

Grass tablet sublingual immunotherapy downregulates the T_H2 cytokine response followed by regulatory T-cell generation

Abel Suárez-Fueyo, PhD,^a Tania Ramos, MD,^b Agustín Galán, BSc,^c Lucía Jimeno, PhD,^c Peter A. Wurtzen, PhD,^d Alicia Marin, BSc,^c Consolación de Frutos, MD,^b Carlos Blanco, MD, PhD,^b Ana C. Carrera, PhD,^a Domingo Barber, PhD,^c and Rosa Varona, PhD^a *Madrid, Spain, and Hørsholm, Denmark*

Background: Sublingual administration of *Phleum pratense* allergen immunotherapy (SLIT) tablets is a clinically efficient treatment for grass pollen-induced rhinoconjunctivitis. This immunotherapy downregulates T_H2 immune responses, induces tolerogenic pathways, and increases regulatory T cells. However, associated immune response markers of allergen desensitization remain undefined.

Objective: We sought to characterize the kinetics of individual changes in the immunologic response to grass tablet SLIT.

Methods: We evaluated the systemic effects of SLIT in a longitudinal analysis of humoral and cellular immune parameters in peripheral blood samples.

Results: Grass tablet SLIT administration induced a 2-phase systemic humoral and cellular response. The T_H2 response was initially exacerbated and detected as increased allergen-specific IgE (sIgE) and IgG₄ (sIgG₄) levels and an increase in IL-4–producing cells, followed by downregulation of the T_H2 response with a shift toward a T_H1 cytokine profile. T cells with a regulatory phenotype were also elicited. Statistical correlations between immunologic measurements for each patient throughout therapy indicated that T_H2 response downregulation and reduction of the immediate SLIT-induced IgE response were associated with increased allergen-specific IgG₄ synthesis early in therapy. T_H2 response downregulation by month 4 correlated with increased frequency of CD4⁺ T cells with a regulatory phenotype by 12 months.

Conclusion: Changes in sIgE levels after therapy were linked to a specific IgG₄ response, and production of blocking antibodies correlated with T_H2 response downregulation.

Reduced IL-4⁺ cell frequency was linked to an increase in the frequency of CD4⁺ T cells with a regulatory phenotype. Changes in sIgE levels and reduced IL-4 and blocking antibody levels could thus be used as indicators of a patient's immune response to therapy. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Sublingual immunotherapy, regulatory T cells, allergic rhinitis, IgG₄, IgE, IL-4

Allergen immunotherapy is an effective means of reorienting inappropriate immune responses in allergic patients.^{1,2} Sublingual administration of *Phleum pratense* allergen immunotherapy (SLIT) tablets is a clinically efficient treatment for grass pollen-induced rhinoconjunctivitis, the effect of which persists for at least 2 years after cessation of treatment,^{3,4} suggesting disease modification. Studies of local and systemic immune responses to therapy have proposed several mechanisms to explain its success, including blocked antibody production, mainly through IgG blockade of IgE-mediated responses.^{5,6} Changes in allergen-specific IgG₄ (sIgG₄) levels and IgE blocking factor and inhibition of facilitated allergen presentation are reported in SLIT-treated patients.⁴ T_H2 response downregulation, immunologic switch to T_H1 responses, or both suggest induction of tolerogenic pathways,⁷⁻⁹ suppression of allergic inflammation, and generation of regulatory T (Treg) cells.^{6,8,10-12}

Treg cells have multiple effects in controlling allergen-specific immune and IgE-mediated allergic inflammation.¹³ These cells can induce dendritic cells that support further Treg cell generation and suppress effector T-cell differentiation¹⁴; they also suppress T_H1 and T_H2 cells and induce IgG₄, IgA, or both.^{13,15} T_H2 cytokines (IL-4, IL-5, and IL-13) are necessary for IgE production and for the differentiation, survival, and activity of mast cells, basophils, eosinophils, and mucus-producing cells^{15,16}; a reduction in levels of these cytokines, together with IL-10 and TGF-β production by Treg cells, suppresses effector cell-induced allergic inflammation.¹³ Although these mechanisms are associated with successful immunotherapy, the sequence of events and pathways that connect these response mediators are poorly understood. Defined optimal treatment periods and early identification of responders or surrogate markers could be used to personalize therapy.

To provide support and direction for larger clinical studies, we performed a pilot clinical trial to examine the immune modulations elicited by SLIT in patients with grass pollen-induced allergic rhinitis over a 2-year treatment period. We sought to analyze the time course of the immune response and to determine how this response correlated to markers of allergen

From ^athe Department of Immunology and Oncology, Centro Nacional de Biotecnología (CNB-CSIC), Madrid; ^bServicio de Alergia, Hospital Universitario de La Princesa (IP), Instituto de Investigación Sanitaria Princesa, Madrid; ^cDepartamento de I+D, ALK-Abelló, Madrid; and ^dthe Department of Immunology, ALK-Abelló, Hørsholm. Supported by Genoma España. A.S.-F. was supported by a predoctoral fellowship from the Carlos III Institute, Spanish Ministry of Health.

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Corresponding author: Rosa Varona, PhD, Department of Immunology and Oncology, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, Campus de Cantoblanco, Madrid E-28049, Spain. E-mail: rvarona@cnb.csic.es.

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Abbreviations used

CTLA-4: Cytotoxic T lymphocyte-associated antigen 4
GPS: Grass pollen season
IgE-FAB: IgE-facilitated antigen binding
iTreg: Induced regulatory T
sIgE: Allergen-specific IgE
sIgG: Allergen-specific IgG
SLIT: Sublingual immunotherapy
Treg: Regulatory T

desensitization over time. We addressed the systemic immunologic effects of SLIT by analyzing allergen-induced humoral and cellular immune parameters in the patients' peripheral blood. Our results indicate that the changes in sIgE and sIgG₄ levels after treatment, as well as reduction in IL-4⁺ cell frequency, can be used as indicators of the patient's immune response to therapy.

METHODS**Study population**

Fifty-eight adult patients were enrolled in the study in September 2009 by the Hospital de La Princesa (Madrid, Spain), 44 remained in the study 1 year later, and 40 remained after 2 years of therapy. Inclusion criteria were a clinical history suggesting grass pollen sensitization, a positive specific IgE result (CAP class ≥ 2), and a positive skin prick test response (Soluprick SQ; ALK-Abelló, Hørsholm, Denmark) to *P pratense*. Exclusion criteria included previous immunotherapy with grass allergen extracts, a clinical history of symptomatic perennial allergic rhinitis or asthma, contraindications for immunotherapy as established by the immunotherapy subcommittee of the European Academy of Allergy and Clinical Immunology and by the summary of product characteristics of GRAZAX, and pregnancy. The institutional review board approved the study protocol; all subjects were informed of the aim of the trial and provided consent.

All patients received daily sublingual administration of GRAZAX (*P pratense*, 75,000 SQ-T oral tablets; ALK-Abelló). Immunologic evaluations were performed out of the pollen season and during peak pollination (May-June). The baseline was established in September-October 2009 before beginning immunotherapy; samples were evaluated at days 3 and 8 and months 1, 4, 9 (grass pollen season [GPS] I), 12, 21 (GPSII), and 24.

PBMC separation and immunophenotypic and flow cytometric analysis

PBMCs were separated on Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and incubated (20 hours) with *P pratense* extract protein (20 μ g/mL). For cytokine analyses, protein secretion was blocked with 2 μ g/mL brefeldin A (Calbiochem, Billerica, Mass) in cultures for the last 5 hours.

The cell subset phenotype was determined and cytokine-secreting lymphocytes were quantified by using multiparameter flow cytometric analyses. For a detailed description of reagents and procedures, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

sIgE, sIgG₄, and sIgG₁

sIgE (Phl p 1 and Phl p 5) was determined by using the ADVIA Centaur platform (Bayer HealthCare, Tarrytown, NY), according to standard methods.¹⁷ Phl p 1- and Phl p 5-specific IgG₄ and IgG₁ levels were evaluated by means of ELISA.¹⁸

FAB assay

Analysis was performed according to standard methods.¹⁹ In brief, a defined indicator serum containing high sIgE concentrations was preincubated with allergen alone or patient's serum and further incubated with

EBV-transformed B cells. Surface binding of allergen-IgE complexes was detected by using flow cytometry. Details are provided in the [Methods](#) section in this article's Online Repository.

Data presentation and statistical analysis

Data are represented as boxes showing sample means $\pm$ 95% CIs. All statistical procedures were performed with Prism version 5.0 software (GraphPad Software, La Jolla, Calif). Unless otherwise specified, the 1-way ANOVA test (1-way ANOVA, repeated measures, and the pair-based Dunnett test for longitudinal assays) was used in individual parameters, and Pearson and Fisher exact tests were used for pairwise correlations. *P* values of less than .05 were considered significant.

RESULTS**Grass tablet SLIT modulates allergen-specific humoral responses**

Allergen-specific immunoglobulin levels (sIgE, sIgG₄, and sIgG₁) were measured by quantifying individual patients' serum reactivity to the main *P pratense* allergens (Phl p 1 and 5)^{20,21} throughout treatment to evaluate humoral responses to therapy.

An increase from baseline (up to 30-fold) was observed in sIgE levels (Phl p 1 + Phl p 5) by 1 month of treatment in 92.5% of patients, followed by a progressive reduction and blunting of the seasonal increase in serum IgE levels ([Fig 1, A](#)). In 70% of patients, a reduction of greater than 25% was detected by month 4 compared with values at month 1. At 1 year, 85% of patients showed at least a 50% decrease compared with 1 month, 5% showed no increase in *P pratense*-specific IgE levels during therapy, and the remaining 10% showed a sustained sIgE level increase throughout treatment.

sIgG₄ levels (Phl p 1 + Phl p 5) were quantified in patients' sera throughout therapy; [Fig 1, B](#) shows the *x*-fold change in sIgG₄ levels from baseline. A *P pratense*-specific IgG₄ level increase was detected in 87% of patients. Serum sIgG₄ changes were time dependent within the population; 1 month of treatment was sufficient to detect an increase (≥ 1.5 *x*-fold change) in sIgG₄ levels affecting 58% of patients, which increased to 75% by 4 months and 87% by 24 months; in the remaining 13% of patients, no change was observed during the 2-year treatment.

Allergen-specific IgG₁ levels (Phl p 1 + Phl p 5) were also quantified in patients' sera. sIgG₁ was detectable in only 60% of patients who showed a significant sIgG₁ increase relative to baseline from GPSI (first-year GPS) to the end of the study ([Fig 1, C](#)). By month 1 of treatment, an sIgG₁ level increase (≥ 1.5 *x*-fold change) was detected in 18% of patients; 36% of patients showed increased sIgG₁ levels by month 4, which peaked at 56% of the population at months 9 to 12, when individual patients also registered the highest sIgG₁ levels. During the second year, 75% of these patients showed a gradual reduction in sIgG₁ levels, although these remained significantly greater than at time 0. The remaining 40% of patients had no detectable sIgG₁ at time 0 or at any test point during the 2-year treatment.

Analysis of Phl p 1- plus Phl p 5-specific IgE and sIgG₄ levels in serum showed a significant correlation between increases in sIgE levels at 1 month and sIgG₄ levels at 4 months of therapy ([Fig 1, D](#)) and not with increases in sIgG₁ levels at any time during treatment (not shown). There was no association between sIgE baseline values and changes in sIgG₄ or sIgG₁ levels ([Fig 1, E-H](#)).

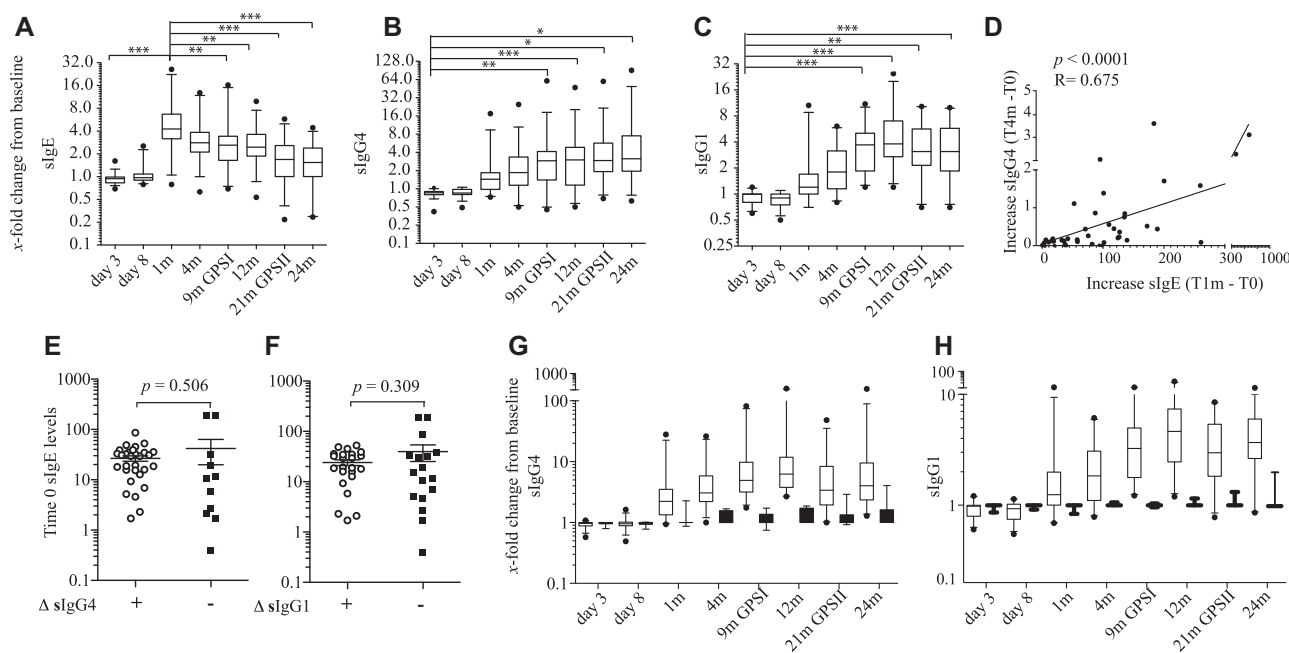


FIG 1. Grass tablet SLIT modulates humoral immune responses. **A-C**, Levels of sIgE ($n = 40$; Fig 1, A), sIgG₄ ($n = 40$; Fig 1, B), and sIgG₁ ($n = 26$ patients with detectable sIgG₁ levels; Fig 1, C) during the 2 years of SLIT are shown as the x-fold change from baseline for the 2 main *P. pratense* allergens (Phl p 1 + Phl p 5). Bars show means $\pm$ 95% CIs. **D**, Correlation between increased sIgE levels (Phl p 1 + Phl p 5) from baseline to month 1 and increased sIgG₄ levels (Phl p 1 + Phl p 5) from baseline to month 4. **E** and **F**, Time 0 sIgE values for patients with (open circles) or without (solid squares) an increase in sIgG₄ (Fig 1, E) or sIgG₁ (Fig 1, F) levels during therapy. The unpaired *t* test with Welch correction was used. **G** and **H**, x-fold changes from baseline in sIgG₄ (Fig 1, G) or sIgG₁ (Fig 1, H) levels in both groups throughout the 2-year treatment period (white bars, increased levels; black bars, no significant increase). **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001.

Grass tablet SLIT modulates the T_H2-type cytokine profile

To determine whether SLIT induced modulation of cytokine production in the periphery, we recorded the frequency of cytokine-producing cells in PBMC subpopulations. Patients' PBMCs showed a biphasic response in IL-4⁺ cell frequency, with a short-term increase at month 1 in 90% of patients (Fig 2, A), followed by a progressive reduction from month 4 in 54% of these patients. During GPSI, 90% of patients showed a reduction in IL-4⁺ cell frequency. Baseline levels were re-established in 68% by 1 year, whereas IL-4⁺ cell frequency remained higher than at time 0 in 32%. During the second treatment year, IL-4⁺ cell frequency was similar to or less than baseline in 95% of patients both in and out of the pollen season. Although the precise contribution by each cell type is patient dependent, CD3⁺ T cells and natural killer CD56^{dim}CD16⁺ cells account for 50% to 60% of IL-4⁺ cells detected at baseline (data not shown).

The percentage of IL-5⁺ lymphoid cells (mainly CD3⁺ T cells) peaked at GPSI; 3 months later, IL-5⁺ cell frequency was reduced compared with that seen at GPSI but remained higher than at time 0 (Fig 2, B). During the second year, there was no increase in the frequency of these cells in or out of GPSII; indeed, the lowest IL-5⁺ cell frequency was registered during this period (Fig 2, B). These findings suggest that SLIT suppressed T_H2-type cytokine responses.

Both IL-4⁺ cell frequency and serum sIgE levels showed an initial short-term increase, followed by a progressive reduction during treatment. Comparison of response kinetics showed

temporal coincidence for both parameters in most patients, with induction at 1 month and subsequent downregulation (Fig 2, C). In peripheral blood from 82.6% of patients, we found a concomitant increase in IL-4⁺ cell frequency and sIgE production as early as month 1 after initiation of treatment, suggesting an increased T_H2 response in PBMCs at 1 month of therapy. Downregulation of this response was evident by month 4 in 48% of patients, whereas 11% showed no reduction in either value, indicating 59% coincidence in the patient response for these 2 factors after 4 months. After 2 years of treatment, 84% of patients showed persistent reductions in both parameters.

We used eosinophil count increase during GPS as an indicator of the patient's allergic response to environmental pollen. In approximately 80% of patients, the significant peak in eosinophil counts in the GPS was reduced after 3 months. In the remaining patients the eosinophil peak did not correspond to the GPS period or no eosinophil reduction was detected. In 65% of patients, eosinophilia was reduced in GPSII compared with that seen in GPSI (Fig 2, D), suggesting allergen desensitization by year 2.

Reduced serum sIgE levels and IL-4⁺ cell frequencies correlate with increased serum-mediated inhibition of allergen-IgE binding to B cells and are linked to increased sIgG₄ levels

The increased T_H2 response, followed by its progressive reduction, as well as an increase in levels of specific antibodies other than IgE in patients undergoing therapy, prompted us to

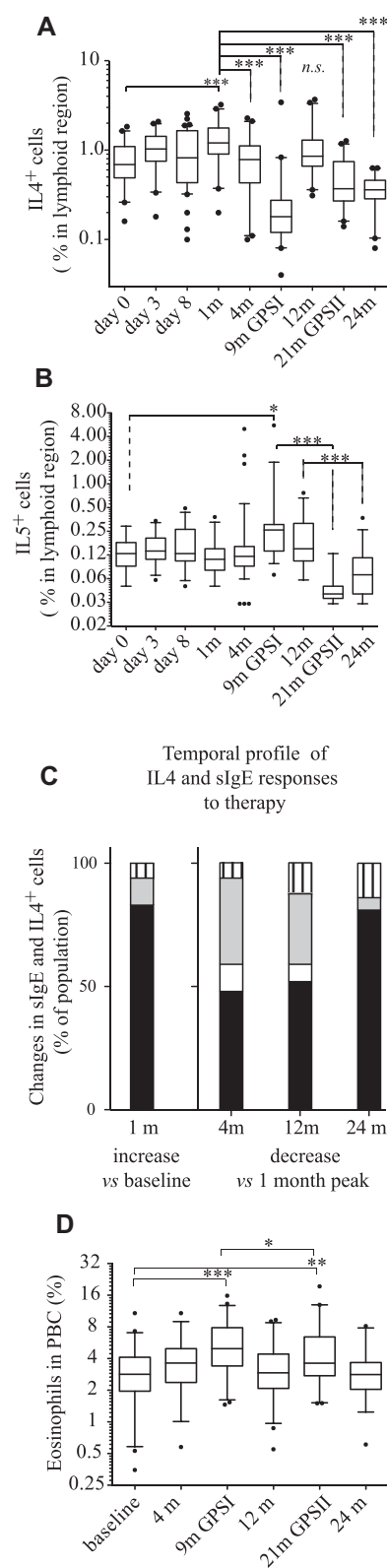


FIG 2. Modulation of the T_H2 cytokine profile during therapy. **A** and **B**, Percentages of IL-4⁺ (n = 40; Fig 2, A) and IL-5⁺ (n = 40; Fig 2, B) cells in patients during SLIT. Data are shown as means $\pm$ 90% CIs. **C**, Temporal profile showing the percentage of patients with IL-4 and sIgE level increases from baseline to the month 1 peak and reduction from the month 1 peak to months 4, 12, and 24. Patients with IL-4 and sIgE level increases (black bars) or no changes (white bars) and patients with changes in IL-4 (gray bars) or

consider a modulatory role for the allergen-sIgE complex through binding to B cells. We used the *in vitro* IgE-facilitated allergen binding (IgE-FAB) assay to determine whether patients' sera inhibited allergen-IgE binding to B cells.

Allergen-sIgE complex binding decreased progressively when B cells were incubated with sera from patients at 4 months of therapy or longer (Fig 3, A). The capacity of patients' sera to inhibit IgE binding to B cells thus appeared to increase with treatment time.

To study a possible association between sIgE levels, IL-4⁺ cell frequency, and blockade of allergen-sIgE binding to B cells in patients' sera, we evaluated these variables using the Pearson correlation coefficient. Comparison of the *x*-fold change in inhibition of allergen-sIgE-B-cell binding correlated significantly with the decrease in sIgE levels (Fig 3, B) and in IL-4⁺ cell frequency (Fig 3, C) from the peak at month 1 to 4 months.

The blocking effect might be caused by a therapy-induced specific immunoglobulin other than sIgE; to study these relationships, we compared changes in sIgG₄ and sIgG₁ levels with sIgE levels and IL-4⁺ cell frequencies. The *P pratense*-specific IgG₄ level increase from baseline to month 4 correlated with the sIgE level decrease from month 1 to all times tested (Fig 3, D). The sIgG₄ level increase from baseline to month 1 also correlated with the IgE level decrease thereafter (not shown); there was no significant correlation by the second year of treatment. Inverse correlations were found between the sIgG₄ level increase and the percentage of B cells with bound allergen-sIgE complexes in treated patients' sera at 4 (data not shown) and 12 (Fig 3, E) months. The reduction in IL-4⁺ cell frequency at month 4 was restricted to patients with increased sIgG₄ levels at 1 and 4 months of treatment (Fig 3, F). There were no differences in the percentage IL-4⁺ cell increase between patients who had an sIgG₄ response by month 1 and those who did not (Fig 3, G). We found no association between changes in sIgG₁ levels and sIgE levels. The sIgG₄ level increase from baseline to months 1 and 4 and the sIgE level decrease from month 1 to the end of treatment correlated with subjective clinical improvement (see Fig E1 in this article's Online Repository at www.jacionline.org).

Increased frequency of IFN- γ -producing cells in grass tablet SLIT-treated patients

To determine whether the reduction in the T_H2 response was accompanied by a shift to a T_H1 response during therapy, we tested for changes in the frequency of IFN- γ -producing cells. IFN- γ ⁺ cell frequency increased in PBMCs in 65% of patients, with a peak at month 12 (3 months after GPSI) that persisted during the second year (Fig 4, A). In 50% of patients, the increase in IFN- γ ⁺ cell numbers was significant by month 4, whereas approximately 15% of patients showed a significant increase only after 12 months, which then persisted. Some patients (35%) showed no persistent increase in these cell numbers (Fig 4, B).

Flow cytometric analysis indicated that approximately 60% of IFN- γ ⁺ cells are CD56⁺CD3⁻ natural killer cells of both subtypes (CD16⁺, 26% to 30%; CD16⁻, 70%), 26% of which

IgE only (dashed bars) are shown. **D**, Percentages of patients' peripheral blood eosinophils (n = 40, mean $\pm$ 95% CI) over the 2-year assay period. n.s., Not significant. **P* $\leq$.05, ***P* $\leq$.01, and ****P* $\leq$.001.

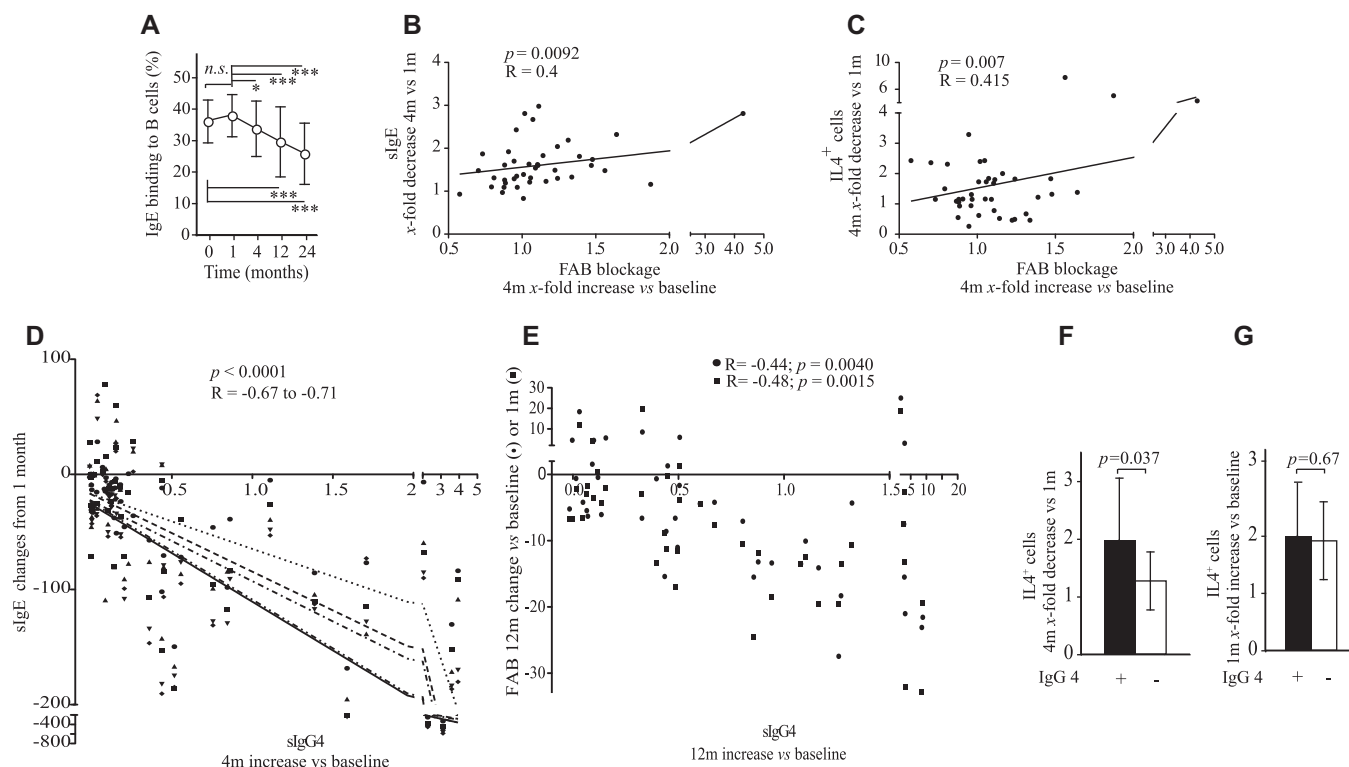


FIG 3. A, SLIT induced an increase in the blocking effect of patients' sera on sIgE binding to B cells. The percentage of B cells with allergen-IgE bound over time is shown as means $\pm$ SDs. B and C, The x-fold reduction in sIgE levels (Fig 3, B) and IL-4⁺ cell frequency (Fig 3, C) from months 1 to 4 are directly associated with the serum-mediated x-fold inhibition of allergen-IgE binding to B cells. D, sIgE level decrease from months 1 to 4 (circles), 9 (squares), 12 (triangles) and 24 (diamonds) correlates with increased sIgG₄ levels. E, Decrease in allergen-IgE complex binding to B cells from baseline (circles) or 1 month of treatment (squares) correlates with increased sIgG₄ levels. F and G, The reduction in IL-4⁺ cell frequency at month 4 is related to increased sIgG₄ levels (Fig 3, F; patients with [open bars] or without [solid bars] an sIgG₄ level increase at month 1), whereas no differences were detected in the increase in IL-4⁺ cell frequency at month 1 of therapy between both patient groups (Fig 3, G). n.s., Not significant. * $P \leq .05$ and *** $P \leq .001$.

are CD8⁺. Double-positive CD11c/CD11b cells also contribute to approximately 20% of the IFN- γ ⁺ cell pool. Only approximately 10% of IFN- γ ⁺ cells are detected as CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells (representative plots in Fig 4, C). The precise contribution of each cell type to the IFN- γ ⁺ cell pool is patient specific. Enrichment in the frequency of IFN- γ -producing cells during therapy corresponded to non-CD4⁺ cells. In culture (up to 5 days), 85% of IFN- γ ⁺ cells were allergen stimulation independent at time 0 and at months 9 (GPSI) and 12 of treatment (Fig 4, D). Our data indicate that the increase detected in IFN- γ ⁺ cell numbers cannot be attributed to the generation of an allergen-specific T_H1 response.

Therapy induces an increase in the frequency of CD4⁺ T cells with a regulatory phenotype

Because SLIT suppressed grass pollen-associated T_H2 responses (sIgE, sIgG₁, IL-4, and IL-5) in most patients by the second year, we wondered whether treatment induced a T cell-mediated regulatory response. We used flow cytometry to fractionate patients' CD4⁺ T cells based on surface IL-7 and IL-2 α -chain receptors, as well as CD45RA expression (Fig 5, A). To further define patients' T cells with a regulatory phenotype (CD4⁺CD127⁻CD25⁺),^{22,23} we used

cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), CD278, and IL-10 as indicators. In the memory/induced T-cell population, the CD4⁺CD127⁻CD45RA⁻CD25^{high} cell subset (resembling activated memory Treg [amTreg] cells) showed the highest levels of CTLA4 and CD278, as well as IL-10, production (Fig 5, A). The ability to suppress conventional T cells is well defined for the CD4⁺CD127⁻CD45RA⁺CD25⁺ (fraction I, resembling resting T cells) and CD4⁺CD127⁻CD45RA⁻CD25^{high} (fraction II) cell subsets.^{22,23} Fraction III cells had intermediate CTLA-4 and CD25 levels and a CD278^{+/+}IL-10^{+/+} pattern, although their suppressive ability remains to be confirmed. Although the CD4⁺CD127⁺CD45RA⁻CD25⁺ T-cell subset (putative memory T cells) did not change substantially throughout treatment (Fig 5, B) and T effector cell numbers (CD4⁺CD127⁻CD45RA⁻CD25⁻) increased only during GPSI (Fig 5, C), SLIT induced an increase in the CD4⁺CD127⁻CD45RA⁻CD25⁺ T-cell pool (fractions II plus III). Their frequency increased significantly from GPSI to the end of therapy (Fig 5, D). These cells were detected in 36% of patients at month 4, which increased to 44% by month 12 and persisted for the remainder of the treatment; 29% of patients showed an increase only in year 2, and 21% of patients showed no increase in this cell subset (Fig 5, E). Because

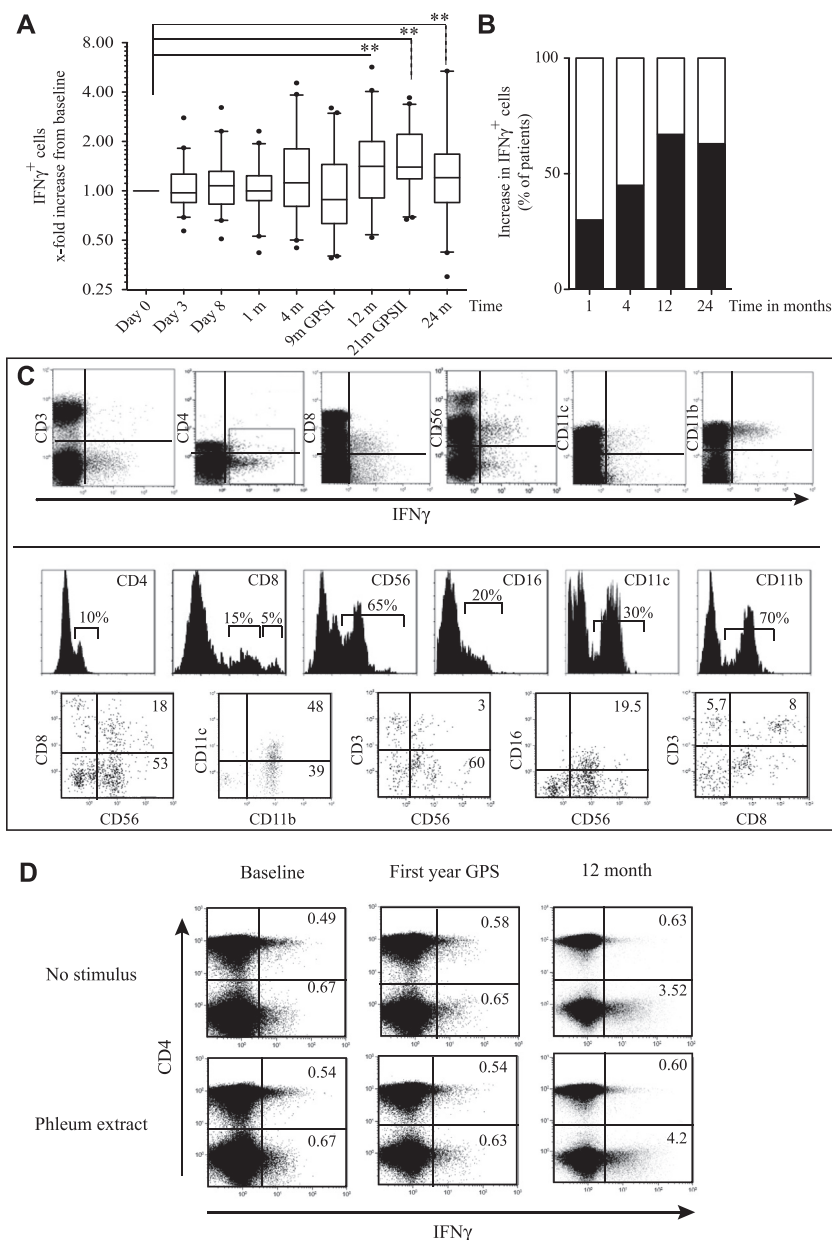


FIG 4. Frequency of IFN- γ -producing cells during treatment. **A**, Data are shown as the x-fold increase from baseline throughout therapy (mean $\pm$ 95% CI, n = 40). **B**, Percentage of patients with increased IFN- γ ⁺ cell frequency over time. **C**, Representative cytometric plots and phenotypic characterization of cell types. Numbers in outlined areas indicate the percentage of cells in each throughout. **D**, The increase in IFN- γ ⁺ cell frequency is allergen stimulation independent *in vitro*. Representative cytometric plots of IFN- γ ⁺ cells from patients' PBMCs, either unstimulated or stimulated *in vitro* with grass extract, from baseline and months 9 (GPSI) and 12. ** $P \leq .01$.

recently activated T cells show a transient loss of CD127 expression, we cannot rule out contamination by some activated non-Treg-like cells in this fraction at the GPS. The CD4⁺CD127[−]CD45RA⁺CD25^{high} cell subset showed a significant increase in frequency during the second year (Fig 5, F). Percentages of CD4⁺CD127[−]CD45RA⁺CD25⁺ T cells were unchanged relative to baseline values by the end of treatment, although their frequency was clearly reduced during GPSI (Fig 5, G).

Reduced CD4⁺IL-4⁺ cell frequency is linked to the increase in CD4⁺CD127[−]CD45RA[−]CD25⁺ T-cell percentage

Induced regulatory T (iTreg) cell generation is extremely sensitive to effector cytokines, such as IL-4, which block their differentiation. Because the allergen-induced IL-4⁺ cell frequency was reduced in 54% of patients by month 4, we tested whether Treg cell generation was linked to this change. The x-fold decrease in IL-4⁺ cell frequency from month

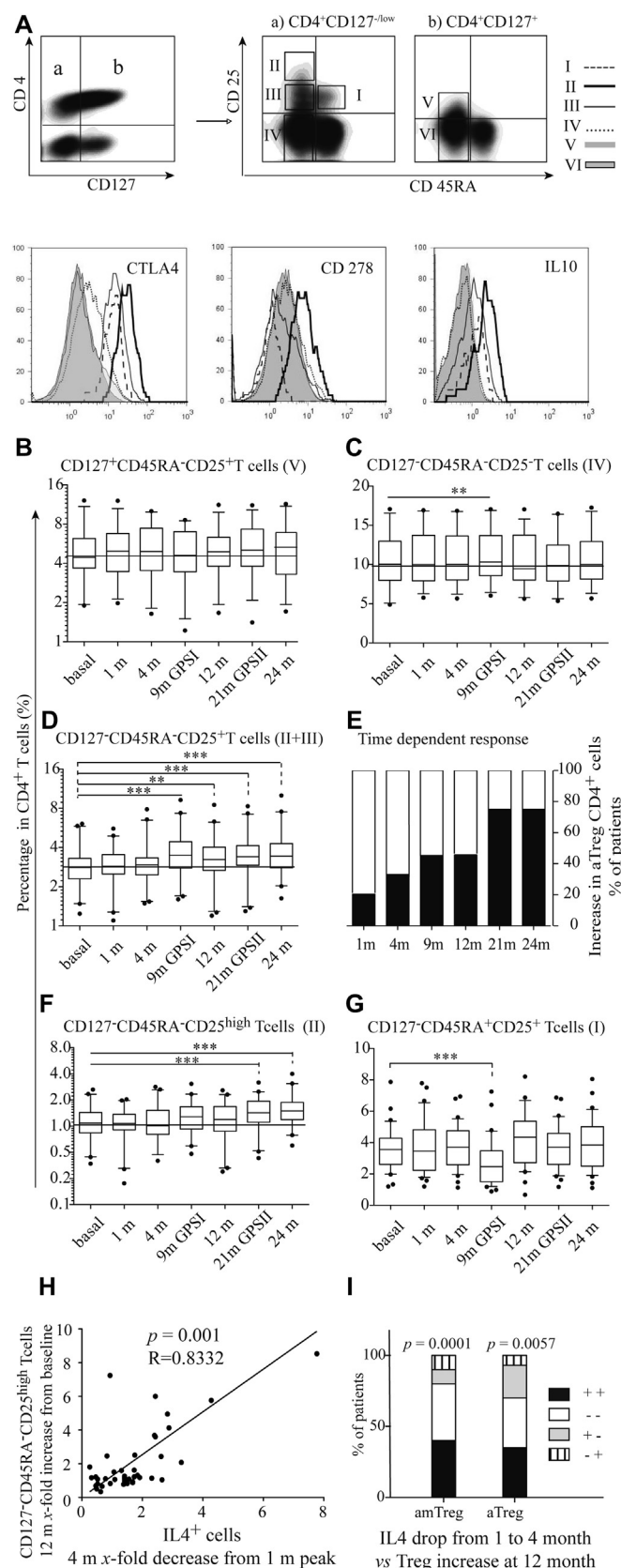


FIG 5. A, CD4⁺ T-cell fractionation and phenotypic characterization of cell subsets. B–G, Generation of a Treg-like cell-mediated response (n = 40). Fig 5, B and C, Percentages of CD4⁺ T-cell populations showing a memory

1 to month 4 correlated with the x -fold increase in the CD4⁺CD127⁻CD45RA⁻CD25^{high} T-cell pool (Fig 5, H). These data suggest that IL-4⁺ T-cell frequency must be reduced for generation of a Treg cell population. These parameters coincided in 70% to 75% of patients, with a reduction in IL-4⁺ cell frequency between months 1 and 4 and an increase in CD4⁺CD127⁻CD45RA⁻CD25⁺ T-cell percentage by month 12 (Fig 5, I).

DISCUSSION

We evaluated the systemic immunologic effects of sublingual allergen administration by analyzing the humoral and cellular immune response to therapy in patients' peripheral blood samples. Our aim was to determine the kinetics of individual immunologic changes in response to grass tablet SLIT over the 2-year treatment period and to provide useful information for large-scale clinical trials. This focus does not require a control group of untreated patients but limits the evaluation of the contribution of treatment-unrelated environmental factors.

The immediate response to therapy was an increase in allergen-associated T_H2-type responses (IL-4, sIgE, and sIgG₄) that was clearly defined by month 1. In sensitized patients complex formation between sIgE levels, allergen, and the low-affinity IgE receptor on B cells is hypothesized to lead to activation of cell-mediated responses through facilitated antigen presentation.²⁴ Activation of allergen-specific T cells could induce expansion of the T_H2 cell population, causing more B cells to switch to IgE production, exacerbating allergy-associated immune parameters. Our data support this hypothesis and provide an explanation for the immediate increase in sIgE and sIgG₄ levels and in IL-4⁺ cells detected in patients early in treatment (1 month).

Cell-activated responses to therapy at month 1 were suppressed in 48% of patients by month 4. Downregulation of these T_H2 responses (sIgE levels and IL-4⁺ cell frequency) appears to be directly related to patients' serum inhibitory activities for sIgE-FAB, as well as to increases in sIgG₄ antibody levels. After grass tablet SLIT, the sIgG₄ level increase from baseline to months 1 and 4 correlated with the decrease in sIgE levels from month 1 to month 4 and thereafter, as well as with the reduction in allergen-IgE complex binding to B cells. In addition, by month 4, IL-4⁺ cell frequency decreased only in patients with increased sIgG₄ levels. The results suggested that sIgG antibodies compete with IgE for allergen-binding sites.

Several studies^{25,26} show a blocking effect of allergen-specific non-IgE serum antibodies on competitive binding with IgE for

(Fig 5, B) or effector (Fig 5, C) phenotype in patients during therapy. Fig 5, D–F, Increased frequency of CD4⁺ T cells with regulatory phenotype after treatment. Percentages of CD127⁻CD45RA⁻CD25⁺ T cells (Fig 5, D) and of patients showing enrichment in this T-cell pool (Fig 5, E, black bars); increased frequency of the CD127⁻CD45RA⁻CD25^{high} subset (Fig 5, F) in CD4⁺ T cells. Data shown are means $\pm$ 95% CIs. Fig 5, G, The CD4⁺CD127⁻CD45RA⁺CD25⁺ T-cell subset showed no significant changes during therapy, except at GPSI. H, Correlation between the x -fold reduction in IL-4⁺ cells from months 1 to 4 and the x -fold increase in the CD127⁻CD45RA⁻CD25^{high} cell subset detected by month 12. I, The percentage of patients showing decreased IL-4⁺ cells at month 4 and an increased CD4⁺CD127⁻CD45RA⁻CD25⁺ T-cell pool at month 12 (black bars), neither (white bars), or only 1 condition (decreased IL-4⁺ cells [gray bars] or increased CD4⁺CD127⁻CD45RA⁻CD25⁺ T-cell frequency [dashed bars]). ** $P \leq .01$ and *** $P \leq .001$.

allergen. Although the mechanisms by which sIgG contributes to desensitization are not completely understood, there is now ample evidence that these antibodies prevent CD23-mediated allergen–IgE–FAB.^{24,27} In particular, sIgG₄ is considered an effective blocking antibody; it is speculated that its immunologic characteristics render this IgG subclass a more effective inhibitor of IgE-mediated reactions.^{28–30} sIgG₄ antibody titers are not directly related to clinical improvement. Nonetheless, functional assays of serum IgG-associated activity, such as inhibition of IgE–allergen interactions and of CD23-dependent IgE–FAB, correlate with clinical outcome and might serve as response biomarkers.^{4–6,31,32} Our results suggest that increases in sIgE levels in the first 4 weeks of treatment indicate patients' sIgG₄ responses and that the systemic increase in sIgG₄ levels at months 1 and 4 are closely related to the downregulation of allergic responses (sIgE, FAB, and IL-4) detected later in therapy, as well as to the patient's subjective clinical improvement.

Inhibition of IgE-facilitated antigen presentation might abolish activation of allergen-specific T_H2 cells and subsequent IL-4 production. This change leads to T-cell activation through allergen presentation by professional antigen-presenting cells, which require higher allergen concentrations, and could promote T_H1 and/or Treg cell differentiation. In our study SLIT led to a time-dependent increase in the IFN- γ ⁺ cell frequency and Treg cell population. The increase in IFN- γ ⁺–producing cell types other than CD4⁺ T cells, which is allergen independent *in vitro*, prompted us to consider this increase the result of an immune shift caused by IL-4 downregulation rather than derived from the generation of a genuine allergen-specific T_H1 response.³³

A CD4⁺ T-cell pool showing the phenotype described for activated memory/induced regulatory cells^{22,23} had been generated in 50% of patients by month 12 and persisted throughout year 2; this increase correlated with downregulation of the T_H2 response. iTreg cell generation *in vitro* is very sensitive to effector cytokines, such as IL-4, which blocks iTreg cell differentiation.^{34–36} Our data suggest that IL-4 downregulation is followed by the development of a Treg cell-mediated response to therapy.

These data, together with the lack of a seasonal sIgE peak, decreased sIgG₁ levels, reduced or baseline IL-4⁺ cell frequency, lack of seasonal IL-5 production by lymphocytes, and reduction in eosinophilia relative to that at GPSI, provide evidence of allergen desensitization during the second year of therapy.

Previous studies have indicated that successful immunotherapy involves downregulation of the T_H2 response, production of blocking antibodies, and generation of Treg cells. Here we show that grass tablet SLIT leads to increased humoral (sIgE and sIgG₄) and T_H2 responses during the first 4 weeks of therapy. Heightened sIgE production during month 1 is indicative of an sIgG₄ response in patients. Production of blocking antibody and sIgG₄ correlates with a decrease in sIgE synthesis and IL-4⁺ T_H2 responses. A reduction in IL-4⁺ cell frequency correlates with increased frequency of T cells with a regulatory phenotype. By the second treatment year, allergen desensitization is evident.

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Key messages

- SLIT induces increased sIgE and sIgG levels and IL-4⁺ cell frequency by month 1 of therapy.
- Production of blocking allergen-specific antibodies other than sIgE is associated with downregulation of patients' T_H2 responses by month 4.
- Reduced IL-4⁺ cell frequency was linked to an increase in CD4⁺ T cells with a regulatory phenotype.

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METHODS

PBMC separation and stimulation

PBMCs were obtained from whole blood samples collected by means of venipuncture in Vacutainer tubes containing sodium heparin (BD Biosciences, Franklin Lakes, NJ). Separation of PBMCs was performed on Ficoll-Hypaque density gradient (GE Healthcare Bio-Sciences AB). PBMCs were incubated (20 hours) with allergen (20 μ g/mL GRAZAX); for cytokine analyses, protein secretion was blocked by adding 2 μ g/mL brefeldin A (Calbiochem) to cultures for the last 5 hours.

Immunophenotypic analyses, mAbs, and flow cytometric analysis

Lymphocyte subsets from whole blood samples were analyzed by using multiparameter flow cytometry. Peripheral blood (100 μ L) was treated with fluorochrome-labeled mAb (20 minutes at room temperature), erythrocytes were lysed, and cells were fixed with Versalyse (all reagents from Beckman-Coulter, Brea, Calif). Eosinophils were quantified as CD15⁺CD14⁺CD16⁺CD11b⁺CD66b⁺CD294⁺ cells in peripheral blood cells.

Fractionated cell subsets were stained with Live/Dead dye (Invitrogen, Carlsbad, Calif) and identified by using the following anti-human mAb: AmCyan-labeled anti-CD45 and anti-CD4 (mIgG1; Becton Dickinson, San Diego, Calif); Pacific Blue-labeled anti-CD15 (mIgM) and anti-CD127 (mIgG1; eBioscience, San Diego, Calif); fluorescein isothiocyanate-labeled anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD16, and anti-CD66b (all mIgG1); phycoerythrin-labeled anti-CD11c and anti-CD56 (mIgG1); phycoerythrin-Texas Red-labeled anti-CD14 (mIgG_{2a}) and anti-CD45RA (mIgG1; all from Beckman Coulter); PC5-labeled anti-CD4, anti-CD19 (both mIgG1), anti-CD152 (mIgG_{2a}) and PE-CY7-labeled anti-CD278, anti-IFN- γ , and anti-IL-4 (all mIgG1; eBioscience); allophycocyanin-labeled anti-IL-5 (rat IgG_{2a}; BioLegend, San Diego, Calif); Alexa Fluor 647-labeled anti-CD294 (rat IgG_{2a}, BD Biosciences), Alexa Fluor 647-labeled anti-IL10 (rat IgG₁; BioLegend); and APC-PC7-labeled anti-CD25 (rat IgG₁; eBioscience). Cells were surface stained according to the manufacturer's protocols. For intracellular staining for CTLA-4 (CD152) expression and cytokine-producing capacity, we used the Intraperm Fix/Perm kit (BD Pharmingen).

For multidimensional flow cytometric analyses of phenotype and cytokine-secreting lymphocytes, we used a Gallios flow cytometer (Beckman-Coulter). Flow-Check Fluorospheres (Beckman-Coulter) were used daily to assess flow cytometric optical alignment and fluidics system performance. Standardization was performed every round of visits by using Flow-Set Fluorospheres (Beckman-Coulter) to re-establish settings of all fluorescence parameters to ensure maintenance of instrument settings and consistency over time. Compensation was performed with an aliquot of frozen human PBMCs cultured overnight and stained with anti-human CD4 labeled with each marker for each dye combination. ADC software (Beckman-Coulter) was used for automatic compensation and verified by using the same PBMC sample labeled with the antibodies in our protocols to ensure satisfactory automatic coefficient calculation.

Kaluz software (Beckman-Coulter) was used to analyze listmode data. One million events were acquired, and lymphocytes were gated by using side-scatter properties.

Allergen-specific IgG₄ and IgG₁

Phl p 1- and Phl p 5-specific IgG₄ and IgG₁ levels were evaluated by means of ELISA.^{E1} Patients' serum samples (1:10, 1:30, and 1:90 dilutions) were

incubated with Phl p 1- and Phl p 5-coated plates (2 μ g/mL) for 1 hour at room temperature. For ELISA determination, we used monoclonal anti-human IgG₄^{E2} and anti-human IgG₁ antibodies (Southern Biotech, Birmingham, Ala), both of which were peroxidase conjugated. Results were obtained from a *P pratense* extract standard curve and expressed in arbitrary units per milliliter.

FAB assay

Analysis was performed with 0.1 and 0.3 μ g/mL *P pratense* allergen extract for complex formation, as previously described.^{E3} In short, a stock indicator serum pool from 3 donors with grass pollen allergy containing high concentrations of sIgE (>100 KU/mL, as measured by the Advia Centaur method; ALK-Abelló) was preincubated with *P pratense* allergen extract (0, 0.1, and 0.3 μ g/mL; 37°C and 1 hour) alone (volume of allergen/indicator/buffer = 1:1:1) or with patients' serum (volume of allergen/indicator/patients' serum = 1:1:1). EBV-transformed B cells were added (10⁵ cells per sample, volume of allergen/indicator/patients' serum/B cells = 1:1:1:1) and further incubated at 4°C for 1 hour before washing. Surface binding of allergen-IgE complexes was detected by using flow cytometry with a fluorescein isothiocyanate-labeled anti-IgE polyclonal antibody (STAR147F; Serotec, Dusseldorf, Germany). All patients' samples were analyzed in duplicate.

Clinical improvement in patients undergoing grass tablet SLIT

To analyze clinical efficiency as an indicator of therapy success, we used each patient's self-assessment. Subjective clinical evaluation during a postseasonal visit was based on comparison between the most recent grass season and the previous one. A numeric value was assigned to all questions, ranging from -1 (worsening in symptoms) to 0 (no change) to 1 and 2 (degree of improvement). Cumulative values from the year 1 plus 2 assessment yielded a scale from -2 (worse symptoms in both years) to 4 (much better symptoms in both years). The relationship between clinical scores and immunologic parameters was analyzed by using the Spearman coefficient. Positive correlation was found between the cumulative clinical score after 2 years of therapy and the decrease in sIgE levels at all time tested compared with 1 month (Fig E1, A).

Positive correlation was also detected between the 2-year cumulative clinical score and increase in sIgG₄ levels at months 1 and 4 compared with baseline (Fig E1, B); there was no significant correlation when compared with sIgG₄ level increases at months 12 (Spearman r = 0.3022, P = .065) or 24 (Spearman r = 0.19, P = .22).

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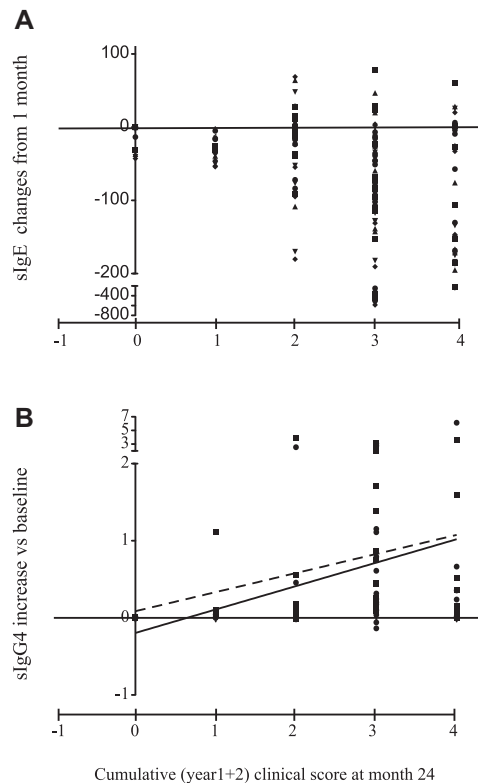


FIG E1. Correlation between changes in sIgE and sIgG₄ levels with clinical improvement in patients receiving grass tablet SLIT. **A**, Cumulative clinical score at the end of therapy correlates with an sIgE level decrease from months 1 to 4 (*circles*), 12 (*squares*), 21 (*triangles*), and 24 (*diamonds*; Spearman $r = -0.4466$ at month 4 [$P = .0049$]; Spearman $r = -0.3551$ at 12 months and -0.3405 at 24 months [2-tailed P values from .029-.039]). **B**, Cumulative clinical score at the end of therapy correlates with an sIgG₄ level increase from baseline to months 1 (*circles*; Spearman $r = 0.3684$, $P = .022$) and 4 (*squares*; Spearman $r = 0.423$, $P = .008$).