ORIGINAL ARTICLE

Leukocyte Phenotype Changes Induced by Specific Immunotherapy in Patients With Birch Allergy

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Abstract

Background: The underlying mechanisms of allergen-specific immunotherapy (SIT) are not fully understood.
Objectives: The present study aimed to investigate how leukocyte phenotypes are affected by SIT.
Methods: Blood samples were taken from 10 patients with birch pollen–induced allergic rhinitis before, during, and immediately after SIT. Further samples were obtained after 1 year and 3 years. All samples were analyzed by flow cytometry and leukocyte differentiation.
Results: SIT caused a decrease in cell-bound immunoglobulin (Ig) E on granulocytes, along with a corresponding increase in the high-affinity IgG receptor. Accordingly, a lower level of allergen-specific IgE was found after 3 years. The treatment induced a decrease in neutrophil CD11b levels, a shift in monocyte subsets, and an increase in the number of activated T lymphocytes, manifested as an upregulation of CD69 and CD98, and an expansion of the $CD4^+CD25^+$ T-cell pool.
Conclusion: The present study shows that the clinical effects of SIT are mirrored by systemic changes in cellular events and in antibodies, and offers new targets for immunomodulation.

Key words: Allergic rhinitis. Flow cytometry. IgE. Specific immunotherapy. Regulatory T cells.

Resumen

Antecedentes: Los mecanismos subyacentes de la inmunoterapia específica con alérgenos (ITE) no se conocen completamente.
Objetivos: El objetivo de este estudio fue investigar el efecto de la ITE sobre los fenotipos leucocitarios.
Métodos: Se obtuvieron muestras de sangre de 10 pacientes con rinitis alérgica inducida por polen de abedul antes, durante e inmediatamente después de la ITE. Se obtuvieron más muestras al cabo de uno y tres años. Todas las muestras se analizaron mediante citometría de flujo para la diferenciación leucocitaria.
Resultados: La ITE causó una disminución de IgE unida en granulocitos, además del aumento correspondiente del receptor de alta afinidad para la IgG. Asimismo, se observó un menor nivel de IgE específica del alérgeno al cabo de tres años. El tratamiento produjo una reducción del nivel de CD11b en neutrófilos, un cambio en los subconjuntos de monocitos y un aumento del número de linfocitos T activados, que se manifestó como un incremento de CD69 y CD98 y una expansión del conjunto de linfocitos T CD4+CD25+.
Conclusión: Este estudio demuestra que los efectos clínicos de la ITE se reflejan en cambios sistémicos en los acontecimientos celulares y los anticuerpos, y ofrece nuevas dianas para la inmunomodulación.

Palabras clave: Rinitis alérgica. Citometría de flujo. IgE. Inmunoterapia específica. Linfocitos T reguladores.

Introduction

Allergen-specific immunotherapy (SIT) is an alternative therapeutic approach for carefully selected patients with immunoglobulin (Ig) E–mediated allergic diseases, such as allergic rhinoconjunctivitis and asthma [1,2]. It induces long-term remission following discontinuation, prevents onset of new sensitization, and helps to prevent the development of asthma in children with allergic rhinitis [2-5]. Even though the clinical improvements observed during SIT are well documented, the mechanisms underlying its beneficial effects are still not fully understood [6]. Previously reported effects include suppressed infiltration of effector cells (type 2 helper T [T_H2] cells, eosinophils, basophils, and neutrophils), reduced serum IgE levels in favor of an increase in inhibitory allergen-specific IgG1 and IgG4 antibodies, a shift from a T_H2-biased response towards a T_H1-biased response, and induction of interleukin (IL) 10 and transforming growth factor ß producing $CD4^+CD25^+$ regulatory T cells (Tregs) [1,3,7]. The present-study aimed to further characterize SIT-induced short-term and long-term changes in leukocyte phenotypes by following a group of patients with birch pollen–induced allergic rhinitis over a 3-year period.

Methods

Patients

Ten patients (6 men, 4 women) suffering from severe allergic rhinoconjunctivitis, with or without seasonal asthma, were included in the study. Median age was 36 years (range, 26-46 y). All patients had a minimum 2-year history of birch pollen–induced rhinoconjunctivitis confirmed by a positive skin prick test result (>3 mm) and a median specific IgE value of 13.7 kU_A/L (range, 1.9-41.9 kU_A/L). They had all experienced severe symptoms during previous pollen seasons (itchy nose and eyes, sneezing, rhinorrhea, and rhinitis). Blood samples were taken outside the pollen season, and all patients were free of infections at the time of the study. Patients were asked to record how they experienced symptoms during the pollen season compared with before SIT using an arbitrary scale of 0 to 10, with 0 representing no improvement and 10 representing complete recovery. They were also asked to record whether their total use of medication during the season—including oral antihistamines, intranasal corticosteroids, eye drops, inhaled $ß_2$-agonists and corticosteroids, as well as combinations thereof—had changed compared with before the start of SIT. The study was approved by the local ethics committee and all patients provided their written informed consent.

Immunotherapy Protocol and Blood Sampling

A standardized, aluminum hydroxide-adsorbed, depot birch-pollen vaccine (Alutard, ALK Abelló, Hørsholm, Denmark) was administered subcutaneously. The patients received incremental doses of allergen over 3 consecutive days, reaching the maintenance dose (100,000 SQ-U) on day 3 (day 1 with 100, 1 000, and 5 000 SQ-U; day 2 with 10 000 and 20 000 SQ-U; day 3 with 40 000 and 60 000 SQ-U). Thereafter, maintenance injections were given regularly every 6-8 weeks for 3 years. The fast updosing used here is usually referred to as rush immunotherapy [8]. Blood samples were taken before and during updosing, and at 12 weeks, 1 year, and 3 years after the start of treatment. The samples were obtained before the injection of allergen. Tubes containing EDTA (Vacuette 454209, Greiner Bio-One, Frickenhausen, Germany) were used for analyzing total leukocyte differential counts on a Coulter LH750/GenS cell counter (Beckman Coulter, Marseille, France), and tubes containing buffered tri-sodium citrate solution (BD Vacutainer 367704, Becton Dickinson, Franklin Lakes, New Jersey, USA) were used for flow cytometry analysis.

FACS Analysis

Flow cytometry was performed using a Coulter Epics XL flow cytometer and Expo32 ADC analysis software (Beckman Coulter). A total of 30 000 events were collected. To ensure standardization of flow cytometry, the voltage settings were updated daily to the target channels using FlowSet calibration beads (Beckman Coulter). All antibodies were titrated before use, and staining intensity was controlled in healthy blood on a weekly basis. For detection of cell surface markers, 50 µL blood was incubated with antibodies for 15 minutes at room temperature. Erythrocytes were lysed by mixing with 600 µL of 0.1% (v/v) formic acid for 3-4 seconds. Ionic strength

Table. Antibody Panels Used for Flow Cytometry Analysis

Antibody Panel	FL-1 (FITC)	FL-2 (PE)	FL-3 (ECD)	FL-4 (PCy5)
I. IgE on granulocytes	IgE	CRTH2	CD45	CD16
II. Granulopoiesis	CD11b	CD16	CD45	CD64
III. Monocyte subsets	CD14	CD16	CD45	CD64
IV. T-cell activation	CD98	CD69	CD45	CD2
V. T cells	CD71	CRTH2	CD45	CD2
VI. Regulatory T cells	CD71	CD25	CD45	CD4

Abbreviation: CRTH2, chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes; Ig, immunoglobulin.

was rendered iso-osmotic by addition of 280 µL of 51 mM Na_2CO_3, 0.20 M Na_2SO_4, and 0.22 M NaCl. Cells were then washed in phosphate-buffered saline (PBS) and resuspended in 1% formaldehyde (in PBS) prior to analysis. Leukocytes were separated into monocytes, lymphocytes, basophils, eosinophils, and neutrophils by gating intact leukocytes based on their forward scatter and side scatter properties as well as by CD16 and CD45 staining. Basophils were further classified as positive for the chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTH2).

Antibodies

The mouse monoclonal antibodies (mAbs) used for FACS analysis were as follows: CD45-ECD (clone J.33), CD11b-FITC (Bear1), CD16-PCy5 (3G8), CD4-PC5 (13B8.2), CD69-PE (TP1.55.3), CD2-PC5 (39C1.5), and CRTH2-PE (BM16) obtained from Immunotech (Beckman Coulter); CD16-PE (DJ130c), CD14-FITC (TÜK4), and CD64-PECy5 (10.1) from DakoCytomation (Copenhagen, Denmark); and CD71-FITC (YDJ1.2.2), CD25-PE (B1.49.9), and CD98-FITC (44D7) from Serotec (Oxford, UK). Goat anti-human IgE-FITC was obtained from Caltag Laboratories (Burlingame, California, USA). The different antibody combinations used for flow cytometry are presented in the Table.

Statistical Analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). All data are expressed as median and range. Nonparametric data were analyzed for statistical differences using the Mann-Whitney test. *P* values <.05 were considered statistically significant.

Results

Clinical Evaluation of the Immunotherapy

One patient was excluded due to a severe allergic reaction during the updosing phase. The remaining 9 patients tolerated the treatment well. Their rhinoconjunctivitis symptoms improved and their medication usage decreased over time. Using an arbitrary scale of 0 to 10, a slight improvement was seen during the first year (3.7 [0.6]). This change was further emphasized during the second year (6.4 [0.5], $P<.01$) and third year (6.7 [0.4], $P<.001$). The clinical improvements were accompanied by a reduction in serum allergen-specific IgE levels 3 years after initiation of SIT (9.8 [2.4] vs. 16.2 [4.2] kU_A/L; $P<.05$).

Total Leukocyte Count and Cell Distribution

The total number of leukocytes and the individual numbers of neutrophils, basophils, monocytes, and lymphocytes did not change during treatment. An increase in eosinophil count was seen during the initial phase, although cell counts had returned to the baseline level 12 weeks after reaching the maintenance dose (Figure 1, A-E).

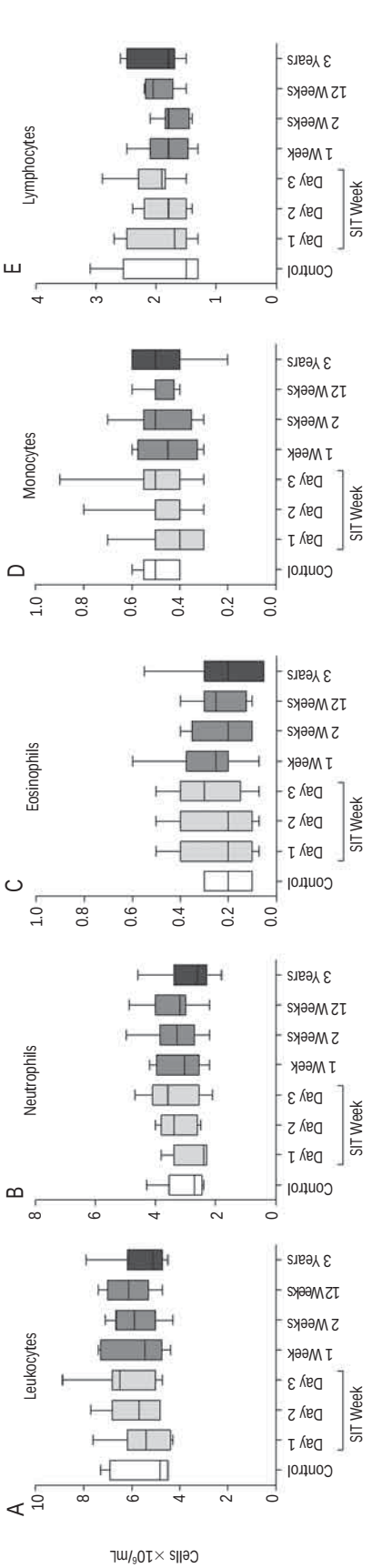

Figure 1. Number of (A) total leukocytes, (B) neutrophils, (C) eosinophils, (D) monocytes, and (E) lymphocytes in blood before (control), during, and 3 years after SIT. Data are presented as median and range (n=9). SIT indicates specific immunotherapy.

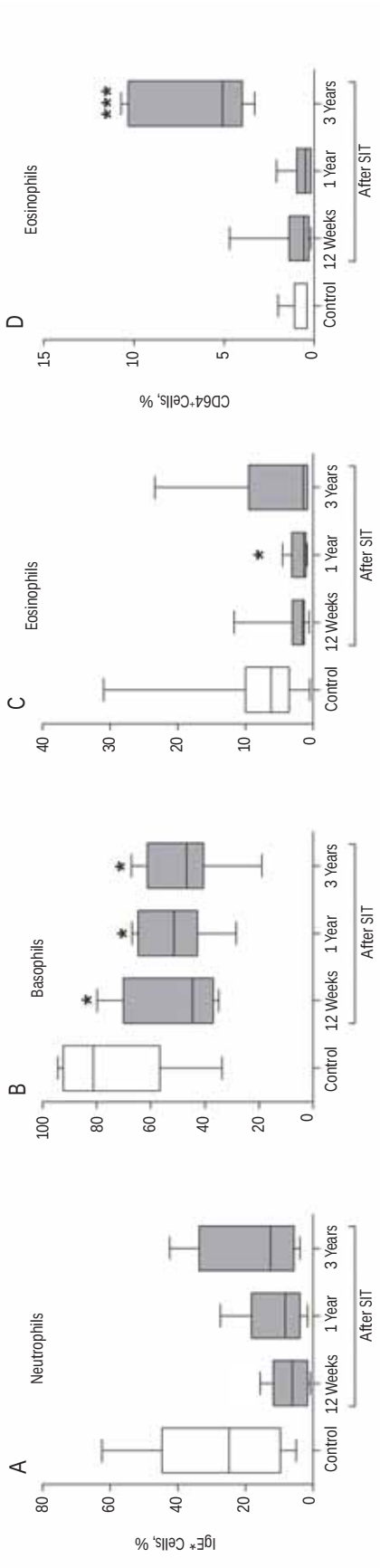

Figure 2. Expression of membrane-bound IgE on (A) neutrophils, (B) basophils, and (C) eosinophils, and (D) CD64 on eosinophils. Peripheral blood was obtained from patients prior to (control) as well as 12 weeks, 1 year, and 3 years after SIT before being stained with antibodies against IgE or CD64 and analyzed by flow cytometry. Data are presented as median and range (n=9)

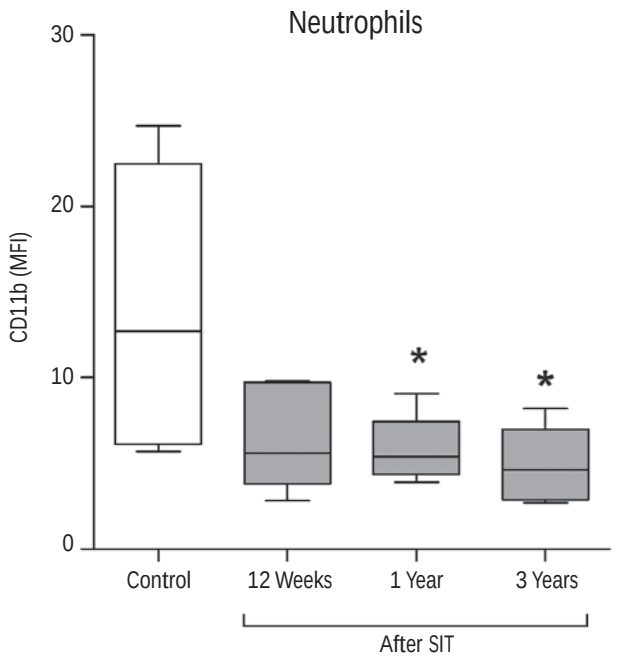

Figure 3. Expression of CD11b on neutrophils from peripheral blood obtained from patients before (control) and 12 weeks, 1 year, and 3 years after SIT. Cells were stained with antibodies against CD11b and analyzed by flow cytometry. Data are presented as MFI. Data are presented as median and range (n=9; *, $P<.05$). MFI indicates mean fluorescence intensity; SIT, specific immunotherapy.

Decrease in Cell-Bound IgE and Increase in High-Affinity IgG Receptor Expression

In the first set of experiments, the effects of SIT on cell-bound IgE levels and expression of the high-affinity IgG receptor was investigated. Eosinophils, neutrophils, and basophils, separated by their expression of CD45, CD16, and CRTH2, respectively (Table, panel I), all exhibited membrane-bound IgE. The percentage of IgE-positive cells decreased for all cell types as a result of SIT (Figure 2, A-C). In addition, the expression of CD64, the high affinity IgG receptor (FcγRI), was examined on neutrophil and eosinophil granulocytes (Table, panel II) and monocytes (Table, panel III). FcγRI was expressed on eosinophils and monocytes, and an increased proportion of $CD64^+$ cells could be distinguished on eosinophils 12 weeks and 3 years after SIT (Figure 2D). A trend towards upregulation was also observed in monocytes, although this did not reach statistical significance (P=.08).

Downregulation of CD11b on Neutrophils

Next, we explored expression of the adhesion molecule CD11b (Table, panel II), which is involved in cellular migration and activation [9]. CD11b was found on neutrophil and eosinophil granulocytes. CD11b staining on eosinophils remained unchanged throughout the study (data not shown), whereas the mean fluorescence intensity (MFI) of CD11b decreased on neutrophils (Figure 3).

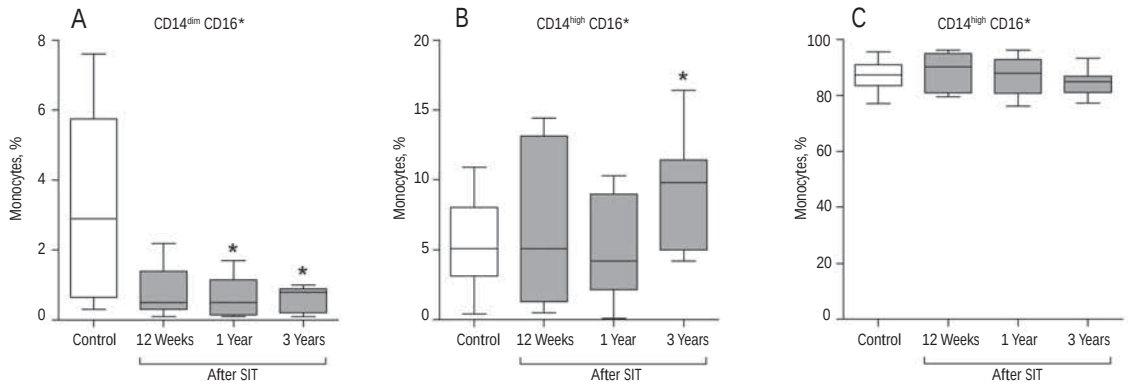

Figure 4. Composition of monocyte subsets in blood obtained before (control) and 12 weeks, 1 year and 3 years after SIT. Cells were stained with antibodies against CD14 and CD16 and analyzed by flow cytometry. The percentage of (A) $CD14^{dim}CD16^{+}$, (B) $CD14^{high}CD16^{+}$, and (C) $CD14^{high}CD16^{-}$ monocytes. Data are presented as median and range (n=9; *, $P<.05$). SIT indicated specific immunotherapy.

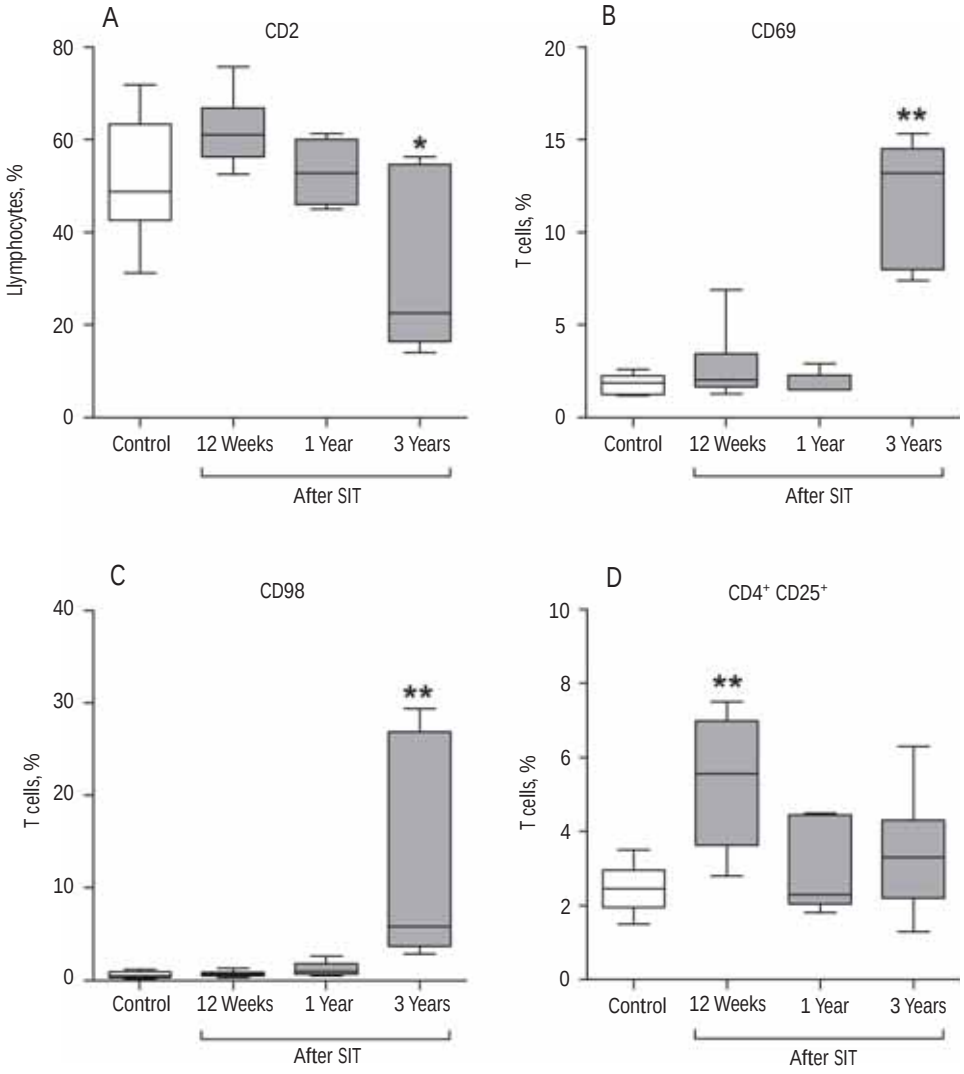

Figure 5. (A) Total number of $CD2^{+}$ T lymphocytes. Activation of $CD2^{+}$ T cells was investigated by staining with antibodies against (B) CD69 and (C) CD98. (D) Number of $CD4^{+}CD25^{+}$ T cells. Blood samples, obtained before (control) and 12 weeks, 1 year, and 3 years after SIT, were stained with the various antibodies and analyzed by flow cytometry. Data are presented as median and range (n=9; *, $P<.05$; **, $P<.01$). SIT indicates specific immunotherapy.

Shift in Monocyte Populations

Based on the expression of CD14 and CD16, we were able to identify 3 different monocyte subsets in peripheral blood: $CD14^{high}CD16^{-}$, $CD14^{high}CD16^{+}$, and $CD14^{dim}CD16^{+}$ cells. By comparing the composition of monocyte subsets prior to and after SIT treatment (Table, panel III), we observed a stable reduction in $CD14^{dim}CD16^{+}$ monocytes 12 weeks, 1 year, and 3 years after SIT (Figure 4A). In addition, the $CD14^{high}CD16^{+}$ monocyte subset was higher 3 years after SIT (Figure 4B). $CD14^{high}CD16^{-}$ cells were found to be the predominant monocyte subset in blood, although the level remained unchanged during treatment (Figure 4C). They were also the only subset that expressed notable levels of FcγRI (CD64) (data not shown).

Induction of T-Cell Activation

Lastly, the effect of SIT on T-cell differentiation and activation was explored (Table, panels IV-VI). Fewer $CD2^{+}$ T lymphocytes were seen 3 years after treatment started (Figure 5A). However, treatment affected neither the percentage of $CD4^{+}$ helper T cells nor the proportion of CRTH2-positive T_H2 cells (data not shown). CD69, an early activation marker [10], and CD98, which is strongly expressed on activated and proliferating T lymphocytes [11,12], were upregulated on $CD2^{+}$ T cells after 3 years (Figure 5, B and C). No significant effect was seen on CD71, a transferrin receptor known to be upregulated on proliferating cells [13] (data not shown). Finally, the proportion of $CD4^{+}CD25^{+}$ T cells was higher 12 weeks after updosing, although their numbers fell back towards baseline in samples obtained after 1 and 3 years (Figure 5D).

Discussion

Allergen immunotherapy has been administered for nearly a century and has proven successful in the treatment of hypersensitivity to a range of allergens, including birch and grass pollen, house dust mite, insect venom, and animal dander [1]. However, the mechanisms behind its protective effects are not fully characterized. In this study, the short-term and long-term effects of SIT were analyzed by following a group of birch-allergic patients for 3 years. We showed that patients given SIT experienced lasting reductions in symptom severity and medication requirements during the pollen season following the start of treatment. Reduced serum IgE levels were observed along with these clinical improvements. By investigating the effects of SIT on leukocyte numbers and phenotypes, we show that the number of eosinophils increased. In addition, SIT induces a decrease in cell-bound IgE on granulocytes and a simultaneous increase in the high-affinity IgG receptor on eosinophils, a decrease in neutrophil CD11b levels, and a shift in monocyte populations in favor of $CD14^{high}CD16^{+}$ cells. We also report an increase in activated T lymphocytes, manifested as upregulation of CD69 and CD98 and expansion of the $CD4^{+}$ $CD25^{+}$ T-cell pool.

The level of peripheral blood eosinophils increased during the first week of SIT, but returned towards baseline after 12 weeks. This observation is consistent with the results of a previous study reporting no differences in eosinophil counts before and 1 year after immunotherapy [14]. In addition, both serum IgE levels and cell-bound IgE on eosinophils, basophils, and neutrophils decreased as a result of treatment. Previous studies have demonstrated an initial rise in serum allergen-specific IgE followed by a slow decline during the course of SIT [15,16]. Along with the reduced IgE levels, titers of allergen-specific IgG—mainly the IgG1 and IgG4 isotypes—are known to increase during treatment. Antibodies belonging to the latter isotype are believed to function as blocking antibodies, since they have the ability to bind and neutralize the allergen before it interacts with the cell-bound IgE [1,5]. We also demonstrated an increase in FcγRI expression on eosinophils and monocytes. Other studies have reported a SIT-induced reduction in levels of the soluble low-affinity IgE receptor (FcεRII), which is important in regulating IgE production [17,18]. Thus, the decrease in cell-bound IgE we observed might be a consequence of the lower level of IgE antibodies present in serum. The reduced level of soluble FcεRII in favor of higher levels of the high-affinity IgG receptor FcεRI could also play a role.

Expression of CD11b was downregulated on neutrophils after SIT. CD11b is a membrane-bound glycoprotein that is involved in neutrophil migration and activation. It is upregulated following neutrophil activation and plays a pivotal role in the capture and rolling of cells [9]. We previously reported that migration of neutrophils from the blood into the nasal mucosa increases during the grass pollen season. In the same study, we found a seasonal increase in neutrophil CD11b expression [19]. This finding, together with those of other reports, suggests the direct involvement of neutrophils and neutrophil-related products in the allergic reaction [20,21]. The decreased expression of CD11b following SIT might reflect a lower degree of neutrophil activation. Hence, the cells might be less disposed to migrate from the bloodstream into the nasal mucosa to participate in the inflammatory process.

It has been proposed that the monocyte pool in blood consists of 3 heterogeneous cell subsets with a different morphology, phenotype, and function: $CD14^{high}CD16^{-}$, $CD14^{high}CD16^{+}$, and $CD14^{dim}CD16^{+}$ [22,23]. The data we present indicate that SIT has the ability to change the association between the different monocyte subsets, which is manifested primarily by inducing $CD14^{high}CD16^{+}$ and repressing $CD14^{dim}CD16^{+}$ cells. A recently published study has shown that $CD14^{high}CD16^{+}$ cells are the main monocytes responsible for the production of IL-10 [24]. IL-10 has several important anti-inflammatory properties, including inhibition of T-cell cytokine production and activation of mast cells and eosinophils [25,26]. Furthermore, this monocyte subset was found to be the only subset expressing the high-affinity IgG receptor, which tended to increase as a result of SIT. Furthermore, $CD14^{dim}CD16^{+}$ cells are known to expand during infection and inflammatory responses [27,28]. Consequently, the reduction in airway inflammation attained during SIT might explain the decrease in $CD14^{dim}CD16^{+}$ cell numbers. Novak et al [29] support this finding by demonstrating a decrease in the percentage of $CD14^{dim}CD16^{+}CD64^{-}$ cells in patients suffering

from the allergic form of atopic eczema/dermatitis syndrome after, as compared to before, topical treatment.

In the present study, we investigated the effect of SIT on T-cell subsets and activation. Treatment did not affect the number of $CD4^+$ cells or $CD4^+CRTH2^+$ cells. However, SIT was found to induce T-cell activation, seen as an increase in CD69 and CD98 expression along with an increased proportion of $CD4^+CD25^+$ T cells. The antigen CD69 is expressed very early after T-cell activation and has been associated with the production of TH1 cytokines [10,30]. CD98 is also expressed on activated and proliferating T cells. However, its cellular function is currently not fully understood [31]. The increased number of peripheral $CD4^+CD25^+$ T cells in patients undergoing immunotherapy is supported by the findings of previous studies [32,33]. These cells might be part of the Treg pool, comprising naturally occurring, thymic-derived $CD4^+CD25^+Foxp3^+$ T cells, inducible $CD4^+CD25^+$ T cells, IL-10–producing Tregs, and transforming growth factor ß–producing Tregs, all with immunosuppressive properties [34]. Francis et al [7] have demonstrated that patients undergoing immunotherapy have higher numbers of $CD4^+CD25^+$ T cells in peripheral blood mononuclear cells than untreated atopic patients or healthy controls, and that this population is the main producer of allergen-induced IL-10 [35], known to modulate the function of effector cells involved in the allergic immune response [32]. Other authors have reported that, although the $CD4^+CD25^+$ T-cell response is impaired in allergic disease, it can be boosted by SIT [36,37]. In contrast, a recently published study showed that patients receiving SIT and birch pollen–allergic controls have similar levels of total $CD25^+$ Tregs in the bloodstream during the pollen season [38]. Nevertheless, there appears to be a positive correlation between $CD4^+CD25^+$ T cells and the outcome and effectiveness of SIT. Even though the exact phenotype of the cells identified in the present study is unknown, the different Treg subsets are indeed enriched within the $CD4^+CD25^+$ T-cell pool.

Although the present study is based on a relatively small group of patients, several interesting findings both confirm and complement those of previous studies. First, the reduction in IgE titers in favor of IgG during immunotherapy is further corroborated by serum and receptor levels. Second, the present findings support a role for $CD4^+CD25^+$ T cells in the protective effects induced by SIT. Lastly, we extend current knowledge on the mechanisms behind SIT by proposing a shift in monocyte subsets as a novel event.

The immunological mechanisms behind allergen immunotherapy are not fully understood. Our results confirm and extend current knowledge on the cellular events that might be linked to the protective effects of allergen immunotherapy, thereby offering new targets for immunomodulation.

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