

Human Leukocyte Antigen-G and Regulatory T Cells during Specific Immunotherapy for Pollen Allergy

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Key Words

Allergy · Human leukocyte antigen-G · Regulatory T cells · Immunotherapy

Abstract

Background: T_H2-biased immune responses are important in allergy pathogenesis. Mechanisms of allergen-specific immunotherapy (SIT) might include the induction of regulatory T cells (Tregs) and immunoglobulin (Ig) G₄ blocking antibodies, a reduction in the number of effector cells, and skewing of the cytokine profile towards a T_H1-polarized immune response. We investigated the effects of SIT on T cells, on immunomodulation of human leukocyte antigen (HLA)-G, which has been associated with allergy, on regulatory cytokine expression, and on serum allergen-specific antibody subclasses (IgE and IgG₄). **Methods:** Eleven birch and/or grass pollen-allergic patients and 10 healthy nonatopic controls were studied before and during SIT. Tregs, chemokine receptors, soluble HLA-G (sHLA-G), Ig-like transcript (ILT) 2, specific IgE, and IgG₄ were studied. Peripheral blood mononuclear cells (PBMCs) were stimulated with pollen extract in vitro and immune factors were evaluated. **Results:** During

SIT, the main changes in the peripheral blood were an increase in CXCR3⁺CD4⁺CD25⁺CD127^{low/-} Tregs and a decrease in CCR4⁺CD4⁺CD25⁺CD127^{low/-} Tregs, an increase in allergen-specific IgG₄, and a decrease in sHLA-G during the first half of the treatment period. In the PBMC in vitro experiments, the following changes were observed upon allergen-stimulation: an increase in CD4⁺CD25⁺CD127^{low/-} Tregs and ILT2⁺CD4⁺CD25⁺CD127^{low/-} Tregs, an increase in IL-10 and IL-2 levels, and an increase in sHLA-G that was most pronounced at the start of SIT. **Conclusions:** The changes in CXCR3⁺CD4⁺CD25⁺CD127^{low/-} Treg, IgG₄, and sHLA-G levels in the peripheral blood and in ILT2⁺ Treg, IL-10, IL-2, and sHLA-G levels upon in vitro allergen stimulation suggest an upregulation in immunomodulatory factors and, to some degree, a shift towards T_H1 during SIT.

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Introduction

Allergies are estimated to affect approximately 20–30% of the global population, and the prevalence of allergic disease is increasing [1, 2]. Type 1 allergy is char-

acterized by inappropriate immune responses towards harmless antigens such as pollen and is mediated by allergen-specific immunoglobulin (Ig) E antibodies [3]. Furthermore, T helper (T_H) 2 cells and inflammatory cytokines such as interleukin (IL)-4, IL-5, and IL-13 play important roles in allergy pathogenesis [4]. An imbalance between T_H 1- and T_H 2-mediated immune responses, the so-called ' T_H 2 polarization', has been postulated to be one of the underlying pathogenic mechanisms in IgE-mediated allergy [3].

Recent studies of regulatory T cells (Tregs) in allergy have shown that these cells can skew T_H 2 responses towards a more cell-mediated T_H 1-response [5], and defective or diminished Treg activity has been associated with allergy [6]. One proposed mechanism of action is the production of the immunoregulatory cytokine IL-10 [7, 8]. Furthermore, Tregs have been suggested to suppress allergic effector cells such as mast cells and basophils, as well as the production of allergen-specific IgE from B cells, while promoting the production of allergen-specific IgG₄ to function as a blocking antibody [4]. However, results from other studies suggest opposite effects of Tregs with regard to allergy and related treatment [9, 10].

Two main classes of CD4⁺ Tregs can be identified based on the site of development. Natural CD4⁺ Tregs (nTregs) develop in the thymus and are released into the peripheral lymphoid tissues after positive thymic selection, while inducible/adaptive CD4⁺ Tregs (iTregs) develop from mature, naive CD4⁺ T cells in the periphery after antigen-specific stimulation [11]. An important regulator of Treg development and function is the transcription factor Forkhead box p3 (Foxp3). Based on this intracellular marker, Tregs can be further subdivided into Foxp3-expressing or non-Foxp3-expressing Tregs [12, 13].

Different T cell subsets (e.g. T_H 1 and T_H 2 cells) express different chemokine receptors. C-C chemokine receptor type 4 (CCR4) is preferentially expressed on T_H 2 lymphocytes and CD4⁺CD25⁺ T cells [14]. Studies have shown that CCR4 is involved in the antigen-specific trafficking of T_H 2 cells and in T_H 2-polarized allergic immune responses [15, 16]. However, there are conflicting views regarding the role of CCR4 in allergic diseases [9, 17]. Allergic individuals express lower levels of the T_H 1-chemokine receptor chemokine (C-X-C) motif receptor 3 (CXCR3). Furthermore, the number of CD4⁺CXCR3⁺ cells was found to correlate strongly with a T_H 1-skewed immune response [18]. Therefore, it can be speculated that CXCR3 might help to reinforce and maintain the T_H 1/ T_H 2 balance that is observed in healthy nonatopic

individuals [18]. Upon allergen stimulation, peripheral blood mononuclear cells (PBMCs) from healthy individuals showed increased levels of CD4⁺CXCR3⁺ cells when compared to PBMCs from allergic individuals [19].

Nicolae et al. [20] reported that the human leukocyte antigen (HLA)-G gene is an asthma susceptibility gene and speculated that HLA-G could be involved in allergy pathogenesis. HLA-G is a nonclassical HLA class Ib molecule with immune-modulating and tolerogenic functions, with limited polymorphism, and with restricted tissue expression [21, 22]. The primary HLA-G gene transcript generates 7 different protein isoforms of HLA-G via alternative splicing; of these isoforms, 4 are membrane bound (HLA-G1 through HLA-G4), while 3 are soluble isoforms (sHLA-G5 through sHLA-G7). The soluble isoforms of HLA-G are generated by alternative splicing, in which intron 4 or intron 2 is retained, as both contain a stop codon. However, sHLA-G1 can also be generated by proteolytic cleavage and shedding of the membrane-bound HLA-G protein [23–28]. Ig-like transcript (ILT) 2 and ILT4 are receptors for HLA-G and other HLA class I molecules [29]. The presence of HLA-G can induce a T_H 2-like cytokine profile in PBMCs and trigger the production of cytokines such as IL-4 and IL-13 [30] that promote antibody isotype switching to IgE. Recent studies have shown that sHLA-G levels are significantly higher in atopic asthmatic and allergic patients compared to healthy nonatopic individuals [31, 32]. Furthermore, serum levels of sHLA-G decrease after sublingual immunotherapy (SLIT), while production of the T_H 1-cytokine IFN- γ increases [33].

Currently, there is no cure for allergy. However, allergy can be managed with proper prevention and treatment. Elucidating the underlying mechanisms behind allergen sensitization and allergen-specific immunotherapy (SIT) is of great importance. Therefore, the aim of this study was to evaluate whether allergen-SIT changed the frequency or phenotype of Tregs, the level of sHLA-G, and the concentration of allergen-specific serum antibodies, as well as certain cytokines that are relevant to allergy pathogenesis.

Materials and Methods

Study Population

Eleven atopic patients (5 females and 6 males) with clinical histories of *Betula verrucosa* allergy, *Phleum pratense* allergy, or both were included in this study. The mean patient age was 36 years (range 20–47). Clinical examination, a conjunctival provocation test, a lung function test, and a skin prick test for the 10 most com-

mon inhalant allergens, including birch and grass, were performed on the allergic individuals. Furthermore, the allergic individuals were sensitized to birch allergen (n = 4), grass allergen (n = 3), or both (n = 4). Subcutaneous injections were given over a period of 6–8 months. During the first 12–14 weeks incremental doses of allergen were given; thereafter, maintenance doses were given. Patients and controls were followed during a period of 7 months.

Ten nonatopic individuals (9 females and 1 male) with a mean age of 43 years (range of 23–63) were enrolled as controls. A negative skin prick test (mean wheal diameter <3 mm was defined as negative) and negative total and specific IgE tests were acquired. The nonatopic control individuals did not receive any SIT treatments. The study and thus the SIT treatment of the patients was initiated in late autumn to avoid the pollen season. Venous blood samples (whole blood, plasma, and serum) were collected from the patients and the controls during a period of approximately 7 months at the following 8 time points: immediately prior to the first subcutaneous injection of the specific allergen extract [30 standardized quality units (SQ)], and just before the allergen extract injections at approximately 3-week intervals (visit 2: 300 SQ; visit 3: 2,000 SQ; visit 4: 40,000 SQ; visit 5: 80,000 SQ; visit 6: 100,000 SQ; visit 7 at 4 weeks after visit 6: 100,000 SQ, and approximately 6 weeks after visit 7: 100,000 SQ, named the 'maintenance phase'). In total, the patients received allergen injections at 15 visits during the 7-month period; however, blood samples were only collected at 8 of the visits, as outlined above. This study was approved by the Danish National Committee on Biomedical Research Ethics (approval No. SJ-137) and informed consent was obtained from all participants. This study was conducted according to the principles expressed in the Declaration of Helsinki.

Peripheral Blood Mononuclear Cells

PBMCs were isolated at 6 different time points, prior to, during, and after the 7-month treatment course, from 9 ml of heparinized whole blood, and isolated on a Lymphoprep gradient according to the manufacturer's instructions (Axis-Shield, Oslo, Norway). PBMCs were subjected to flow cytometric analysis as described below. PBMCs for cell culture experiments were isolated from 36 ml of whole blood collected at 2 different time points prior to SIT and in the maintenance phase after 7 months of treatment; cells were isolated on a Lymphoprep gradient (Axis-Shield).

Cell Culture

PBMCs collected at the beginning or in the maintenance phase of SIT were resuspended in minimum essential medium alpha (MEM- α ; GIBCO™, Invitrogen, Paisley, UK) supplemented with 1% penicillin/streptomycin and 5% autologous human serum. Resuspended PBMCs were plated in 10 different conditions, or setups, at a density of 5.0×10^5 cells per well in round-bottomed 96-well plates (Nunc, Roskilde, Denmark) in triplicate or hexaplicate. The plates were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 6 days. Four of the 10 experimental setups were performed in hexaplicate to permit flow cytometry analysis on the cells as described below. The concentrations of the whole extract birch (Bet v) or grass (Phl p) (ALK-Abello, Hørsholm, Denmark) allergens in the final culture volumes were 2, 5, or 15 µg/ml. Patient PBMCs were cultured with the same allergen that had been administered to the patient. Control PBMCs were given one of the two allergens (the same allergen was given both times). Additionally, PBMCs were cultured in the presence of mycobacterial purified

protein derivative (PPD), tetanus toxoid (TT) (25 and 10 µg/ml, respectively; National Institute for Health Data and Disease Control, Copenhagen, Denmark), or medium alone. Furthermore, some cultures were incubated with allergen extract and blocking antibodies against the IL-10 receptor (5 µg/ml; BD Biosciences, San Jose, Calif., USA) or HLA-G (20 µg/ml; Exbio, Prague, Czech Republic). Baseline experiments were not performed on all patients because of random initial practical and organizational limitations.

Flow Cytometric Analysis

Flow cytometric analysis was performed on freshly isolated and cultured PBMCs. Freshly isolated PBMCs were stained with anti-ILT2-FITC, anti-CXCR3-FITC, anti-CD127-PE, anti-HLA-G-PE, anti-ILT4-PE, anti-CCR4-PE, anti-CD4-ECD, anti-CD25-PC5, anti-CD8-PC5, anti-CD127-PC7, and anti-CD3-PC7. All of the antibodies were obtained from Beckman-Coulter (Brea, Calif., USA), except for anti-HLA-G-PE (Exbio), anti-CXCR4-FITC, anti-CCR4-PE (R&D Systems, Minneapolis, Minn., USA), and anti-ILT2 (BD Pharmingen, San Jose, Calif., USA).

After 6 days of incubation, the PBMCs from all of the participants which were cultured with medium alone, the highest allergen concentration, the intermediate allergen concentration, anti-HLA-G, and PPD were harvested for flow cytometric analysis. Briefly, PBMCs were stained with anti-ILT2-FITC, anti-CD127-PE, anti-CD4-ECD, anti-CD25-PC5, anti-CD3-PC7, anti-mHLA-G-PE, and anti-CD8-PC5. Appropriate isotype controls were included to estimate the amount of nonspecific binding. Cells were incubated for 20 min in the dark at room temperature, washed twice with isoton buffer (a nonfluorescent, azide-free balanced electrolyte solution), and centrifuged at 300 g for 5 min. Cells were then analyzed on a flow cytometer (Cytomics™ FC-500; Beckman-Coulter).

Gates were positioned to include 0.5% of the positive events in the isotype control. Cells were first visualized in an SS log/FS linear density plot, and the defined lymphocyte population was gated for further analysis. Tregs were defined as CD4⁺CD25⁺CD127^{low/-} cells from the lymphocyte cell population. Afterwards, defined Tregs were analyzed for expression of the HLA-G receptor ILT2, as well as the chemokine receptors CCR3 and CCR4.

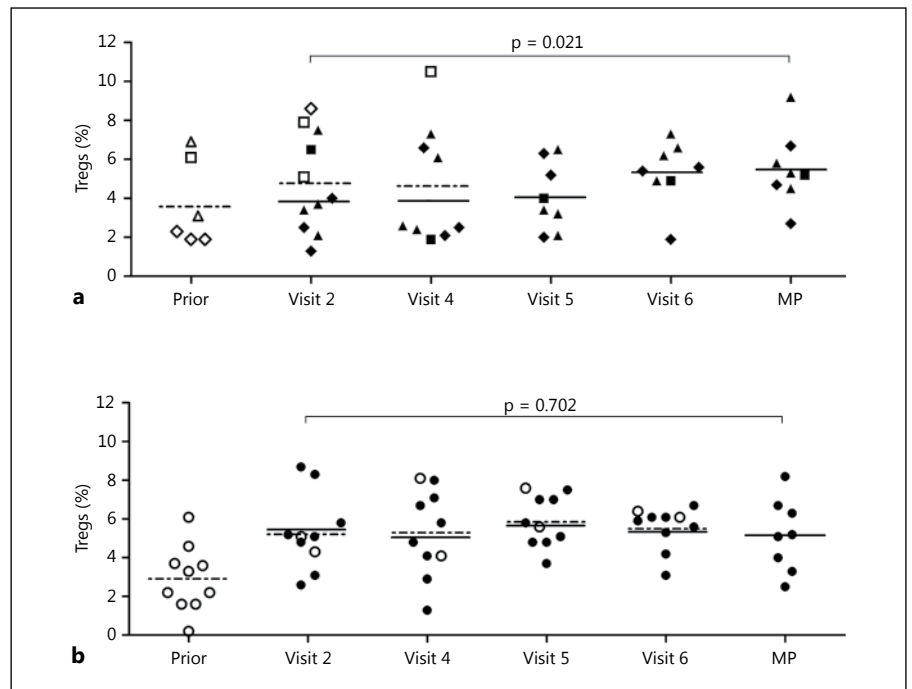
sHLA-G Measurements

The levels of sHLA-G1 and sHLA-G5 molecules in the culture medium or in the subject plasma samples were determined by a commercially available sandwich enzyme immunoassay (Exbio) according to the manufacturer's instructions. Six plasma samples were obtained prior to, during, and after 7 months of treatment. Culture medium samples were also collected in triplicate after 6 days of incubation, and sHLA-G levels were determined with a BEP2000 ELISA instrument (Siemens, Tarrytown, N.Y., USA) before treatment initiation and after 7 months of treatment. Undiluted conditioned culture medium was tested.

Measurement of Allergen-Specific Ig

Serum samples from the nonatopic and allergic individuals were collected before the initiation of SIT treatment, during the treatment, and after 7 months of treatment. Measurements of allergen-specific IgE and IgG₄ levels were analyzed with two different assays, i.e. ImmunoCAP ISAC® and Phadia 250®, according to the manufacturers' instructions (Phadia, Allerød, Denmark).

Fig. 1. Frequency of CD3⁺CD4⁺CD25⁺CD127^{low/-} Tregs in peripheral blood samples from patients (**a**) during 7 months of allergen-SIT and from controls (**b**) during the 7-month period. Statistical analysis was performed using a two-way repeated measures ANOVA [n = 7 for the patients (▲ = grass and birch, ■ = grass, ◆ = birch) and n = 8 for the controls (●); see Results for details] and a paired samples t test. The p values shown are from the paired t test [visit 2 vs. maintenance phase (MP); n = 8]. Closed symbols illustrate the data points that were included in the ANOVA. Dotted lines indicate the mean for all data points at the specific visit; solid lines indicate the mean for the data points included in the two-way ANOVA.



Data were collected with Phadia Microarray Image Analysis (MIA) software and Phadia Information Data Manager (IDM) software, respectively.

Measurements of Cytokine Concentrations in the Cell Culture Supernatants

Supernatants from both nonatopic and allergic PBMCs that were cultured under 10 different in vitro conditions were collected before and after 7 months of treatment. The supernatants were stored at -80°C until quantification of IL-2, IFN- γ , IL-10, and IL-17 levels by the Luminex 100 platform (Luminex Corporation; MAP assays; R&D Systems). Analyses were performed according to the manufacturer's suggested protocol.

Statistical Analysis

Statistical calculations were performed with SPSS software version 20 for Windows XP, and graphs were made with GraphPad Prism version 5.04. Variables were tested for normal distributions, but only a few fulfilled the criteria and Student's t test was used in these cases. Otherwise, either paired (Wilcoxon signed-rank test) or nonpaired (Mann-Whitney test) nonparametric tests were used. A two-way 'mixed between-within subjects' ANOVA (two-way repeated measures ANOVA) was used for serial measurements within cases and controls over time. In cases of nonparametric data, Friedman's test was used for serial measurements in either cases or controls. Clear a priori hypotheses were formulated; therefore, no correction for multiple comparisons was performed. Statistically, two things were of specific interest. First, the difference in the levels of a specific immune parameter at the start of SIT and in the maintenance phase for the patients and for the controls, respectively, was assessed. This was determined by a comparison of visit 2 and the visit in the maintenance phase using a paired t

test or Wilcoxon's signed-rank test. Visit 2 was chosen instead of visit 1 (prior to the initiation of SIT) because a number of individual measurements were missing at visit 1. Second, the difference between the patients and the controls in the changes of a specific parameter during SIT was determined. This was analyzed with the use of a two-way repeated measures ANOVA or Friedman's test. The significance level was set at 0.05.

Results

Tregs in Peripheral Blood during SIT

Freshly isolated PBMCs collected prior to, during, and after 7 months of SIT were analyzed to determine the frequency of CD3⁺CD4⁺CD25⁺CD127^{low/-} Tregs (fig. 1). During the course of SIT, there was a trend towards increased Treg frequencies in the patient group but not in the control group. However, this trend was not statistically significant according to a two-way repeated measures ANOVA that explored the impact of group (case or control) and time/visit on the Treg frequencies. $p = 0.449$ for the main effect during the treatment period (Wilks' lambda = 0.734), $p = 0.189$ for an interaction effect between the patients and controls (a difference over time between the two groups; Wilks' lambda = 0.598), and $p = 0.281$ for a group effect. There was a significantly higher Treg frequency in the maintenance phase, compared to

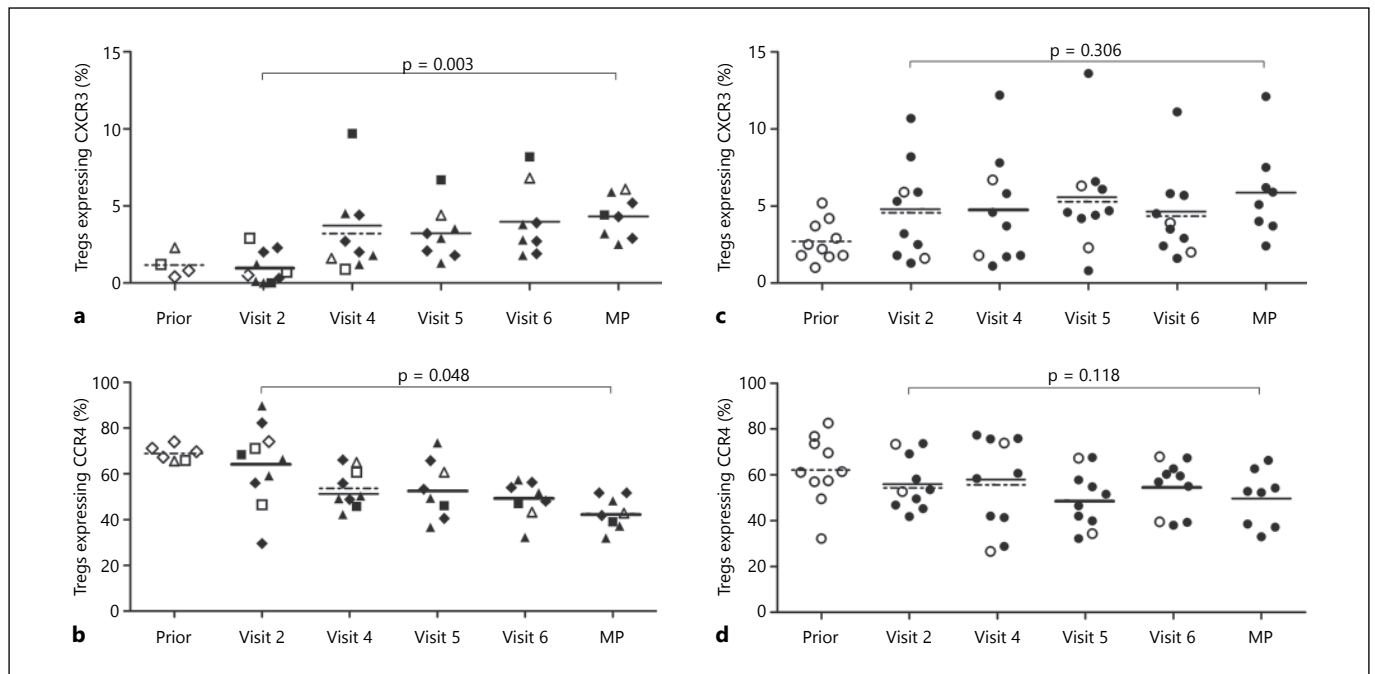


Fig. 2. Frequency of CXCR3⁺CD4⁺CD25⁺CD127^{low/-} and CCR4⁺CD4⁺CD25⁺CD127^{low/-} Tregs in peripheral blood samples from patients (**a, b**) during 7 months of allergen-SIT and controls (**c, d**) during the 7-month study period. The frequency of CXCR3⁺ Tregs increased significantly in the patients during the treatment period (▲ = grass and birch, ■ = grass, ◆ = birch) but not in the controls (●). Statistical analysis was performed with a two-way repeated measures ANOVA (n = 7 for the patients and n = 8 for the controls; see Results for details) and a paired samples t test. **b,**

d There was a significant decrease in the frequency of CCR4⁺ Tregs during the treatment period in the patients but not in the controls (two-way repeated measures ANOVA; n = 7 for the patients and n = 8 for the controls). The p values shown correspond to the paired t test [visit 2 vs. maintenance phase (MP); n = 8]. Closed symbols illustrate the data points that were included in the ANOVA. Dotted lines indicate the mean for all data points at the specific visit; solid lines indicate the mean for the data points included in the two-way ANOVA.

the beginning of the treatment phase (visit 2) in the patients [$p = 0.021$, $3.9 \pm 2.1\%$ (mean $\pm$ SD) vs. $5.5 \pm 1.9\%$, paired t test, n = 8]; however, this was not the case for the controls (visit 2 vs. maintenance phase: $p = 0.702$, $5.3 \pm 2.0\%$ vs. $5.2 \pm 1.9\%$, paired t test, n = 8).

CXCR3⁺-Expressing Tregs Increase and CCR4⁺-Expressing Tregs Decrease in the Peripheral Blood after SIT

Further phenotypic analysis of the CD4⁺CD25⁺CD127^{low/-} Tregs in the patient peripheral blood samples revealed that the frequency of Tregs that were positive for the T_H1-associated chemokine receptor CXCR3 increased during the course of the treatment; a similar increase in CXCR3⁺ Tregs was not observed in the controls (fig. 2a, c). Statistical analysis with a two-way repeated measures ANOVA revealed a statistically nonsignificant trend (n = 7 for patients and n = 8 for controls). $p = 0.066$ for the main effect during the treatment period (Wilks' lambda =

0.445), $p = 0.124$ for an interaction effect between the patients and controls (Wilks' lambda = 0.515), and $p = 0.116$ for a group effect. However, the frequency of CXCR3⁺ Tregs was significantly lower at visit 2 than in the maintenance phase ($p = 0.003$, $1.0 \pm 1.1\%$ vs. $4.3 \pm 1.4\%$, paired t test, n = 7) in the patients, while the frequency remained the same in the controls ($p = 0.306$, $4.6 \pm 3.1\%$ vs. $5.9 \pm 3.0\%$, paired t test, n = 8). Furthermore, a comparison of the CXCR3⁺CD4⁺CD25⁺CD127^{low/-} Tregs in the patients and nonatopic controls at the beginning of the treatment indicated a lower level of CXCR3⁺ Tregs in the patients, which was a borderline significant difference ($p = 0.053$, unpaired t test, visit 2).

Next, the frequency of CD4⁺CD25⁺CD127^{low/-} Tregs that also expressed the T_H2-associated chemokine receptor CCR4 was analyzed (fig. 2b, d). In the patients, a significant decrease in this cell population was observed during the treatment period. The two-way repeated measures ANOVA (n = 7 for patients and n = 8 for controls)

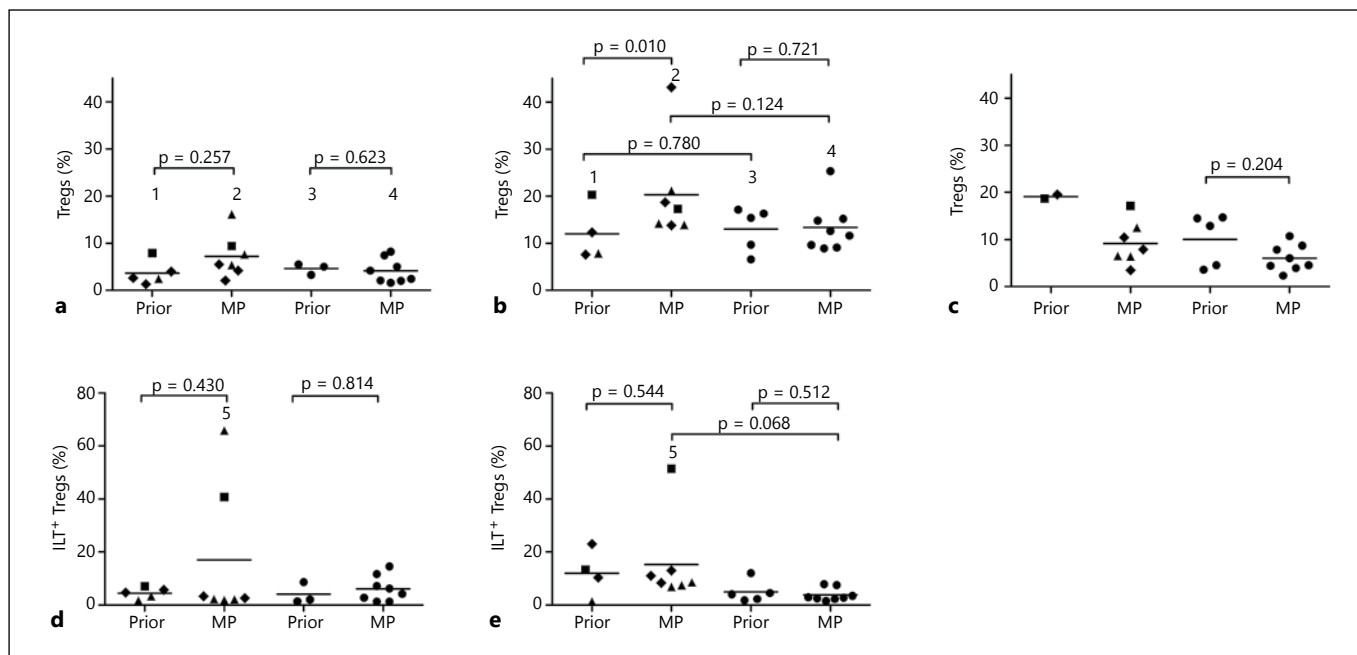


Fig. 3. Effect of different in vitro conditions on the frequencies of $CD3^+CD4^+CD25^+CD127^{low/-}$ Tregs from patients ($\blacktriangle$ = grass and birch, $\blacksquare$ = grass, $\blacklozenge$ = birch) and controls ($\bullet$). The results from 4 different experiments conducted prior to and in the maintenance phase (MP) of SIT treatment are shown. **a** Unstimulated PBMCs. **b** PBMCs stimulated with allergen extract (high concentration, 15 μ g/ml). **c** PBMCs stimulated with PPD (25 μ g/ml). Percentage

of $ILT2^+CD4^+CD25^+CD127^{low/-}$ Tregs in unstimulated (**d**) and allergen-stimulated (high concentration, 15 μ g/ml) (**e**) PBMCs. The lines indicate mean values for all data points shown. Statistical analysis was performed with a paired or unpaired Student's t test. 1: $p = 0.014$ ($n = 4$), 2: $p = 0.019$ ($n = 6$), 3: $p = 0.073$ ($n = 3$), 4: $p < 0.001$ ($n = 8$), 5: $p = 0.001$ ($n = 6$).

revealed $p = 0.040$ for the main effect during the treatment period (Wilks' lambda = 0.398), $p = 0.274$ for an interaction effect between the patients and controls (Wilks' lambda = 0.625), and $p = 0.004$ for a group effect. When the frequencies of $CCR4^+$ Tregs in the patients at the start of the treatment (visit 2) and the maintenance phase were compared, a significant difference was observed ($p = 0.048$, $64.3 \pm 17.5\%$ vs. $43.1 \pm 7.1\%$, paired t test, $n = 7$). This was not true for the controls ($p = 0.118$, $56.4 \pm 11.8\%$ vs. $49.6 \pm 12.2\%$, paired t test, $n = 8$).

In vitro Stimulation with Allergen Extract Increases the Frequency of Tregs and $ILT2^+$ Tregs in PBMCs from Atopic Patients

To investigate whether SIT could induce cellular and phenotypical changes, PBMCs from both atopic patients and nonatopic controls were subjected to 10 different culture conditions. Treg frequency was measured in only 4 of these culture conditions (fig. 3). The frequency of $CD3^+CD4^+CD25^+CD127^{low/-}$ Tregs found in atopic patient PBMCs that were cultured in pure medium alone in-

creased slightly from the start of the treatment to the end of the treatment; however, this increase was not significant ($p = 0.257$, $2.6 \pm 1.1\%$ vs. $7.0 \pm 6.2\%$, paired t test; fig. 3a). Both before SIT and in the maintenance phase of SIT, there was a significant difference between the conditions with medium alone and those with the highest allergen concentrations in both the patients and the controls [$p = 0.014$ and $p = 0.019$ vs. $p = 0.073$ and $p < 0.001$ (controls), respectively; paired t test; fig. 3a, b]. The addition of specific allergen extract to patient PBMCs resulted in an increase in Treg frequency that was even stronger at the end of SIT than at the start; a similar increase was not observed in control PBMCs ($p = 0.010$ and $p = 0.721$, respectively; $10.0 \pm 3.3\%$ vs. $16.3 \pm 3.5\%$, and $13.3 \pm 5.8\%$ vs. $11.8 \pm 3.0\%$, paired t test; fig. 3b). The elevated Treg levels indicate that, as a result of SIT, the patients were better able to activate Tregs upon specific allergen stimulation. The frequency of Tregs in response to the allergen seemed to increase in the patients after SIT when directly compared to the controls, although this difference was not statistically significant ($p = 0.780$ and $p = 0.124$, respectively; $12.0 \pm 5.9\%$ vs. $13.0 \pm$

4.6%, and $20.3 \pm 10.4\%$ vs. $13.4 \pm 5.4\%$, unpaired t test; fig. 3b). There were no significant differences in Treg frequencies prior to treatment or after 7 months of treatment with regard to responses towards PPD (fig. 3c).

Comparisons of the frequencies of ILT2⁺-expressing Tregs found in the PBMCs from the patients and the controls revealed a significant increase in the frequency of ILT2⁺ Tregs in patient PBMCs upon specific allergen stimulation ($p = 0.001$, $8.8 \pm 15.6\%$ vs. $16.5 \pm 17.2\%$, paired t test; fig. 3d, e), which was not observed in the controls. Furthermore, there was also a trend towards a higher frequency of ILT2⁺ Tregs in the patient PBMCs in the maintenance phase compared to the control PBMCs ($p = 0.068$, $15.2 \pm 16.1\%$ vs. $3.8 \pm 2.4\%$, unpaired t test).

Plasma Levels of sHLA-G during Allergen-SIT

The concentration of sHLA-G in the blood plasma samples from patients were high before treatment and decreased during SIT (fig. 4). There was a trend towards higher plasma sHLA-G levels in the patients prior to SIT, compared to the first visit for the controls, although this trend was not significant [$p = 0.190$, Mann-Whitney test, 298 U/ml (72–2,049); median (range) vs. 197 U/ml (39–456)]. Plasma levels of sHLA-G reached the lowest concentrations after approximately 4 months of treatment (visit 5). However, the levels were not significantly lower than those prior to SIT [$p = 0.327$, Wilcoxon's test, 246 U/ml (72–737) vs. 368 U/ml (132–1,569), $n = 8$; $p = 0.122$, Mann-Whitney test], although the patients had significantly lower levels of plasma sHLA-G at visit 5 compared to the controls [$p = 0.012$, Mann-Whitney test, 187 U/ml (69–360) vs. 490 U/ml (198–1,852); fig. 4]. Interestingly, the concentrations of sHLA-G rose at the end of the treatment, and the plasma sHLA-G concentrations in the patients were the same as those in the controls at the end of SIT (fig. 4). Furthermore, none of the changes in plasma sHLA-G concentrations during the 7 months were significant.

IgG₄ Levels in Serum Samples Increased after SIT

The levels of IgE and IgG₄ were measured quantitatively and semiquantitatively. As expected, the patients did not show changes in specific IgE levels during SIT (fig. 5a, b). However, the quantitative specific IgG₄ measurements were significantly increased for both pollen allergens and showed clear increases in IgG₄ levels in the patients after SIT (fig. 5c, d). The controls were also analyzed quantitatively and, as expected, there were no differences in the specific IgE or IgG₄ antibody levels at the first visit compared to those at the last visit (data not shown).

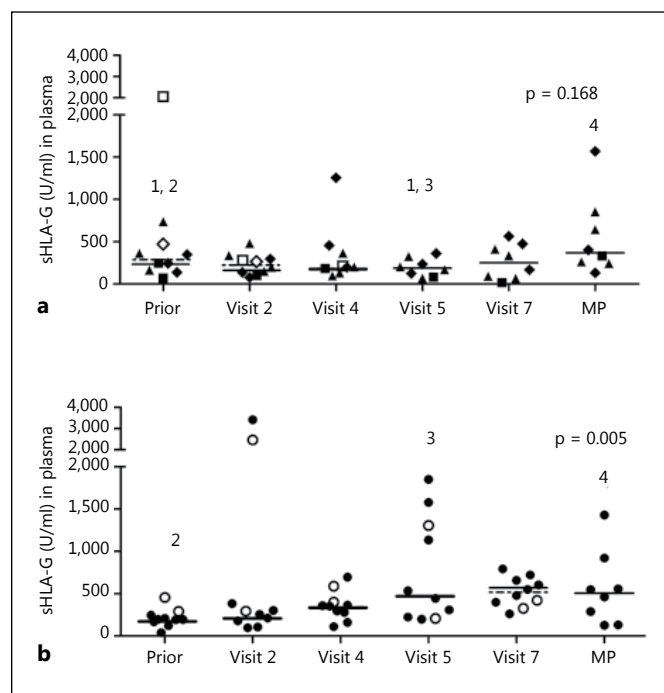


Fig. 4. Concentration of sHLA-G in blood plasma from patients (a) ($\blacktriangle$ = grass and birch, $\blacksquare$ = grass, $\blacklozenge$ = birch) and controls (b) ($\bullet$) collected prior to, during, and after the maintenance phase (MP) of allergen-SIT. Statistical analysis was performed with Friedman's test, Wilcoxon's test, and the Mann-Whitney test ($n = 8$ for patients and $n = 8$ for controls). Closed symbols illustrate data points that were included in Friedman's test. Dotted lines indicate the median for all data points at the specific visit; solid lines indicate the median for the data points included in Friedman's test. 1: $p = 0.327$ (Wilcoxon, $n = 8$) and $p = 0.122$ (Mann-Whitney), 2: $p = 0.190$ (Mann-Whitney), 3: $p = 0.012$ (Mann-Whitney), 4: $p = 1.0$ (Mann-Whitney).

Additionally, the levels of specific IgE and IgG₄ were measured semiquantitatively. The semiquantitatively measured IgG₄ levels increased significantly after SIT and thus supported the results of the quantitative measurements. The controls were also analyzed semiquantitatively and, as expected, there were no differences in specific IgE or IgG₄ antibody levels between the two different time points (data not shown).

Allergen-SIT Reduces the Level of sHLA-G in Vitro

The levels of sHLA-G before and after SIT in both the patients and the controls are shown in fig. 6. The median level of sHLA-G in the media from PBMCs collected prior to SIT increased with increasing concentrations of added allergen; this increase was not significant, possibly due to the low number of samples. After SIT, a lower me-

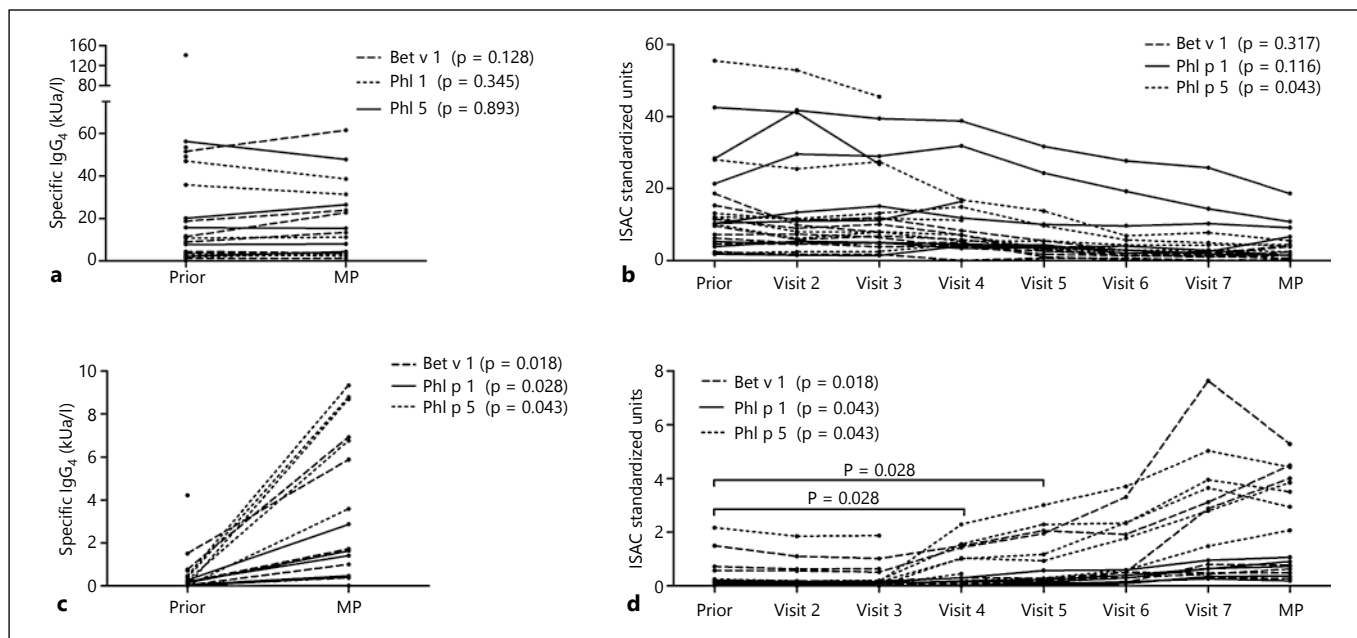


Fig. 5. Quantitative and semiquantitative measurements of specific IgE (**a, b**) and IgG₄ (**c, d**) antibodies in patients with Phadia 250 (**a, c**) and ImmunoCAP ISAC (**b, d**). A significant increase in

IgG₄ was observed. Statistical analysis was performed with Wilcoxon's test ($n = 7$ for Bet v 1 and $n = 6$ for Phl p 1 and Phl p 5). MP = Maintenance phase.

dian level of sHLA-G was observed in the media after allergen stimulation. Comparisons of the culture conditions with the highest allergen concentration and the unstimulated PBMCs both before and after SIT resulted in borderline significant differences ($p = 0.043$, $n = 5$; $p = 0.093$, $n = 8$, Wilcoxon). In general, the levels of sHLA-G were higher in the patients in each condition and at both time points, compared to the nonatopic controls. On the contrary, the median sHLA-G levels in the controls, both before and after SIT, remained fairly stable in each condition and, as expected, a comparison of the unstimulated PBMCs and those treated with the highest allergen concentration both before and after SIT did not yield any significant results ($p = 0.138$, $n = 5$; $p = 0.484$, $n = 8$, Wilcoxon). Blocking of the IL-10 receptor with a specific mAb did not result in any significant differences (fig. 6). Although the median level of sHLA-G decreased in the media from in vitro experiments with patient PBMCs after SIT, the levels never decreased to the levels of sHLA-G observed in the controls.

Cytokine Measurements

The functional characteristics of the PBMCs were investigated by examining the cytokine production from stimulated PBMCs collected before and after (mainte-

nance dose) SIT and cultured under different in vitro conditions to evaluate SIT-induced changes in the production of the following relevant cytokines: IL-10, IL-2, IL-17, and IFN- γ (fig. 7). In general, unstimulated PBMCs from both patients and controls produced almost undetectable levels of the 4 cytokines.

Prior to SIT, stimulation of patient PBMCs with increasing allergen concentrations resulted in increased cytokine levels while the cytokine levels produced by the control PBMCs remained fairly stable regardless of the addition of increasing allergen concentrations and in spite of the time point, with the exception of IL-17. Stimulation of the patient PBMCs collected after SIT revealed differences in responses towards increasing allergen concentrations. Patients produced more IL-10 and IL-2 upon allergen stimulation, whereas the levels of IL-17 and IFN- γ remained stable or decreased. To elucidate how HLA-G and IL-10 influenced the production of the different cytokines, blocking experiments with anti-HLA-G and anti-IL-10-receptor were performed (fig. 7b). However, mAb blocking of HLA-G or the IL-10 receptor did not alter the cytokine responses to allergen stimulation. Cultures from both control and patient PBMCs collected before SIT had higher levels of IL-10, IFN- γ , and IL-17 in response to stimulation with the control antigen PPD, but

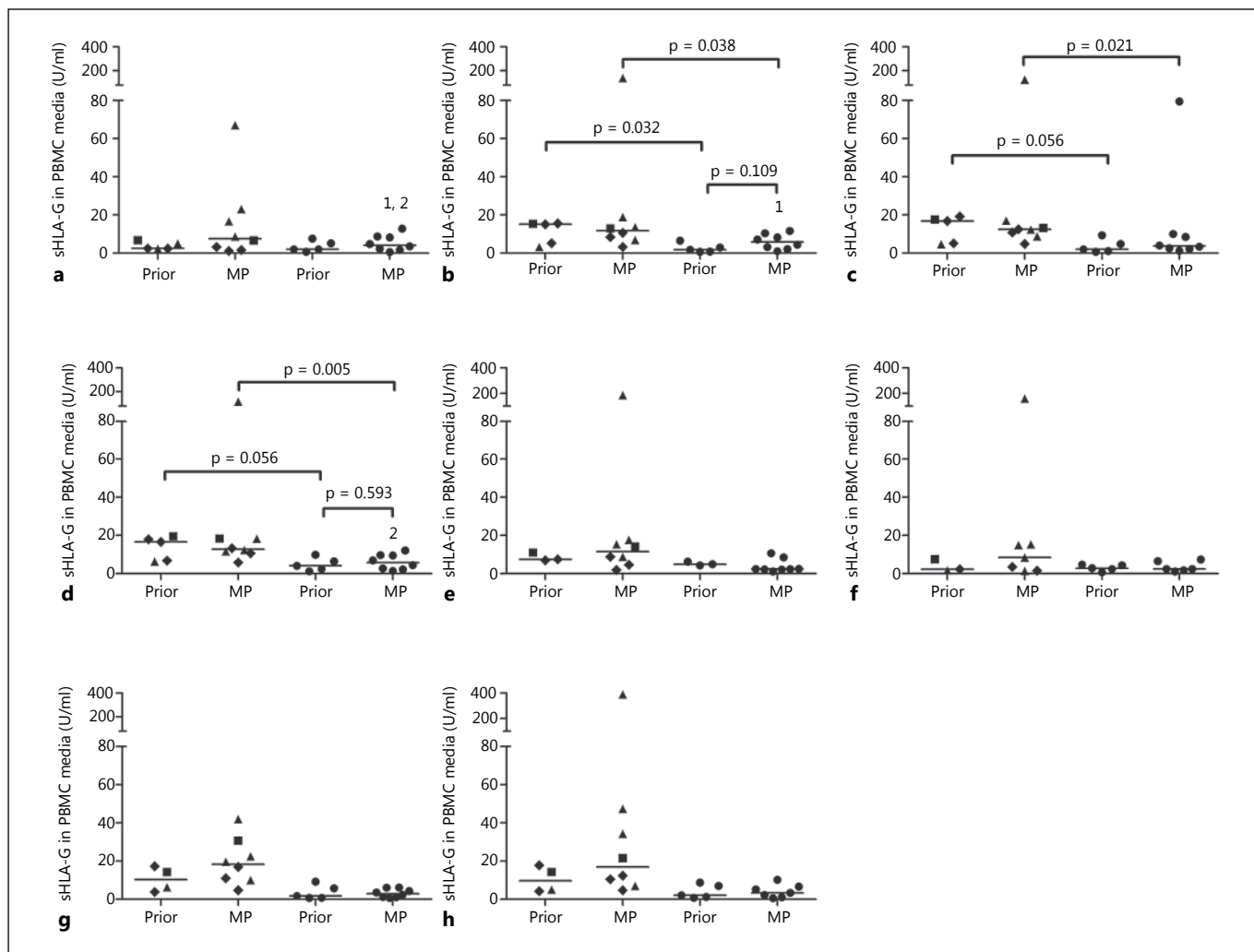


Fig. 6. Concentration of sHLA-G from PBMCs cultured in 8 different in vitro conditions prior to and during the maintenance phase (MP) of allergen-SIT in patients ($\blacktriangle$ = grass and birch, $\blacksquare$ = grass, $\blacklozenge$ = birch) and controls ($\bullet$). **a** No allergen. **b** Low allergen concentration (2 μ g/ml). **c** Intermediate allergen concentration (5 μ g/ml). **d** High allergen concentration (15 μ g/ml). **e** PPD

(25 μ g/ml). **f** TT (10 μ g/ml). **g** Allergen concentration (5 μ g/ml) + anti-IL-10 receptor. **h** Allergen concentration (5 μ g/ml) + anti-IL-10 receptor isotype control. The lines indicate the median level of sHLA-G. Statistical analysis was performed with Wilcoxon's test or the Mann-Whitney test. 1: $p = 0.176$, 2: $p = 0.484$ (Wilcoxon, $n = 8$).

very low or undetectable concentrations of the cytokines were observed in PBMCs collected after allergen-SIT. IL-2 was the only cytokine that was detected both prior to and after SIT in both groups.

Discussion

Immunological dysfunction is a central part of allergy pathophysiology and is characterized by a T_H2 -polarized cytokine profile and the production of IgE antibodies.

Allergen-SIT has been shown to induce changes in cellular and humoral immune responses in allergic patients. These changes include the induction of immune Tregs as well as changes in the cytokine profile [7]. Furthermore, some studies have reported changes in the immunoregulatory HLA class Ib protein HLA-G [31–35]. The overall objective of the present study was to investigate changes in these parameters in patients who received SIT as an allergy treatment and to compare the changes in the parameters to those observed in healthy nonatopic controls.

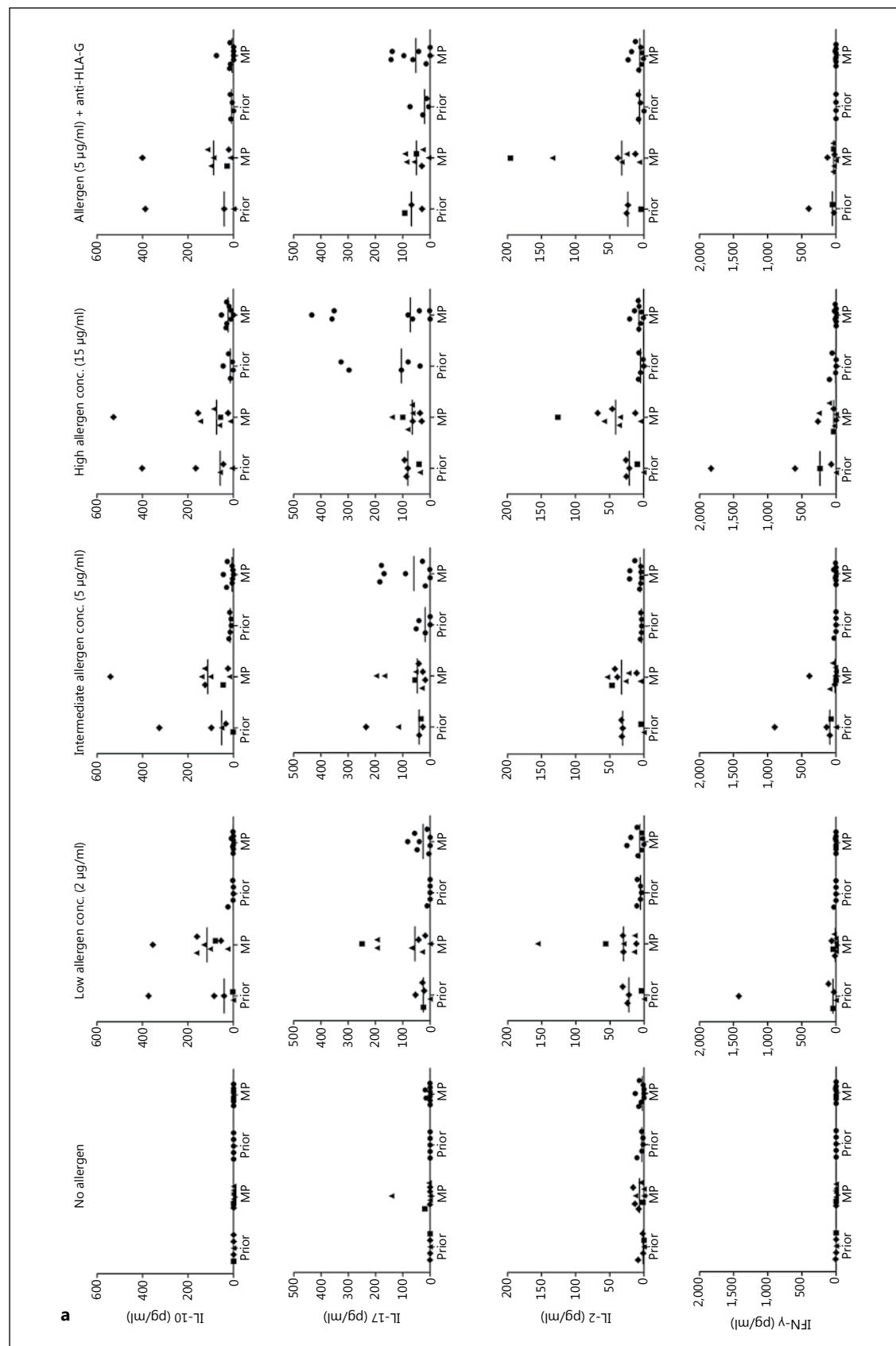


Fig. 7. a Levels of the cytokines IL-10, IL-17, IL-2, and IFN-γ in the different in vitro culture conditions prior to and during the maintenance phase (MP) of allergen-SIT in patients (▲ = grass and birch, ◆ = grass, ■ = birch) and controls (●). conc. = Concentration.

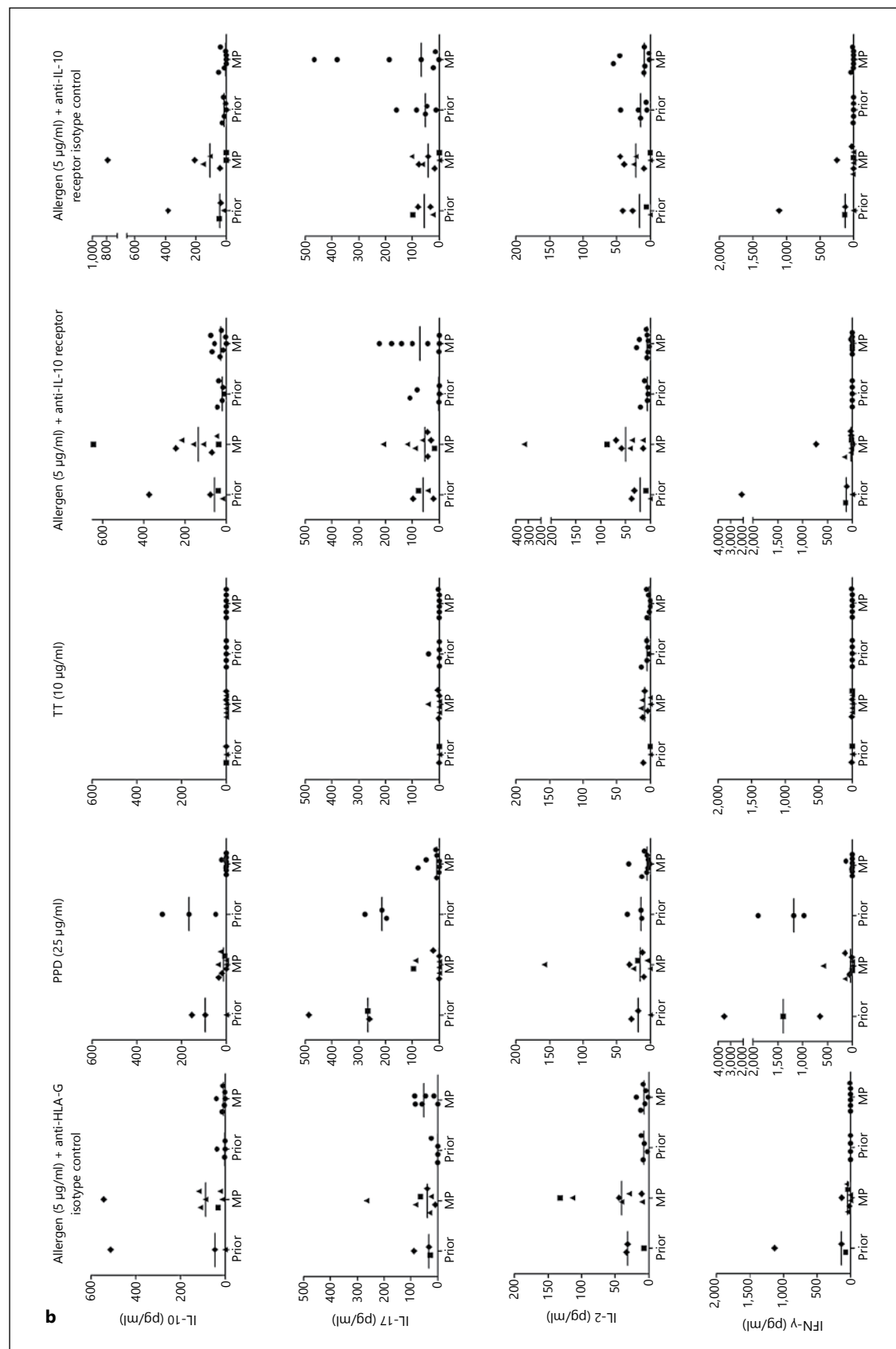


Fig. 7. b Levels of the cytokines IL-10, IL-17, IL-2, and IFN- γ in the different in vitro culture conditions prior to and during the maintenance phase (MP) of allergen-SIT in patients (▲ = grass and birch, ◆ = grass, ■ = birch) and controls (●). conc. = Concentration.

Table 1. Clinical data of the atopic patients treated with allergen-SIT

Clinically important allergy/allergies	Sex	Age, years	Skin prick test (wheal diameter), mm	Conjunctival provocation test positive, SQ/ml	SIT	Specific IgE Phl 1 (g205), kU/l	Specific IgE Phl 5 (g215), kU/l	Specific IgE Bet v 1 (t215), kU/l	Subjective symptoms of allergy (scale 1–10, 10 = worst)	
									last season before SIT ^a	first season after SIT ^a
G	1 f, 2 m	21 (20–27)	12 (9–15)	1,000–10,000	G	35.76 (9.11–53.38)	20.05 (11.68–141.16)		9 (8–10)	6 (6–6)
B	3 f, 1 m	38 (35–47)	5 (4–8)	1,000–100,000	B			11.37 (3.43–51.56)	8 (5–10)	3 (2–4)
G and B	1 f, 3 m	42 (35–47)	G: 8 (4–9) B: 5 (3–8)	G: 1,000–10,000 B: 10,000–100,000	G and B	7.04 (3.37–46.99)	11.69 (2.12–56.25)	6.57 (1.11–18.74)	9 (7–10)	4 (2–7)

Data are presented as medians (range) unless otherwise stated. G = Grass; B = birch; f = female; m = male. ^a $p = 0.003$ (all 3 groups, Wilcoxon signed rank test).

All atopic patients included in this study reported subjective reductions in symptoms during the first pollen season after SIT. However, the reductions were most pronounced in patients who were allergic to birch pollen alone or to both birch and grass pollen (table 1).

The frequency of Tregs in atopic patients might change during the course of allergen-SIT, and possible differences between healthy, nonatopic controls and atopic individuals might elucidate some of the mechanisms in the allergic immune response and in the effects of SIT. However, in vivo and in vitro induction of Tregs in patients suffering from pollen allergies who have received SIT is a controversial subject. Different in vivo experiments have shown that the frequencies of CD4⁺CD25⁺CD127^{low/-} Tregs do not differ between the initiation and maintenance phases of SIT and that, furthermore, similar frequencies of CD4⁺CD25⁺ Tregs can be found in healthy nonatopic controls and atopic patients [9, 36]. However, other in vitro experiments have found a significant increase in the frequency of CD4⁺CD25⁺Foxp3⁺/CD127^{low} Tregs in allergen-stimulated cultures of PBMCs collected prior to and after 24 months of SLIT [37]. Similarly, in the current study, isolated and stimulated PBMCs that were collected from patients during the maintenance phase (after 7 months of SIT) showed significantly increased frequencies of CD3⁺CD4⁺CD25⁺CD127^{low/-} Tregs compared to PBMCs collected from the patients prior to the treatment ($p = 0.010$, paired t test; fig. 3b). This was not true for the controls (fig. 3a, b). In the peripheral blood, there was an increase in the percentage of Tregs in PBMCs collected from patients during the SIT treatment (fig. 1).

In a grass pollen allergy study, Mittag et al. [38] found that the induction of FoxP3^{high} Tregs upon pollen stimulation was reduced in allergic subjects. It was concluded

that the generation of FoxP3^{high} Tregs in response to allergen might be a mechanism that controls potential allergic reactions in nonatopic individuals and that this mechanism might be impaired in allergic individuals. In the current study, the frequency of Tregs in response to specific pollen allergen stimulation did not seem to be directly impaired in the allergic patients, compared to the nonatopic control individuals, but the Treg response in the allergic patients was greater after SIT. However, although the overlap between CD4⁺CD25⁺CD127^{low/-} Tregs in our study and FoxP3^{high} Tregs is large (data not shown), the subpopulations are not completely identical. Mittag et al. [38] observed that the frequency of CD25⁺FoxP3⁺ cells in the total CD4⁺ PBMC pool was the same in both non-SIT-treated allergic subjects outside pollen season and nonatopic controls. However, allergen-induced CD25⁺FoxP3⁺ Tregs were lower in PBMCs from the allergic subjects compared to the controls, which is in contrast to our results [38]. Möbs et al. [36] also studied changes in CD4⁺CD25⁺CD127^{low} Treg frequencies in (birch) pollen-allergic patients who underwent SIT and did not detect any significant changes in the peripheral blood during a 12-month study period. Furthermore, the in vitro Treg results in the current study are similar to those from a study of Japanese cedar pollen-allergic individuals who underwent SLIT [39].

An important point to make is that the expression of several immune parameters can change in the peripheral blood T cell compartment in response to seasonal variations, maybe partly reflecting changes in vitamin D₃ levels. One study found significantly higher levels of Tregs during the winter and spring versus the autumn [40]. Therefore, it is important in studies of changes in immune parameters during SIT to include a control that is

evaluated during the same time period and to compare measurements over time within the patient group and between the patients and controls. Ideally, two types of control groups should be included. The purpose of the optimal SIT treatment is to normalize the immune factors involved in the pathogenesis and pathophysiology of allergy to a state comparable with nonallergic healthy individuals during a specific time of year. Therefore, it is clearly relevant to compare patient data with that of nonatopic healthy control subjects as was performed in the current study. Furthermore, with the inclusion of another control group of untreated allergic individuals, it would be possible to monitor and compare the changes in immune parameters in response to SIT during the treatment in a more direct way. However, in the current study, the SIT-treated patients were also their own controls because we have *a priori* data before SIT as well as data from the maintenance phase.

Peripheral T-cell trafficking is regulated by specific chemokine receptors, which are selectively expressed by the various T-cell subsets [41]. We investigated the frequency of CXCR3⁺ cells among the CD4⁺CD25⁺CD127^{low/-} Tregs in both allergic patients and nonatopic controls. The CXCR3⁺ Treg frequency increased during SIT in the patients but not in the controls (fig. 2a, c). Casas et al. [19] reported a reduced percentage of CXCR3-positive CD4⁺ cells in birch-allergic patients compared to healthy controls. That finding is somewhat in line with the current study, although we specifically investigated CXCR3⁺ Tregs. CXCR3 is an important factor in the control of T_H1 cell migration, and the upregulation of CXCR3 during SIT might promote a T_H1 cytokine response and the concomitant inhibition of T_H2 cytokines and eosinophilia, which might have antiallergic effects [42]. Interestingly, one study has shown that sHLA-G downregulates CXCR3-positive T cells; in the present study, the upregulation of CXCR3 on peripheral CD4⁺CD25⁺CD127^{low/-} Tregs is paralleled by a reduction in sHLA-G levels in peripheral blood plasma from the allergic patients, as discussed below [41] (fig. 4).

The CCR4 chemokine receptor has been detected on T_H2 cells [43]. The frequency of CCR4-expressing CD4⁺CD25⁺CD127^{low/-} Tregs in peripheral blood from the allergic patients was significantly reduced during SIT but was not reduced in the nonatopic controls, suggesting downregulated Th2 cell migration. However, there was also a trend towards such downregulation in the controls, and thus some of the differences might be due to seasonal variation (fig. 2b, d). Similarly, Khoo et al. [40] reported a significantly higher frequency of CCR4⁺ Tregs in pe-

ripheral blood samples collected in autumn compared to winter and spring. In a study of birch-allergic patients versus healthy controls, Casas et al. [19] found a similar CCR4 expression on T cells.

Changes in allergen-specific non-IgE antibody levels are often reported in SIT studies. In the present study, no clear alterations were observed in the median IgE level during the 7-month treatment period (fig. 5a, b); however, the IgG₄ level increased over time in the patients but not in the healthy controls (fig. 5c, d and data not shown). IgG₄ is thought to compete with IgE for allergens, thus preventing the activation of effector cells [44, 45]. There is some controversy regarding the effects of IgG₄ and its correlation with clinical improvements, due to conflicting results that were obtained in different studies [44]. However, a recent study of more than 200 patients suggested that correlations between clinical scores and antibody changes during SIT might be demonstrated in IgE/IgG competition assays [46].

An experiment by Kapasi et al. [47] showed that, in a mixed lymphocyte reaction, a high HLA-G concentration resulted in a T_H2-like cytokine profile, while a low HLA-G concentration resulted in a T_H1-like cytokine profile. Similarly, high sHLA-G levels have been associated with pollen-allergic patients and asthmatic children, and decreased sHLA-G levels have been observed after SLIT [31–34]. The current study investigated sHLA-G levels in peripheral blood plasma from pollen-allergic patients and controls during subcutaneous SIT. Furthermore, this study also evaluated the sHLA-G levels in different *in vitro* cultures of patient and control PBMCs and the possible association between the effects of SIT and sHLA-G both before and during the maintenance phase of SIT. Prior to SIT, there was a trend towards higher peripheral blood plasma sHLA-G levels in the allergic patients when compared to the controls, which was in line with previous published studies (fig. 4). The sHLA-G plasma concentrations decreased during the first 3–4 months of SIT treatment in the patients but not in the controls; in fact, the median sHLA-G concentration increased in the latter group (fig. 4). However, the sHLA-G level also increased in the patients during the maintenance phase of SIT. It can be speculated that seasonal variations in blood sHLA-G levels might exist, with higher levels in the winter and early spring versus the autumn, and that this might influence both the patient and the control data. The decrease in sHLA-G levels during the first months of SIT agrees with an earlier study by Ciprandi et al. [33]. Interestingly, the decrease in blood sHLA-G levels in the allergic patients after 3–4 months of SIT is paralleled by the presence

of specific IgG₄ antibodies in the peripheral blood from the patients (fig. 4, 5c, d). In the in vitro experiments, increasing concentrations of the allergen extracts resulted in increased sHLA-G concentrations in the patient PBMCs but not in the control PBMCs, indicating that sHLA-G somehow contributes to the allergic response upon allergen stimulation. Generally, patients exhibited higher sHLA-G concentrations, regardless of the different conditions or time points, compared to the controls. However, after the 7-month treatment period, the sHLA-G levels after allergen stimulation were reduced in the PBMC culture media from the patients, thus supporting studies which showed that successful SIT resulted in decreased sHLA-G levels and perhaps a shift from a T_H2-polarized immune response towards a T_H1-polarized one.

It is difficult to clarify the role of HLA-G in allergic immune responses. HLA-G is a tolerance-inducing molecule but it is also a stimulus for T_H2 response [22, 28]. Some studies have shown a link between sHLA-G and Tregs. In one study, the actively secreted soluble form of HLA-G (HLA-G5) induced suppressive CD4⁺CD25^{high}FoxP3⁺ Tregs in an IL-10-dependent manner [48]. In the present study, we identified an ILT2⁺ subpopulation of CD4⁺CD25⁺CD127^{low/-} Tregs that increased in frequency upon allergen stimulation. The ILT2 receptor binds HLA-G and other HLA class I molecules [49]. Whether such a connection exists between sHLA-G, IL-10, and Tregs in the early phases of allergen-SIT should be clarified in future studies. However, in the present study, in vitro stimulation of PBMCs with allergen extract resulted in clear increases in the levels of sHLA-G and IL-10 and in the frequency of CD3⁺CD4⁺CD25⁺CD127^{low/-} Tregs in the allergic patients both prior to and in the maintenance phase of SIT (fig. 3, 6, 7). However, upon allergen stimulation the increase in sHLA-G production was less in PBMCs collected during the maintenance phase of or after SIT than in those collected prior to SIT, and the increases in IL-10 production and Treg frequencies were stronger. We have previously shown an inverse relationship between the concentrations of sHLA-G and IL-10 in lipopolysaccharide-activated PBMCs [50]. In the present study, IL-2 levels were also increased in the PBMC media after allergen stimulation (fig. 7). These changes were not observed in PBMCs from the nonatopic controls. When a blocking antibody against the IL-10 receptor was added to the specific allergen-stimulated PBMC cultures, the level of IL-2 increased significantly, and the level of IL-10 showed an increasing trend compared to the cultures that were treated with an isotype antibody control ($p = 0.018$ and $p = 0.237$, respectively, Wilcoxon).

These data are in agreement with a previously published study [9]. Furthermore, IL-10 can block the production of IL-2 in an indirect manner [51]. In most of the in vitro PBMC experiments, it was not possible to detect IFN- γ . Furthermore, the patterns of changes in IL-17 expression upon stimulation with allergen were the same in PBMCs collected prior to and at the end of the SIT treatment from both patients and controls (fig. 7). Generally, an increase in IL-17 production was observed upon allergen stimulation. Some studies have observed increased levels of IL-17 levels in allergic patients [52, 53].

In summary, IL-10 production increased after allergen stimulation; this response was stronger after SIT, especially in the presence of low pollen allergen extract concentrations. An inverse trend toward lower IL-10 production after stimulation with higher allergen concentrations was observed (fig. 7a). Furthermore, trends toward increases in IL-2, CXCR3, Tregs, and ILT2⁺ Tregs and a reduction in sHLA-G were observed upon allergen stimulation after SIT. These findings might indicate both an increase in immunomodulatory factors and a shift towards a T_H1 response.

In conclusion, the findings of the present study show changes in the frequencies of CXCR3⁺ Tregs, CCR4⁺ Tregs, and ILT⁺ Tregs as well as the production of certain key cytokines, IgG₄, and sHLA-G during allergen-SIT for pollen allergy.

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