

Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation

Barbara Bohle, PhD,^a Tamar Kinaciyan, MD,^b Marianne Gerstmayr, MSc,^a Astrid Radakovics,^a Beatrice Jahn-Schmid, PhD,^a and Christof Ebner, MD^c Vienna, Austria

Background: The immunologic mechanisms underlying sublingual immunotherapy (SLIT) are still unclear, particularly the role of regulatory T cells.

Objective: We sought to characterize allergen-specific T-cell responses during successful birch pollen SLIT.

Methods: Proliferation of PBMCs and PBMCs depleted of CD25⁺ cells obtained from 9 patients before, after 4 weeks, and after 52 weeks of SLIT was assessed in response to the major birch pollen allergen Bet v 1, the homologous apple allergen Mal d 1, or tetanus toxoid. Allergen-induced cytokine responses and FoxP3 expression of T cells were analyzed by using real-time PCR. The role of IL-10 for regulatory activity of T cells was investigated.

Results: After 4 weeks, higher frequencies of circulating CD4⁺CD25⁺ T cells were detected together with increased FoxP3 and IL-10 and reduced IL-4 and IFN- γ mRNA expression levels compared with those before SLIT. Proliferation to all 3 antigens was markedly reduced but increased significantly after depletion of CD25⁺ cells or addition of anti-IL-10 antibodies. After 52 weeks, proliferation in response to Mal d 1 or tetanus toxoid returned to pre-SLIT levels, whereas Bet v 1-induced proliferation remained significantly suppressed and was enhanced by neither depletion of CD25⁺ cells nor addition of anti-IL-10 antibodies. In parallel, increased IFN- γ and reduced IL-4, IL-10, and FoxP3 mRNA expression was detected. Neither TGF- β levels nor cell-cell contact-mediated suppression of CD4⁺CD25⁺ cells changed during the course of SLIT.

Conclusion: SLIT induces regulatory T-cell suppression through IL-10 during the early phase and specific nonreactivity

and immune deviation of allergen-specific T cells during the later phase of therapy.

Clinical implications: SLIT induces immune mechanisms comparable with subcutaneous specific immunotherapy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Specific immunotherapy, sublingual immunotherapy, T-cell tolerance, immune deviation, regulatory T cells, Bet v 1, IL-10

Conventional specific subcutaneous immunotherapy (SCIT; ie, subcutaneous administration of allergen extract in an incremental dose regimen) is an effective treatment for IgE-mediated respiratory allergic disorders.¹ Successful SCIT has been associated with an increase in allergen-specific IgG4 antibodies.²⁻⁵ Furthermore, the induction of peripheral T-cell tolerance plays a crucial role in SCIT, and reduced proliferative and cytokine responses in allergen-stimulated PBMCs were accompanied by immune deviation (ie, the shift from the disease-eliciting T_H2-like phenotype toward a more nonpathogenic T_H0/1-like phenotype).⁶⁻¹¹ In parallel, it has been shown that the number of peripheral blood T cells that produce IL-10 in response to allergen stimulation increases during SCIT and that IL-10 can induce specific unresponsiveness (anergy) in peripheral T cells.^{5,11-16} Moreover, CD4⁺CD25⁺ T cells actively suppressed allergen-specific T-cell responses through the release of IL-10 and thus were considered as regulatory T (Treg) cells.^{11,17,18} The balance between allergen-specific IL-10-producing Treg cells and T_H2 cells has also been proposed to be pivotal for a physiologic immune response to allergens.¹⁹

Sublingual immunotherapy (SLIT) is an effective, safe, and convenient alternative to SCIT.²⁰⁻²³ However, there is only limited information on the modulation of the allergen-specific T-cell response to sublingual administration of allergens. Similar to SCIT, SLIT caused the reduction of allergen-induced proliferation in PBMCs from adult patients.²⁴⁻²⁶ Recent data showing that allergen-stimulated PBMCs from patients undergoing SLIT produced increased levels of IL-10 and IFN- γ indicated that immune deviation and the induction of Treg cells might also represent immune mechanisms operative in SLIT.^{25,27} However, the precise immune mechanisms underlying successful SLIT still remain to be elucidated.

From ^athe Department of Pathophysiology, Center for Physiology, Pathophysiology, and Immunology, and ^bthe Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Medical University of Vienna, and ^cAllergy Clinic Reumannplatz, Vienna.

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Reprint requests: Barbara Bohle, PhD, Medical University of Vienna, Center for Physiology and Pathophysiology, Department of Pathophysiology, Waehringer Guertel 18-20, AKH-3Q, A-1090 Wien, Austria. E-mail: barbara.bohle@meduniwien.ac.at.

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Abbreviations used

Ct: Cycle number at which detected fluorescence exceeds threshold
 SCIT: Specific subcutaneous immunotherapy
 SLIT: Sublingual immunotherapy
 Treg cell: Regulatory T cell

More than 96% of persons with birch pollen allergy are sensitized to the major birch pollen allergen Bet v 1, which is one of the best-characterized allergens regarding protein structure and B-cell and T-cell epitopes.²⁸⁻³⁴ We previously investigated the humoral and cellular response to Bet v 1 in 9 patients with improved nasal provocation scores to birch pollen before and after 1 year of birch pollen SLIT. Successful SLIT significantly increased Bet v 1-specific serum IgG4 levels and significantly decreased Bet v 1-specific T-cell responses, including T cells specific for all relevant epitopes of the major birch pollen allergen.²⁶ In the present study we aimed at a more detailed analysis of the immune mechanisms operative in SLIT. For this purpose, proliferative and cytokine responses to rBet v 1 before and after either 4 or 52 weeks of SLIT were monitored, and the involvement of Treg cells at each of the time points was investigated in parallel. The highly cross-reactive Bet v 1 homologue from apple, Mal d 1,³⁴⁻³⁸ and the unrelated antigen tetanus toxoid served as controls to elucidate the allergen specificity of immune mechanisms induced by SLIT.

METHODS**Patients**

The clinical data of the patients with birch pollen allergy included in the study have been published previously.²⁶ Briefly, all patients had a clear history of birch pollen allergy, positive skin prick test responses ($>5 \text{ mm}^2$) to birch pollen extract (Soluprick; ALK Abelló, Hørsholm, Denmark), and specific serum IgE levels of greater than 0.7 kU/L to birch pollen and Bet v 1 (CAP/FEIA; Phadia & Upjohn, Uppsala, Sweden). All patients showed subjective and objective (improved nasal provocation test results to birch pollen) improvement of birch pollen allergy after 52 weeks of Pangramin SLIT BU (221; ALK-Abelló, Allergie-Service GmbH, Linz, Austria). According to the manufacturer's protocol, increasing numbers of drops were self-administered at home daily during 4 weeks and held under the tongue for 2 minutes before swallowing. Thereafter, the maintenance dose of 10 drops ($= 4.5 \mu\text{g}$ of Bet v 1) was applied daily. To monitor the patients' adherence to the daily treatment schedule, patients met the clinician weekly during the increasing phase and every 8 weeks during the maintenance phase. At each meeting, the use of the therapy solutions was controlled. All individuals showed a very high compliance. Heparinized blood (50 mL) was collected before (November 2002), after 4 weeks (December 2002), and after 52 weeks (November 2003) of SLIT. The study was approved by the local Medical Ethical Committee of Vienna.

Antigens

Recombinant allergens were purchased from Biomay (Vienna, Austria), and tetanus toxoid was purchased from Calbiochem

(Darmstadt, Germany). Birch pollen extract was prepared as described previously.³⁹ Throughout the entire study, the same batches of antigens were used. Endotoxin levels were less than $2.5 \text{ EU}/\mu\text{g}$, as determined by using the LAL assay (BioWhittaker, Walkersville, Md).

Proliferation assays and cytokine ELISA

Freshly isolated PBMCs (2×10^5) were cultured in triplicates in medium alone or in the presence of rBet v 1 or rMal d 1 ($3.18\text{--}25 \mu\text{g}/\text{mL}$), tetanus toxoid ($0.06\text{--}0.5 \mu\text{g}/\text{mL}$), or rIL-2 ($10 \text{ U}/\text{mL}$).²⁶ After 6 days, supernatants were harvested, and proliferation was determined by means of tritiated thymidine incorporation. Data are expressed as Δ counts per minute, which is defined as counts per minute in stimulated cultures minus counts per minute in cultures containing medium alone. IL-10 and TGF- β were measured in supernatants by using ELISA with matched antibody pairs (Endogen, Woburn, Mass), as previously described,⁴⁰ or a TGF- β 1 ELISA kit (IBT, Reutlingen, Germany), according to the manufacturer's instructions. Cytokine levels in unstimulated cultures were subtracted from levels in allergen-stimulated PBMCs. For inhibition experiments, thawed PBMCs were incubated with allergens in the presence of a neutralizing anti-IL-10 and an isotype-matched control antibody (both $50 \text{ ng}/\text{mL}$; BD Biosciences Pharmingen, San Jose, Calif).

Depletion of CD4⁺CD25⁺ cells

Freshly isolated PBMCs were stained with CD4-fluorescein isothiocyanate, CD25-phycoerythrin, CD3-peridinin-chlorophyll-protein, and CD8-phycoerythrin (all from BD Biosciences) to determine percentages of T-cell subsets by means of flow cytometry. To deplete CD25⁺ cells, $30 \mu\text{L}$ of anti-CD25-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were suspended together with 1×10^7 PBMCs and incubated for 15 minutes on ice. After 2 washings, CD25⁺ cells were eluted by means of magnetic sorting on MS columns. The obtained cell fraction contained consistently less than 2% CD25⁺ cells, as determined by using flow cytometry.

Measurement of cytokine and FoxP3 levels by means of real-time PCR

Freshly isolated PBMCs ($10 \times 10^6/\text{mL}$) were cultured in 6-well plates (Costar, Inc, Cambridge, Mass), either in the presence or absence of birch pollen extract ($37.5 \mu\text{g}/\text{mL}$). After 6 hours, T cells were purified with anti-CD3 Dynal magnetic beads (Dynal, Hamburg, Germany), according to the manufacturer's protocol. RNA was isolated from purified T cells by using the RNeasy Mini Kit (Qiagen, Hamburg, Germany) and reverse transcribed with TaqMan reverse transcription reagents by using random hexamers (Applied Biosystems, Foster City, Calif). Quantitative real-time PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and cDNA-specific Assays-on-Demand to analyze 18S rRNA, IL-4, IL-13, IL-10, IFN- γ , TGF- β , and FoxP3 (Applied Biosystems). A negative control without a template and a positive control with a template of known amplification were included in each run. All amplifications were performed in triplicates. During the PCR, the cycle number at which the detected fluorescence exceeds the threshold (Ct value) was determined. The levels of 18S rRNA as a measure of a control housekeeping gene were not altered during allergen stimulation (mean Ct $\pm$ SD unstimulated = 9.39 ± 0.99 ; mean Ct $\pm$ SD stimulated = 9.14 ± 0.86). Relative quantification and calculation of the range of confidence was performed by using the comparative threshold cycle ($\Delta\Delta\text{CT}$) method (Applied Biosystems). The cytokine stimulation indices

at t4 and t52 were calculated by using the following formula:
 $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ for stimulated culture t4/52} - \Delta Ct \text{ for stimulated culture t0})}$.

FoxP3 was evaluated in unstimulated cells.

Coculture experiments of autologous CD4⁺CD25⁻ and CD4⁺CD25⁺ cells

CD4⁺ cells were purified from cryopreserved PBMCs with a Dynabead CD4 negative selection kit (DynaL Biotech ASA, Oslo, Norway). CD4⁺CD25⁻ and CD4⁺CD25⁺ cell fractions were further isolated by using anti-CD25-coated beads (3 μ L/10⁷ cells) with LS columns for magnetic sorting (Miltenyi Biotech). Thereafter, CD4⁺CD25⁻ (1×10^4 cells/well) or CD4⁺CD25⁻ plus CD4⁺CD25⁺ cells were cultured in triplicate at ratios of 1:1, 1:3, and 1:6, together with irradiated autologous PBMCs (1×10^5 /well) in 96-well round-bottom plates (Nunc, Rochester, NY) and 1 μ g/mL OKT3. Proliferation was determined after 72 hours by using tritiated thymidine incorporation.

Statistics

Statistical significances of differences were determined by using the Wilcoxon signed-rank test. Differences were considered statistically significant at a *P* value of less than .05.

RESULTS

Allergen-specific proliferative responses during SLIT

PBMCs obtained before and after either 4 or 52 weeks of therapy were stimulated with rBet v 1, rMal d 1, or tetanus toxoid. The concentration inducing the highest proliferative responses before SLIT of each antigen was individually determined and used for evaluating the course of T-cell reactivity during the study period. At 4 weeks, rBet v 1-induced proliferation was significantly decreased (*P* = .042, Fig 1). In parallel, rMal d 1-induced proliferation was also significantly decreased (*P* = .015). Tetanus toxoid-induced proliferation at 4 weeks was reduced, but this effect was not statistically significant. At 52 weeks, only rBet v 1-induced proliferative responses remained significantly reduced (*P* = .038), whereas rMal d 1- and tetanus toxoid-induced responses returned to the levels observed before therapy. IL-2-induced proliferation of PBMCs did not change significantly during SLIT (data not shown).

Depletion of CD25⁺ cells increases proliferation after 4, but not after 52, weeks of SLIT

PBMCs depleted of CD25⁺ cells were stimulated in parallel to PBMCs, as described above (Fig 1). Before SLIT, the proliferation of CD25⁺-depleted PBMCs was less pronounced in response to both rBet v 1 and rMal d 1 and was significantly less to tetanus toxoid (*P* = .022). In contrast, after 4 weeks of SLIT, CD25⁺-depleted PBMCs showed significantly higher proliferative responses to rBet v 1 (*P* = .009), rMal d 1 (*P* = .013), and tetanus toxoid (*P* = .005). However, after 52 weeks of SLIT, the proliferation of CD25⁺-depleted cells was again less pronounced than that of nondepleted cells. In parallel, the percentage

of circulating CD4⁺CD25⁺ cells was evaluated in freshly isolated PBMCs by means of flow cytometry and revealed an increased frequency of this cell type after 4 weeks of SLIT. The median values of CD4⁺CD25⁺ cells in PBMCs of 9 patients were 15% (2.6% to 37%) before SLIT, 35% (4.5% to 43%) at 4 weeks, and 21% (3.2% to 39%) at 52 weeks. No changes in the numbers of CD8⁺CD25⁺ cells were detected.

Cytokine responses of allergen-stimulated T cells during SLIT

CD3⁺ T cells were purified from birch pollen-stimulated and unstimulated PBMCs from 5 of 9 individuals, and mRNA expression for IL-4, IL-13, IFN- γ , IL-10, and TGF- β was determined by means of quantitative real-time PCR. In addition, the expression of mRNA for FoxP3 was determined in T cells from unstimulated PBMCs as a marker for Treg cells.⁴¹ The relative changes in mRNA expression after 4 and 52 weeks of SLIT compared with values before therapy were calculated for each individual, and median values of the 5 patients are shown in Fig 2, A. Compared with values before therapy, mRNA expression for IL-4 and IFN- γ was reduced at 4 weeks, whereas IL-10 mRNA expression was clearly increased. At 52 weeks, mRNA expression for IL-4 and IL-10 was less than that before SLIT. However, at this time point, a significant increase of mRNA expression for IFN- γ was observed (*P* = .043). During the course of SLIT, no significant changes of mRNA expression of TGF- β were detected. Unstimulated T cells of the 3 time points showed no alteration of mRNA for cytokines (data not shown). An increased expression of FoxP3 was observed at 4 weeks, but this was no longer evident at 52 weeks (Fig 2, A). In parallel to real-time PCR, IL-10 and TGF- β levels were analyzed in supernatants of PBMCs from 7 of 9 patients stimulated with rBet v 1, rMal d 1, or tetanus toxoid. Compared with values before SLIT, enhanced levels of secreted IL-10 in response to both allergens were only observed at 4 weeks (Fig 2, B). Tetanus toxoid-stimulated PBMCs synthesized very low amounts of IL-10. No significant changes of TGF- β levels were detected at either time point (data not shown). These data confirmed, at the protein level, the results obtained from mRNA expression analysis.

IL-10 mediates the suppression of proliferation

To evaluate whether IL-10 was involved in the suppression of allergen-specific T-cell responses at 4 weeks, cryopreserved PBMCs of each time point from 4 donors were stimulated with antigens in the presence of a neutralizing anti-IL-10 antibody or an isotype-matched control antibody. The addition of anti-IL-10 antibody significantly increased antigen-induced proliferative responses after 4 weeks of SLIT but not before SLIT or after 52 weeks of SLIT (Fig 3). To evaluate whether the regulatory function of CD4⁺CD25⁺ T cells at 4 weeks was due to changes of suppressive surface molecules, this population was purified from cryopreserved PBMCs of

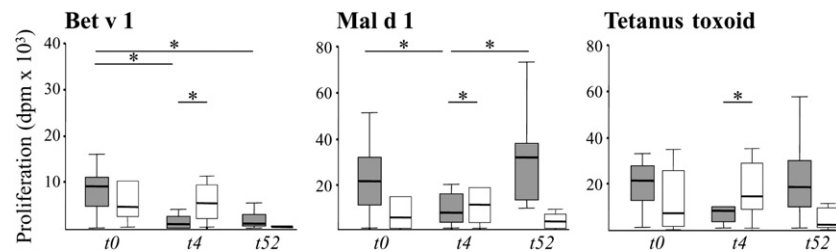


FIG 1. Antigen-stimulated proliferation of PBMCs (shaded boxes) and CD25⁺-depleted PBMCs (open boxes) before and after 4 or 52 weeks of SLIT (n = 9). Box plots are depicted. Each box represents the interquartile range containing 50% of values. The line across the box indicates the median. The whiskers extend from the highest and lowest values. *P < .05.

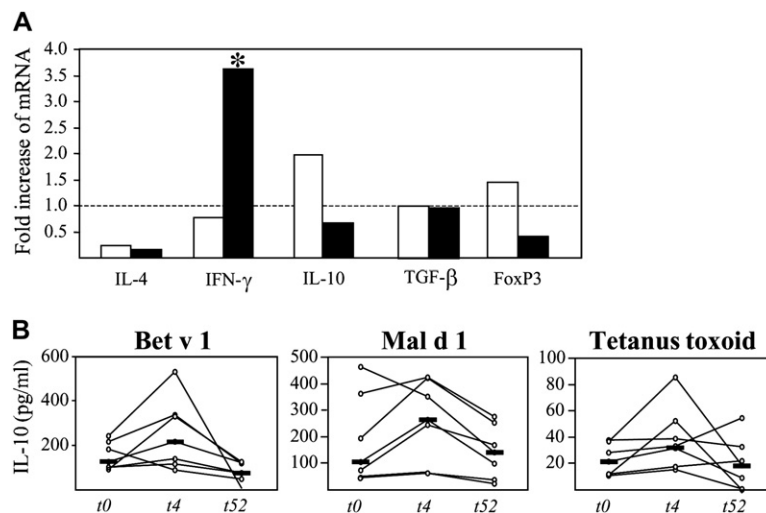


FIG 2. Allergen-specific cytokine and FoxP3 expression during SLIT. **A**, Median values (n = 5) of the relative mRNA expression for IL-4, IFN- γ , IL-10, TGF- β , and FoxP3 in CD3⁺ cells at 4 (open bars) and 52 (solid bars) weeks compared with values before SLIT. *P < .05. **B**, IL-10 levels in supernatants (n = 7) of antigen-stimulated PBMCs. The black lines indicate median values.

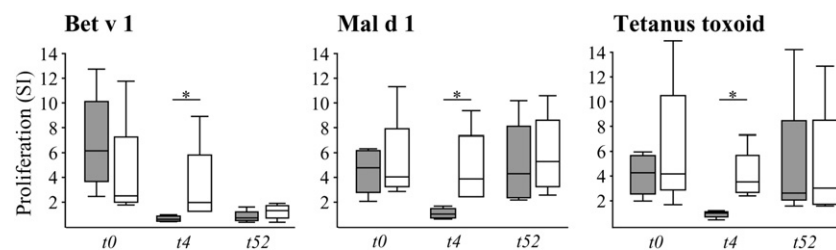


FIG 3. Effects of anti-IL-10 on antigen-induced proliferation before and after 4 or 52 weeks of SLIT. PBMCs of 4 patients were stimulated with antigens in the presence of an isotype control (shaded boxes) or anti-IL-10 antibody (open boxes). *P < .05.

3 additional patients at all time points and cocultured with autologous CD4⁺CD25[−] cells in ratios of 1:1 and 1:3 in the presence of irradiated PBMCs isolated before therapy and anti-CD3 antibodies. At all time points, addition of CD4⁺CD25⁺ cells resulted in a very similar downregulation of proliferation of the CD4⁺CD25[−] cells, thereby indicating that the suppressive capacity per cell did not change during the course of SLIT (Fig 4).

DISCUSSION

Although based on a limited number of studied individuals, this longitudinal study demonstrates for the first time that successful SLIT induces IL-10-producing Treg cells and that different immune mechanisms are operative during early and later phases of treatment.

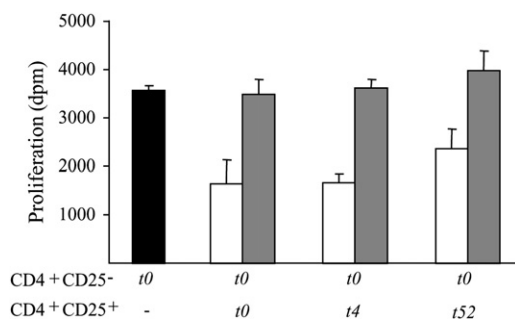


FIG 4. Suppressive activity of Treg cells during SLIT. CD4⁺CD25⁺ cells from cryopreserved PBMCs before and after 4 or 52 weeks of SLIT were cocultured with autologous CD4⁺CD25⁻ T cells isolated before SLIT in a ratio of 1:1 (open bars) or 1:3 (shaded bars) in the presence of anti-CD3 and autologous irradiated PBMCs. The solid bar represents CD4⁺CD25⁻ cells alone. Similar results were obtained in 3 different experiments.

After 4 weeks of SLIT, higher numbers of CD4⁺CD25⁺ cells in PBMCs and increased mRNA expression for FoxP3 in isolated T cells were detected compared with pretreatment levels (Fig 2). Bet v 1- and Mal d 1-induced proliferation was significantly reduced, and tetanus toxoid-induced proliferation was also diminished (Fig 1). Depletion of CD25⁺ cells or neutralization of IL-10 significantly enhanced antigen-induced proliferation (Figs 1 and 3). In parallel, allergen-induced IL-10 production of T cells was increased, whereas IL-4 and IFN- γ synthesis was decreased (Fig 2). Together, these findings provide strong evidence that during the early phase of SLIT, the CD4⁺CD25⁺ subset contained a substantial fraction of Treg cells that actively suppressed T-cell responses *in vitro*. Because neither changes of TGF- β synthesis nor of suppressive activity in coculture experiments with defined numbers of CD4⁺CD25⁺ T cells were observed (Figs 2 and 4), we conclude that IL-10 represents an important factor for suppression by these Treg cells. Thus our findings are in accordance with those of previous studies^{24,25} and suggest that SLIT-induced Treg cells do not fulfill the criteria typical for T_H3 or Treg cells specific for mucosal allergens observed during SCIT.^{11,42} In particular, T_H3 cells have been associated with peripheral tolerance after oral antigen administration. However, this cell type mediates active suppression by the secretion of high levels of TGF- β and variable amounts of IL-4 and IL-10.⁴²

After 52 weeks of SLIT, exclusively Bet v 1-induced proliferation remained significantly diminished, whereas proliferative responses to Mal d 1 and tetanus toxoid returned to levels observed before therapy (Fig 1). Neither the depletion of CD25⁺ cells nor neutralizing anti-IL-10 antibodies increased Bet v 1-specific proliferation (Figs 1 and 3). At the same time, the expression of IL-10 and FoxP3 mRNA was no longer increased (Fig 2). It is noteworthy that birch pollen-stimulated T cells showed a significantly higher IFN- γ mRNA expression (Fig 2). Together, these observations suggest that tolerance

mechanisms other than active suppression by IL-10-producing Treg cells are operative during the later course of SLIT. On the one hand, clonal deletion of allergen-reactive T lymphocytes might have reduced the number of Bet v 1-specific T cells *in vivo*. On the other hand, the increased synthesis of IL-10 during the early phase of SLIT might have induced anergy in Bet v 1-specific T lymphocytes.¹⁴ Our experimental approach, however, does not allow us to provide conclusions regarding which of these mechanisms was decisive for T-cell tolerance at this time point. Nevertheless, the pronounced synthesis of IFN- γ and abrogated expression of IL-4 in birch pollen-stimulated T cells strongly indicate that remaining allergen-reactive T cells at the later time point of SLIT belonged to a more T_H0/1-like subset.

In our opinion the observation that different immune mechanisms are operative during subsequent phases of SLIT matches data obtained by monitoring T-cell responses during longitudinal studies of SCIT. Suppressive Treg cells synthesizing IL-10 and TGF- β were identified within the first 70 days of injection therapy with house dust mite extract.¹¹ In general, evidence for immune deviation was provided after at least 12 months of SCIT with house dust mite or grass pollen extracts.^{6,8,10} Moreover, induced Treg cells were shown to have a short life span and, consequently, are rapidly removed from the periphery.⁴³ We speculate that the predominance of different mechanisms of tolerance induction might be time and dose dependent. Low doses of orally administered antigens were shown to favor active suppression, whereas high doses supported clonal deletion/anergy.^{44,45} Furthermore, stimulation of human T cells with high doses of house dust mite extract promoted the expansion of IFN- γ -producing cells, apoptosis of CD4⁺IL-4⁺ T cells, and T-cell anergy *in vitro*.⁴⁶ In the present study, during the first 4 weeks, a total amount of 20.5 μ g of Bet v 1 was administered, whereas during the maintenance period approximately a 6 times higher amount of Bet v 1 was applied per month. Thus lower allergen doses administered during the early phase might have promoted the induction of allergen-specific IL-10-producing CD4⁺CD25⁺ Treg cells, whereas allergen doses cumulating during the further course of SLIT might have fostered the induction of deletion/anergy and immune deviation of allergen-reactive T cells, respectively. Monitoring to determine whether similar alterations emerge during the different phases of SCIT is currently under investigation in our laboratory.

Analyzing the T-cell response to Mal d 1 during SLIT with birch pollen adds another interesting aspect to this study. This apple allergen shares several cross-reactive T-cell epitopes with the major birch pollen allergen.^{34,37} After 4 weeks of SLIT, proliferative responses to Mal d 1 and tetanus toxoid were reduced, indicating that Bet v 1-specific IL-10-producing Treg cells activated *in vivo* suppressed any T-cell responses in *in vitro* cultures. After 52 weeks, however, Mal d 1-induced, as well as tetanus toxoid-induced, proliferation was no longer abrogated. These findings not only point to the existence

of a Bet v 1-independent T-cell response to Mal d 1^{26,37} but also indicate that the immune mechanisms operative during allergen-specific therapy are restricted to the applied allergens.

This longitudinal study is based on 9 patients with birch pollen allergy who improved nasal provocation scores to birch pollen after 1 year of birch pollen SLIT.²⁶ Because of limitations in blood sampling, it was not possible to perform all experiments at each time point with cells from each individual. Nevertheless, our data indicate that SLIT induces specific T-cell tolerance and immune deviation. Active suppression provided by IL-10-producing Treg cells was predominant during the early course of therapy and was followed by other tolerance mechanisms during the later phase of therapy. Thus SLIT modulates the allergen-specific T-cell response in a similar way as SCIT.

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