Positive Cells Among CD3$^+$ T Lymphocytes[a]

| Donor | Before SLIT | After SLIT |
|---|---|---|
| 1 | 0.68 | 1.22 |
| 5 | 0.71 | 3.29 |
| 7 | 2.18 | 4.63 |
| 8 | 0.90 | 2.98 |
| 11 | 1.42 | 5.49 |
| Median | 0.90 | 3.29 |
| Interquartile range | 0.71–1.42 | 2.98–4.63 |

Abbreviations: IL-10, interleukin 10; SLIT, sublingual immunotherapy.
[a] Results of intracellular staining for CD3 and IL-10 of peripheral blood mononuclear cells stimulated with rPhl p 1. Values indicate the percentage of CD3-positive cells coexpressing intracellular IL-10 and were obtained by subtracting for each donor the score obtained in the sample stained with control antibody to the score obtained with the anti–IL-10 antibody ($P = .008$, before SLIT vs after SLIT).

Interestingly, it has been suggested that the capability of IgG4 to interfere with the IgE facilitation of allergen presentation may be a more crucial parameter than the IgG4 levels per se.[34] Indeed, a functionally relevant interference by blocking antibodies may depend, rather than on the total amount of IgG4, on such hard-to-quantify factors as their affinity and fine epitope specificity to allergen.[35–37]

As for allergen-specific proliferation, most studies on SCIT described a decreased T-cell responsiveness to allergen, whereas contradictory results have been reported in SLIT-treated patients.[18,19,22,38] Indeed, a specific caveat should be kept in mind when comparing biologic parameters that can be referred to the tolerogenic effects of allergoid with that of native allergens due to the different pharmacokinetics profiles of the 2 preparations. In fact, it was reported that although allergoid and allergen from a *P judaica* major allergen (Par j 1) have a similar pattern of absorption at the oral mucosa level, their respective plasma curves are different.[13] The allergoid not only had a higher plasma peak than the allergen, but it was also detected in the circulation in a prevalently undegraded form.[13] This observation was more recently confirmed in a Der p 2 allergen/allergoid immunotherapy study.[14] These data suggest that the allergoid may reach the intestinal tissue in an undegraded form and thereby more efficiently amplify the induction of immunological tolerance by acting with local immunocompetent cells in Peyer patches and mesenteric lymph nodes. Our data encourage further studies based on the direct comparison of native vs modified allergens.

On the whole, the data reported in this pilot study suggest that a short course of SLIT with an allergoid preparation can affect the cellular allergen-specific responses in a fashion compatible with a systemic increase in the regulatory immune response. Our results are in agreement with a recent report by Savolainen et al,[20] which showed the increase of the IL-10 cytokine in children undergoing SLIT for birch allergy. In contrast, no systemic changes were seen in cytokines during

build-up or early maintenance phase by Dehlink et al,[39] but different methods for IL-10 detection and different doses of immunotherapy (below 10 $\mu$g of major allergen) were used in that study. Our data are also in agreement with those of Bohle et al,[40] who reported after 4 weeks of SLIT a reduced proliferative response to allergens and an enhanced IL-10 messenger RNA expression. Furthermore, these authors, who also measured $T_H1$ cytokines, found that at 52 weeks of SLIT, allergen-induced proliferation remained reduced at the same time as signs of immune deviation emerged. According to these data, the inverse correlation between IL-10 production and allergen proliferation we observed may be limited to the initial phase of SLIT.

Among the different biological parameters, which may help to clarify the role of immune regulation vs immune deviation after SLIT, we used in the present work a reductionistic approach focused on the 2 cytokines, which have been more consistently associated with the T-regulatory immune response. More comprehensive investigations, including Treg, $T_H17$, $T_H1$, and $T_H2$ axis cytokines, are needed to clarify the precise immunological mechanisms underlying successful SLIT, which are still unclear.

Incidentally, we used a simplified protocol devoid of any induction phase to treat our patients, who actually received the same dose of vaccine since the first day of treatment. We did not observe any severe unwanted side effects with such a protocol, which has the advantage of remarkably improving the compliance to SLIT while obtaining the same efficacy level. This observation is consistent with previous data from our group.[41,42]

Finally, our data suggest that allergen proliferation assays, which are technologically simple although not suitable to a high-throughput clinical laboratory, may provide an effective immunological marker in the follow-up of allergic patients undergoing immunotherapy.

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*Requests for reprints should be addressed to:*
*Samuele E. Burastero, MD*
*Department of Biotechnology*
*San Raffaele Scientific Institute*
*58, via Olgettina*
*20132 Milan, Italy*
*E-mail: s.burastero@hsr.it*