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Birch Pollen Immunotherapy Leads to Differential Induction of Regulatory T Cells and Delayed Helper T Cell Immune Deviation

Christian Möbs,* Caroline Slotosch,* Harald Löffler,† Thilo Jakob,‡ Michael Hertl,*1 and Wolfgang Pfützner*1

Correction of an imbalance between allergen-specific T cell subsets is considered a critical event in establishing allergen tolerance by specific immunotherapy (SIT). In a comprehensive, longitudinal study, distinct T cell populations and IgG subtypes were analyzed in subjects allergic to birch pollen during decisive time points of SIT (i.e., induction and maintenance phase), as well as in and out of birch pollen season. An increase in Bet v 1-specific, IL-10–secreting T cells, fulfilling the criteria of inducible type 1 regulatory T (Tr1) cells, was observed by the end of the induction phase; this resulted in a decreased ratio of allergen-specific IL-5+ Th2/Tr1 cells. In contrast, CD4+CD25+CD127low regulatory T cell numbers did not change. Furthermore, enhanced concentrations of allergen-specific IgG Abs were observed, whereas allergen-specific IgE and IgA levels remained unchanged. After 1 y of SIT, a reduced ratio of allergen-specific Th2/IFN-γ+ Th1 cells was apparent. Although untreated and SIT-treated allergic subjects developed enhanced Th2 cell responses during birch pollen season, only SIT-treated patients experienced elevated numbers of allergen-specific Tr1 cells, which were associated with reduced skin prick test reactivity and diminished clinical symptoms. In coculture assays, allergen-specific Tr1 cells showed an IL-10– and dose-dependent inhibition of CD4+CD25+ Th1 cells was apparent. Although untreated and SIT-treated allergic patients by suppressing allergen-specific T effector (Teff) cells. Thus, SIT has differential effects on regulatory T cell subsets, resulting in an early induction of allergen-specific Tr1 cells associated with an increase in allergen-specific IgG, and it leads to a delayed shift from an allergen-specific Th2- to a Th1-dominated immune response. The Journal of Immunology, 2010, 184: 2194–2203.

The increased incidence of respiratory allergies, which has reached epidemic proportions in Europe and Northern America, poses a major challenge to Western medicine (1–4). Individuals with allergic rhinitis and bronchial asthma constitute ~20% and 8% of the population, respectively (5–7). In addition, the prevalence of pollen allergy has risen to an estimated 40% in the past decades (8). Among tree pollen allergies, birch (Betula) has a remarkable clinical impact because it is one of the major pollen allergen-producing trees. The prevalence of subjects with a positive skin prick test (SPT) to birch pollen allergen is as high as 54% of the general population in some regions of Europe (8). Among the various components of birch pollens, Bet v 1 was shown to be the major allergen (9, 10). Apparently, >90% of individuals who are allergic to birch pollen are sensitized to Bet v 1.

Specific immunotherapy (SIT) is the only causative treatment shown to modulate the natural course of respiratory allergies (11). SIT leads to improvement or even complete remission of allergic symptoms (12), and it prevents the de novo development of allergen sensitizations (13) and bronchial asthma in patients with allergic rhinitis (14, 15). The therapeutic effect of SIT was demonstrated in various randomized, double-blind, placebo-controlled trials (16–19), with prolonged clinical improvement extending >3 y after discontinuation of treatment (20). Thus, SIT is the treatment of choice for patients with considerable pollen-induced rhinitis and asthma.

Although the clinical efficacy of SIT is well-established, the immunological mechanisms leading to allergen tolerance are the focus of intensive research. Because IgE-mediated allergies are characterized by a Th2 cell-mediated immune response (21), it was believed that immune deviation of allergen-specific Th2 cells to a Th1 cell-driven response is the prevailing immunological process elicited by SIT (11, 22). However, recent data suggest that a distinct group of T cells, called regulatory T (Treg) cells, is a major player in the immunological network responsible for immune tolerance in allergic patients by suppressing allergen-specific T effector (Teff) cells (23–28). Among the known subpopulations of Treg cells, a subset of CD4+CD25+ Treg cells is characterized by constitutive expression of forkhead winged-helix transcriptional factor Box p3 (Foxp3), which negatively correlates with CD127 expression (29, 30) and is thought to represent naturally occurring Treg cells evolving in the thymus or adaptive Treg cells developing in the periphery (31). Another subgroup generated outside the thymus is called inducible type 1 Treg (Tr1) cells, and it can be identified by the secretion of IL-10 (32). Thus, Foxp3+ adaptive Treg and Foxp3+ Tr1 cells are inducible Treg cells that differentiate from naïve T cells in the periphery by antigenic stimulation in a submissive cytokine

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CS, clinical score; Foxp3, forkhead winged-helix transcriptional factor Box p3; NPT, nasal provocation test; SIT, specific immunotherapy; SPT, skin prick test; Teff, T effector; Treg, regulatory T; Tr1, type 1 Treg

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compared with pretreatment parameters, subjects allergic to birch pollen season in the SIT-treated patients were determined and closely monitored clinical and experimental design was chosen to identify changes in the pivotal phases of SIT (i.e. induction and maintenance phase) compared with pretreatment values. Additionally, longitudinal studies that directly focus on the frequencies of distinct T cell populations are rare. For example, the number of IL-10+ CD4+CD25+ T cells was exclusively assessed during the first few weeks of SIT, demonstrating an early induction of allergen-driven T1 cells (25, 33). Also, variable dynamics of IL-10 production were reported in studies with longer observation periods. A decrease in allergen-induced IL-10 synthesis to less than pretreatment levels at the end of the first year of SIT (35) as well as increased amounts of IL-10 secretion by PBMCs from patients after 1 year of SIT compared with controls were found (24).

To further assess the impact of allergen-specific T cells and Treg cells on achieving immunological tolerance against birch pollen allergens by SIT, we performed a longitudinal study in patients with Bet v 1-induced allergic rhinitis and/or bronchial asthma who were followed-up clinically and immunologically on SIT with birch pollen extract. A closely monitored clinical and experimental design was chosen to identify changes in the pivotal phases of SIT (i.e. induction and maintenance phase) compared with pretreatment values. Additionally, potential effects evoked by natural allergen exposure during birch pollen season in the SIT-treated patients were determined and compared with pretreatment parameters, subjects allergic to birch pollen who were not treated with SIT, and healthy controls. The frequencies of allergen-specific T cells and Treg cell subsets, including Bet v 1-specific T1 cells and CD4+CD25+CD127low Treg cells, were monitored before and during SIT over a 12-mo observation period. In addition, to assess potential changes in T cell subpopulations in relation to humoral alterations evoked by SIT, the serum concentrations of allergen-specific IgE, IgA, IgG, and IgG4 Abs were determined. Clinical outcome criteria included SPT reactivity, nasal provocation test (NPT), and a patient questionnaire regarding allergic symptoms and the use of antiallergic drugs.

Materials and Methods

Patients

A cohort of 15 patients with allergy to birch pollen and who were on birch pollen SIT was investigated in the present study. All participants had a history of birch pollen allergy with 1) moderate-to-severe seasonal allergic rhinoconjunctivitis and/or mild-to-moderate allergic asthma, 2) positive SPT to birch pollen extracts (ALK Prick SQ, ALK-Abelló, Hørsholm, Denmark), 3) serum IgE reactive with birch pollen extracts and rBet v 1 (Phadia ImmunoCAP System, Phadia, Uppsala, Sweden), and 4) a positive NPT to aqueous birch pollen extracts (ALK-depot SQ, ALK-Abelló). NPT was performed with Rhinotest2000 (Allergopharma, Reinbek, Germany), according to the guidelines of the German Society for Allergy and Clinical Immunology (36). Exclusion criteria were polysensitization with perennial symptoms, clinically relevant allergies against additional pollen allergens, and chronic nonallergic asthma. Thirteen of 15 patients (87%) completed the first year of the study. Two patients dropped out because of pregnancy and relocation, respectively. The two control groups consisted of seven subjects allergic to birch pollen treated only symptomatically and eight healthy nonallergic volunteers.

Study design

SIT with birch pollen allergen extract was performed according to international guidelines and a safe-dose regimen. Patients received incremental, weekly doses of birch pollen allergen (ALK-depot SQ, ALK-Abelló) injected s.c. until a maintenance dose of 100,000 Standard Units per injection was reached; thereafter, it was given in monthly intervals. Birch pollen extract SPT reactivity was evaluated before (m0) and 1 and 2 (m1) after the initiation of SIT. Furthermore, the nasal response to allergen exposure was assessed in a subgroup of five patients by NPT at the same time points. Global assessment of the patients’ symptoms was performed at several defined time points: at initiation (m0) and after 1 (m1), 3 (m3, end of induction phase), 6 (m6, maintenance phase, birch pollen season), and 12 mo (m12) of SIT. Clinical score (CS) was assessed by patients reporting the extent of allergic symptoms on a scale of 1 (no symptoms) to 7 (most severe symptoms) before and on SIT during birch pollen season. In addition, changes in symptomatic antiallergic drug use were noted. The study was approved by the Ethics Committee of the Medical Faculty of Philipps University; all patients gave written informed consent to participate in the trial.

Blood samples and preparation of PBMCs

Citrato-phosphate-dextrose-adrenaline-containing peripheral blood samples were taken from subjects allergic to birch pollen and the healthy control donors at the defined time points. Unless otherwise specified, PBMCs were isolated by Pansoli (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation, frozen at a concentration of 107 PBMCs/ml in 5% (v/v) heat-inactivated FCS (PAV Laboratories, Pasching, Austria) and 10% DMSO (Sigma, Schnelldorf, Germany), and stored in liquid nitrogen until needed for further experiments.

ELISPOT assay

Frozen PBMCs were thawed and cultured in 24-well plates (Nunc, Langenselbold, Germany) at a concentration of 1 x 106/ml in culture medium consisting of RPMI 1640 (Lonza, Basel, Switzerland) and 10% pooled human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μM l-glutamine (all from PAA Laboratories). PBMCs were stimulated with 10 μg/ml Bet v 1 (endotoxin level of Bet v 1 was 1.94 ng LPS/mg protein using LAL Test Kit QCL-1000, Bio-Whittaker, as specified by the manufacturer; Biomet, Vienna, Austria) and left unstimulated. On day 3, IL-2 (10 U/ml; Roche, Mannheim, Germany) and IL-7 (10 ng/ml; Milteny Biotec, Bergisch-Gladbach, Germany) was added for in vitro expansion of Bet v 1-specific T cells. After 7 d, PBMCs were restimulated with Bet v 1 (10 μg/ml) or PHA (1%; Sigma) as a positive control. After coating the plates with anti-human IFN-γ or anti-IL-5 or anti-10 μg/ml overnight at 4°C, 2 x 105 Bet v 1-specific, IL-10-secreting Tr1 cells were isolated (see below), fixed in paraformaldehyde, and prepared for ELISPOT analysis. The number of spots was counted automatically using an ELISPOT plate reader (A.EL.VIS, Hannover, Germany), and the median number of spots was determined in triplicate after subtracting the number of spots in the unstimulated wells from those in the Ag-stimulated wells.

Flow cytometric analysis

PBMCs were isolated from full blood by ACK lysis. Cell surface staining was performed simultaneously using anti-human CD4-FITC, anti-human CD25-APC, and PE-conjugated mouse anti-human CD127 or appropriate isotype controls (all from BD Pharmingen, Heidelberg, Germany) for 20 min on ice in the dark. Cells were washed and analyzed by flow cytometry analysis (FACS Calibur, BD Biosciences) using CellQuest software. For the analysis of intracellular Foxp3 expression, Bet v 1-specific, IL-10-secreting Tr1 cells were isolated (see below), fixed in paraformaldehyde solution (1%), permeabilized in saponin, and APC-conjugated Foxp3-specific Ab (both from eBioscience, San Diego, CA) were added for 30 min on ice in the dark. Afterwards, cells were washed and quantified by flow cytometry as described before.

Isolation and functional characterization of Bet v 1-specific Tr1 cells

IL-10–secreting Tr1 cells were isolated after ex vivo stimulation of PBMCs with Bet v 1 followed by the addition of IL-2/IL-7 (10 U/ml and 10 ng/ml, respectively) using the IL-10 Secretion Assay Cell Enrichment and Detection Kit (Milteny Biotec), according to the manufacturer’s protocol. CD4+CD25+ T cells were isolated by negative selection using the CD4+CD25+ Regulatory T cell Isolation Kit, human (Milteny Biotec) and cocultured at concentrations of 5, 7.5, and 9 x 106 cells/ml with IL-10–secreting Tr1 cells at ratios of 1:1, 3:1, or 9:1, respectively, in the presence of 4 x 105/ml autologous x-irradiated (2 x 31Gy) PBMCs and anti-CD3 (1 μg/ml; BD Pharmingen). To assess the role of IL-10 secreted by the Tr1 cells, unconjugated neutralizing anti-IL-10 mAb (10 μg/ml; JES3-19Fl; BD Pharmingen) was added to cocultures of T cells and Bet v 1-specific Tr1 cells. Moreover, to analyze allergen-specific T cell
suppression by Tr1 cells. Bet v 1-specific Teff cell lines (1 × 10^5 cells/ml) were also cocultured with autologous Tr1 cells (1–5 × 10^4 cells/ml) at ratios of 2:1, 4:1, and 10:1 and Bet v 1 (10 μg/ml). Bet v 1-specific Teff cell lines were generated as described previously (37) by repeated in vitro stimulation of PBMCs from patients allergic to birch pollen in the presence of 10 μg/ml Bet v 1 and 10 U/ml IL-2. T cell proliferation in the coculture experiments was assessed 5 d later by the uptake of [3H]thymidine (0.5 μCi/well; Amersham Biosciences, Buckinghamshire, U.K.), which was added for the final 18 h of culture.

**Analysis of allergen-specific serum Abs**

Specific IgE, IgA, IgG, and IgG4 Abs against *Betula verrucosa* (birch) pollen allergen and rBet v 1 were measured by the Phadia ImmunoCAP System (Phadia), according to the manufacturer’s instructions.

**Statistics**

Allergen-specific Ab concentrations and T cell frequencies were expressed as median ± range and depicted as box-and-whisker plots because of the nonparametric distribution of the collected patients’ data. Median values are represented by center line, with 25th and 75th percentiles (boxes) containing 50% of the data points. The whiskers extend from the highest and lowest values. Outliers (distance from 25th or 75th percentile >1.5 × length of the box) are depicted as open circles, and extreme values (distance from 25th or 75th percentile >3 × length of the box) are represented by asterisks. Statistical analysis was performed using SPSS. Comparisons of paired samples of patients before and at different time points during SIT were made using the Wilcoxon signed-rank test. Differences were considered statistically significant at p values < 0.05. To allow for the calculation of cell ratios (Th2/Th1 and Th2/Tr1), any individual values of 0 for the number of cytokine-producing cells were arbitrarily replaced by the number 1 in the statistical analysis.

**Results**

**Clinical efficacy of birch pollen SIT**

Upon SIT, clinical improvement was apparent by significantly diminished CSs of SIT-treated patients that resulted in a shift from severe (birch pollen season before initiation of SIT; m5 = 6–8) to mild symptoms (subsequent birch pollen season; m6) on a 7-point-scale in contrast to only symptomatically treated allergic individuals who did not experience a relief of symptoms (p = 0.03; Supplemental Fig. 1A, 1B, Table I). Furthermore, a long-term decrease in symptomatic drug consumption (data not shown) and a trend toward improved nasal symptoms, as determined by NPT (Supplemental Fig. 1C), were noted in subjects receiving SIT. In addition, >80% (11/13) of these patients displayed reduced SPT reactivity to birch pollen allergen compared with pretreatment values (p = 0.003; Supplemental Fig. 1D, Table I).

**Changes in Bet v 1-specific Th and Tr1 cells induced by SIT**

To investigate cellular immunological changes induced by birch pollen SIT, the presence and frequency of allergen-specific Th and Tr1 cells were analyzed by ELISPOT assay. Although there was no change in the numbers of Bet v 1-specific, IL-5–producing Th2 cells during the first 3 mo of SIT, a significant increase was noticed during the birch pollen season (m6; p = 0.025), which returned back to pretreatment values at mo 12 (Fig. 1A). These changes were allergen-specific and were not due to a general activation of Th2 cells upon SIT, because the PBMCs of SIT-treated individuals failed to show substantial variations in cytokine secretion in PHA mitogenesis assays (Supplemental Fig. 2). In contrast to Th2 cells, Bet v 1-specific IFN-γ-producing Th1 cells did not show substantial alterations during the first year of SIT (Fig. 1B).

Unlike the Th cell subsets, allergen-specific IL-10–secreting Tr1 cells were significantly influenced by SIT. Their frequency started to increase by 3 mo of SIT (p = 0.01), peaking at 6 mo during the birch pollen season (p = 0.003), and correlated with the decline in CS, despite the presence of substantially enhanced Bet v 1-specific Th2 cells at the same time point. Although the frequency of Bet v 1-specific Tr1 cells decreased later in the maintenance phase of SIT, it remained significantly increased at 12 mo after the initiation of SIT compared with pretreatment values (p = 0.049; Fig. 1C).

**CD4+CD25+CD127low Treg cell numbers remain stable during SIT**

In contrast to inducible, allergen-specific Tr1 cells, CD4+CD25+Foxp3+ T cells, which have low expression of CD127 (29, 30), are thought to represent Treg cells that suppress Teff cells upon activation in a cell contact- but not cytokine-dependent manner (38, 39). They are generated in the thymus as naturally occurring cells or in the periphery under the influence of TGF-β as adaptive Treg cells (31, 40). Several studies suggested that these cells might be involved in the inhibition of Th2-driven allergic immune responses (41, 42). To determine whether the number of CD4+CD25+CD127low Treg cells in patients allergic to birch pollen was influenced by SIT, the frequency of this Treg cell population was assessed in the peripheral blood of SIT patients. FACS analysis showed that, in contrast to Bet v 1-specific Tr1 cells, no significant changes in the number of CD4+CD25+CD127low Treg cells were seen with SIT. Only slight individual deviations were noticed among the analyzed individuals, whereas the median percentage of CD4+CD25+CD127low Treg cells remained relatively constant during the initiation and maintenance phases as well as during and out of pollen season compared with pretreatment levels (Fig. 2).

**SIT-induced modifications of Bet v 1-specific T cell subsets**

Correction of an imbalance between the different T cell populations (i.e., between the presumably pathogenic Th2 cells and the potentially protective allergen-specific Th1 or Tr1 cells) may be a critical factor for successfully establishing allergen tolerance by SIT (23). To better understand the impact of the observed changes on Bet v 1-specific T cell subsets, the number of pathogenic Th2 cells was directly related to Bet v 1-specific Th1 and Tr1 cells.

### Table I. Clinical characteristics of SIT-treated patients allergic to birch pollens

<table>
<thead>
<tr>
<th>Patients Undergoing SIT</th>
<th>n = 15</th>
<th>Range</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n; female/male)</td>
<td>9/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mean; y)</td>
<td>41</td>
<td>(21–61)</td>
<td></td>
</tr>
<tr>
<td>Total IgE before SIT (kU/l)</td>
<td>117b (3.08–1040)</td>
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<tr>
<td>Birch pollen-specific IgE before SIT (kU/l)</td>
<td>13.6b (0.49–81.7)</td>
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<tr>
<td>Bet v 1-specific IgE before SIT (kU/l)</td>
<td>16.1b (0.48–84.3)</td>
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<tr>
<td>CS 6 mo before SIT (in season; m6–6)</td>
<td>5b (3–12)</td>
<td>p = 0.03</td>
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<tr>
<td>CS 6 mo after initiation of SIT (in season; m6)</td>
<td>3b (1–5)</td>
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<tr>
<td>SPT before SIT (out of season; m0)</td>
<td>2b (1–4)</td>
<td>p = 0.003</td>
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<tr>
<td>SPT 12 mo after initiation of SIT (out of season; m12)</td>
<td>1b (0.5–2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined by the Wilcoxon signed-rank test.

bMedian.
Although the ratio of Bet v 1-specific Th2/Th1 cells remained unchanged within the first 6 mo of SIT, a significant decrease became apparent in the later portion of the maintenance phase ($p = 0.016$; Fig. 3A). In contrast, the proportion of allergen-specific Th2 to Th1 cells had significantly diminished after 3 mo of SIT, the end of the induction phase ($p = 0.016$; Fig. 3B). This decrease persisted over the entire observation period ($p = 0.028$), including birch pollen season, despite the increased frequency of Bet v 1-specific Th2 cells at this time point ($p = 0.022$).

**Influence of natural birch pollen exposure on Bet v 1-specific T cell subsets**

At natural allergen exposure during the birch pollen season, SIT-treated patients experienced a strong increase in Bet v 1-specific Th2 and Th1 cells (Fig. 1A, 1C). To determine whether these enhancements were induced by natural birch pollen exposure or SIT, the frequencies of the different T cell subsets were also assessed by ELISPOT analysis of PBMCs from control populations. These consisted of individuals allergic to birch pollen treated only symptomatically or healthy volunteers. Significant alterations were only found in the allergen-specific Th2 cell subset of subjects allergic to birch pollen. During birch pollen season, a substantial increase in IL-5–secreting, Bet v 1-specific Th2 cells was noticed compared with values out of season ($p = 0.046$; Fig. 4A). In contrast, Bet v 1-specific IFN-γ+ Th1 and IL-10+ Th1 cells revealed only minor changes in this control group. T cells from healthy volunteers did not show a distinct pattern of cytokine secretion upon ex vivo stimulation with Bet v 1, during and out of birch pollen season. Only augmented, but not statistically significant, frequencies of IFN-γ–secreting Th1 cells were observed upon natural birch pollen exposure (Fig. 4B).

**Teff cells are suppressed by Bet v 1-specific Tr1 cells in a cell number- and IL-10-dependent manner**

To evaluate the mechanisms by which Bet v 1-specific Tr1 cells suppress Teff cells, different coculture assays with these T cell populations were performed. First, IL-10–secreting, Bet v 1-stimulated Tr1 cells were cocultured with autologous CD4+CD25− Teff cells that were stimulated with anti-CD3 mAb. Although Bet v 1-specific Tr1 cells isolated from three allergic individuals did not show a robust proliferative response to stimulation with anti-CD3 as determined by [3H]thymidine uptake (no Ag: 118 ± 129 cpm; anti-CD3: 129 ± 117 cpm), peripheral CD4+CD25− Teff cells from the same donors exhibited a strong proliferative response (no Ag: 58 ± 7 cpm; anti-CD3: 15,320 ± 14,988 cpm), which was suppressed up to 95% when equal numbers of Bet v 1-specific Tr1 cells were added (Teff alone: 15,320 ± 14,988 cpm; Teff + Tr1: 586 ± 846 cpm; Fig. 5A). This suppressive effect was directly related to the numbers of Bet v 1-specific Tr1 cells (Fig. 5A). Furthermore, when a mAb against IL-10 was added to these cultures, the suppressive effect of the Tr1 cells was almost completely abrogated (Teff alone: 4784 ± 3651 cpm; Teff + Tr1: 2478 ± 2501 cpm; Teff + Tr1 + anti–IL-10: 4423 ± 3434 cpm; n = 3; Fig. 5B).

Second, in vitro experiments were performed with Bet v 1-specific Teff cell lines generated from four individuals allergic to birch pollen (no Ag: 57 ± 19 cpm; Bet v 1: 19771 ± 4380 cpm). Upon coculture of Bet v 1-stimulated Teff cell lines with allergen-specific Tr1 cells, an almost complete suppression of allergen-driven Teff cell proliferation was noticed (Teff alone: 9771 ± 4380 cpm; Teff + Tr1: 468 ± 750 cpm; Fig. 5C). Again, Tr1 cell-induced suppression of Teff cells was dependent on the ratio of Tr1/Tr1 cells (Fig. 5C).

To exclude that Foxp3+ Treg cells contributed to the suppressive effect, we analyzed the expression of Foxp3 in the Bet v 1-specific Tr1 cells. In fact, when isolated Bet v 1-specific IL-10–secreting Tr1 cells were stained with a mAb against Foxp3, we could not detect Foxp3+ cells (Supplemental Fig. 3). Thus, SIT-induced Bet v 1-specific Tr1 cells confer Teff cell suppression in a cell number- and IL-10–dependent manner.

**FIGURE 2.** CD4+CD25+CD127low Treg cells are not altered by specific immunotherapy. Treg cells were detected by FACS analysis as a subpopulation of CD4+CD25+CD127low cells of total peripheral blood CD4+ lymphocytes.
Changes of allergen-specific humoral parameters during SIT

Finally, to assess relevant changes in the humoral response to birch pollen induced by SIT, allergen-specific serum Ig concentrations were measured by ImmunoCap. Allergen-specific IgE and IgA levels did not change significantly during the first 12 mo of SIT compared with pretreatment levels (Fig. 6A, 6B). In addition, there was no substantial difference between birch pollen-specific and Bet v 1-specific IgE concentrations in the individual patients (Table I). In contrast, allergen-specific IgG, and among these also IgG4, Abs, increased significantly after 3 mo (p = 0.002 and p = 0.007, respectively; Fig. 6C, 6D) and continued to increase during the first year of SIT (m6 and m12; p = 0.001; Supplemental Fig. 4). Likewise, the ratio of allergen-specific IgE/IgG as well as IgE/IgG4 was significantly decreased at the end of the induction phase (m3; p = 0.017 and p = 0.012, respectively) and diminished further during the maintenance phase of SIT (Fig. 6E, 6F).

In contrast to SIT-treated individuals, the amount of allergen-specific IgG and IgG4 of healthy controls and subjects allergic to birch pollen who were not on SIT remained unchanged when comparing values during birch pollen season to out of season values, as did the concentration of allergen-specific IgE and IgA Abs (data not shown).

Discussion

Herein we present a comprehensive analysis focusing on allergen-specific Th cells and distinct Treg cell subsets in a closely meshed study covering the first year of SIT, with emphasis on the induction and maintenance phase as well as on comparing the period during and out of birch pollen season. The present long-term study showed that SIT resulted in a differential induction of Treg cells with an early and prolonged increase of Bet v 1-specific Tr1 but not of CD4⁺CD25⁺CD127low Treg cells, it induced a delayed immune deviation toward a Bet v 1-specific Th1 response and, furthermore, it led to the prolonged production of allergen-specific IgG, particularly IgG4, Abs.

Treg cells can be generated in the thymus by high-avidity interactions or induced in the periphery by foreign Ags. Although the former are characterized by their obligatory expression of Foxp3 and, therefore, are called Foxp3⁺ natural Treg cells, the latter can be subdivided into inducible Foxp3⁺ and Foxp3⁻ Treg cells (31). Our observations suggest that SIT preferentially results in an allergen-induced Treg cell response characterized by increased numbers of Foxp3⁺ Bet v 1-specific Tr1 cells, whereas the frequency of Foxp3⁺ Treg cells remained unchanged [determined by the number of CD4⁺CD25⁺ CD127low Treg cells, which was recently described as a well-suited marker profile for Foxp3⁺ Treg cells (29, 30)]. Bet v 1-specific Tr1...
of SIT. In contrast, Francis et al. (24) observed increased amounts of pollen on SIT only during the induction phase but not after the first year. C abolishment by anti–IL-10 mAb.

The extent of inhibition is expressed as the percentage of Teff cell proliferation induced by autologous allergen-specific Teff cell lines. For each individual patient, the percentage proliferation of Teff cells in the presence of x-irradiated autologous PBMCs and anti-CD3. B, Tr1 cell-induced suppression of Teff cells is abolished by anti–IL-10 mAb. C, Coculture of Bet v 1-specific Tr1 cells and autologous allergen-specific Teff cell lines. For each individual patient, the extent of inhibition is expressed as the percentage of Teff cell proliferation after stimulation by anti-CD3 or Bet v 1 set as 100%. Bars depict mean percentage proliferation ± SD from three (A, B) or four (C) patients.

FIGURE 5. Suppressor activity of Bet v 1-specific Tr1 cells. A, Bet v 1-induced IL-10–secreting Tr1 cells were cocultured at different ratios with autologous CD4+CD25+ Teff cells in the presence of x-irradiated autologous PBMCs and anti-CD3. B, Tr1 cell-induced suppression of Teff cells is abolished by anti–IL-10 mAb. C, Coculture of Bet v 1-specific Tr1 cells and autologous allergen-specific Teff cell lines. For each individual patient, the extent of inhibition is expressed as the percentage of Teff cell proliferation after stimulation by anti-CD3 or Bet v 1 set as 100%. Bars depict mean percentage proliferation ± SD from three (A, B) or four (C) patients.

Cells showed a pronounced suppressor effect on allergen-specific Teff cells mediated by the secretion of IL-10, which is in line with previous studies showing IL-10–dependent inhibition of allergen- or CD28-induced T cell proliferation (23, 33, 35). It is generally agreed that allergen-specific Tr1 cells are key regulators of SIT-induced allergen tolerance (23, 24), which suppress the production of allergen-promoting cytokines, such as IL-4, -5, and -13 (33), and induce a state of allergen-specific anergy (43). Although the early induction of allergen-specific, IL-10–secreting cells after 3 mo of SIT (induction phase) has been reported (25, 33, 35), data on their persistence during the maintenance phase are rare and inconsistent. Bohle et al. (35) reported enhanced IL-10–secreting CD3+ T cells in patients allergic to birch pollen on SIT only during the induction phase but not after the first year of SIT. In contrast, Francis et al. (24) observed increased amounts of IL-10 secreted by PBMCs from patients after the first year of SIT. This discrepancy may be explained by different treatment protocols (i.e., sublingual versus s.c. SIT). In the present study of patients allergic to birch pollen on s.c. SIT, Bet v 1-specific Tr1 cells were continuously increased from the end of the induction phase throughout the first year of SIT compared with pretreatment values, although the numbers started to decrease after 12 mo of SIT. More extended observation periods should help to determine the long-term influence of allergen-specific Tr1 cells on the outcome and sustained efficacy of SIT, which is generally performed over 3 y.

To our knowledge, studies investigating CD4+CD25+Foxp3+ Treg cells in the peripheral blood of patients on allergen-specific SIT have not been performed. Unlike Tr1 cells, CD4+CD25+ Foxp3+ Treg cells are thought to suppress Teff cells in a cell contact-dependent manner (44). Because they are not characterized by a distinct profile of cytokine secretion, these Treg cells are best identified based on membrane-bound and intracellular markers, such as the expression of CD25 and Foxp3, respectively (45–47). Foxp3+ Treg cells can develop in the thymus or arise as adaptive Treg cells from the conversion of naive conventional CD4+ T cells in the periphery (48). Radulovic et al. (49) reported a significant increase in CD25+Foxp3+ cells during grass pollen season in the nasal mucosa of patients allergic to grass pollen after 2 years of SIT by immunohistochemical analysis. In contrast, we could not detect changes in the percentage of CD4+CD25+Foxp3+ Treg cells in the peripheral blood during the first year of SIT. This suggests differences in Treg cell subset distribution (peripheral blood versus effector organ) or a delayed recruitment of Foxp3+ Treg cells during the later phases of SIT. However, because other studies demonstrated that allergic patients show a diminished capacity of CD4+CD25+ Treg cells to suppress allergy-promoting Th2 cell proliferation and cytokine production than reduced cell numbers (50–52), SIT still could have an impact on the suppressive activity of Foxp3+ Treg cells.

Bet v 1-specific Th2 cell responses remained largely unaffected during the first 12 mo of SIT, in contrast to other studies that reported a decrease in allergen-specific Th2 cells (53, 54). In light of the prevailing reduction in the Bet v 1-specific Th2/Tr1 cell ratio by the end of the induction phase, the balance between allergen-specific Th2 cells and Tr1 cells, rather than the absolute number of pathogenic Th2 cells, seems to be critical for the development of allergen tolerance (23). This is also supported by the observation that SIT-treated patients who were allergic to birch pollen experienced a significant relief in symptoms during birch pollen season; a temporary increase in allergen-specific Th2 cells was accompanied by a greater increase in Bet v 1-specific Tr1 cells. This finding may explain why the clinical improvement of patients on SIT occurs despite enhanced Th2 cytokine responses during birch pollen season (55).

Although no significant alteration in Bet v 1-specific Th1 cells was evident, a trend to increased Th1 cell numbers was found after 12 mo of SIT. In vitro data showed that high allergen concentrations, as applied in the maintenance phase of SIT, led to elevated IFNγ+CD4+ T cell numbers and the induction of enhanced IL-4+ T cell apoptosis accompanied by T cell anergy (56). Thus, increased frequencies of allergen-specific Th1 cells may only become apparent at later time points of SIT, upon reaching higher cumulative doses of applied allergen. Of note, a significant decline in the allergen-specific Th2/Th1 cell ratio became apparent by the end of the first year of SIT, indicating a shift from an initial Th2 cell-dominated immune response toward a more pronounced Th1 cell immune response in the later phase of SIT. Other studies reported increased IFN-γ and reduced IL-4 mRNA expression by peripheral T cells after the first year of SIT (35). In addition, during the maintenance phase of SIT, a significant reduction in the IL-4/IFN-γ ratio was found compared...
with the induction phase in subjects who clinically responded to SIT (57), supporting the concept that the allergen-specific Th2 cell response is balanced by SIT-induced activation of Th1 cells (53, 58). Furthermore, recent analysis of T cell responses to Bet v 1 with MHC class II peptide tetramer staining showed mainly IFN-γ–producing Th1 cells and only a small amount of IL-10–producing Treg cells in healthy individuals (59), suggesting that the balance between allergen-specific Th2 and Th1 cells also plays an important role in natural allergen tolerance in healthy individuals. In addition to changes among distinct T cell subsets, humoral alterations were noticed during SIT, which led to an early increase of allergen-specific IgG Abs, with significantly enhanced concentrations of Bet v 1-specific IgG4. Similar changes were described in studies analyzing Ab responses during SIT (60–63). Although we did not evaluate other IgG isotypes in this study, these might also be affected by SIT. For example, a strong induction of allergen-specific IgG1 and, to a lesser degree, IgG2 was recently reported in patients successfully treated by SIT (64, 65). It is assumed that allergen-specific IgG Abs

**FIGURE 6.** SIT results in increased serum concentrations of allergen-specific IgG Abs. Birch pollen-specific IgE (A), IgA (B), IgG (C), and IgG4 (D) Ab concentrations before (m0) and during the first 12 mo (m12) of SIT. Ratio of allergen-specific IgE/IgG (E) and IgE/IgG4 (F) over the 12-mo observation period.
might function as blocking Abs, playing an important role in establishing allergen tolerance (i.e. by inhibiting IgE-mediated release of inflammatory mediators from mast cells and basophils or by preventing IgE-facilitated allergen presentation to T cells) (66, 67). Because SIT with airborne allergens does not induce significant variations in IgG avidity (68–70), the blocking activity of IgG is presumably a quantititative function, which, at best, is reflected by the observed decrease in the serum IgE/IgG4 and IgE/IgG ratio in patients on SIT (71, 72). Furthermore, it seems that the blocking activity is related to the epitope specificity rather than to the isotype. For example, it was shown that allergen-specific IgG1 mAbs were able to inhibit the binding of IgE to Bet v 1, whereas allergen-specific IgG4 mAbs reacting with a different epitope enhanced the IgE binding (73). Because allergen-specific IgG Abs can also be found in sufficient amounts in mucosal secretions (74), they may also function as a blocking factor in the effector organs of patients suffering from allergic respiratory diseases and treated by SIT. Different results have been reported regarding the effect of SIT on allergen-specific IgA serum Abs. Similar to a former study (65), we could not detect significant changes in allergen-specific IgA, whereas other investigators detected increased serum titers during SIT (34, 75).

Although the main focus of our study was the effect of SIT on T cell subsets, an interesting question is how the observed early increase in Bet v 1-specific, IL-10–secreting Tr1 cells contributes to clinical allergen tolerance in the patients allergic to birch pollen who still experienced a strong increase in allergen-specific Th2 cells during birch pollen season. Tr1 cells suppress Teff cells in an IL-10–dependent manner; however, they might also act on other important cell types orchestrating the immediate-type allergic immune response. For example, in vitro experiments showed that IL-10 inhibits the IgE-induced activation of human mast cells, with the subsequent reduced release of histamine and other inflammatory cytokines (76). Furthermore, Tr1 cells could promote the production of allergen-specific blocking IgG Abs by B cells. Recent studies demonstrated the impact of allergen-specific Foxp3+ Tr1 cells and CD4+CD25+Foxp3+ Treg cells on B cells, leading to the induction of IgG4 through cell contact- and cytokine-dependent mechanisms (77, 78). In this context, although IL-10 is not a switch factor for IgG4, it was shown that IL-10 can increase the synthesis of IgG4 by potentiating IL-4–induced IgG4 switching (79). Thus, the early development of Tr1 cells during SIT might prevent a hypersensitive immune response among allergen contact by pleiotropic effects. Future studies should shed more light on these aspects, as well as on the long-term function of Treg cells in establishing allergen tolerance. Of note, at the end of the first year of SIT, Tr1 cells were not as markedly increased as during the induction phase, similar to what was reported by Bohle et al. (35), whereas allergen-specific IgG4 Abs continued to be strongly enhanced. Although the latter may be due to the existence of long-lived plasma cells (80), it could also point to other control mechanisms independent of the persistent presence of Tr1 cells.

In summary, this study showed that immune regulatory changes elicited by birch pollen SIT are characterized by the differential induction of Treg cells, accompanied by the production of allergen-specific IgG and a delayed Th2/Th1 cell immune deviation. Further studies are needed to better understand the role that the different Treg subsets play in achieving and maintaining immune tolerance, with a focus on the mechanisms that regulate their activation and functional properties.

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