

Specific Immunotherapy to Birch Allergen Does not Enhance Suppression of Th2 Cells by CD4⁺CD25⁺ Regulatory T Cells During Pollen Season

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Abstract

Introduction The aim of this study was to investigate the suppressive capacity of CD25⁺ regulatory T cells on birch allergen-induced T-cell responses during the first birch pollen season after initiation of specific immunotherapy (SIT).

Methods CD25^{pos} and CD25^{neg} T cells were purified from blood of birch-allergic SIT patients and birch-allergic controls, stimulated with birch pollen extract, and analyzed for T-cell proliferation and production of interferon gamma (IFN-γ), interleukin (IL)-5 and IL-10.

Results We show that allergen-induced proliferation and IFN-γ production were suppressed equally well by CD25^{pos} T cells from SIT patients and controls, while the IL-5 production was not suppressed by either of the groups. IL-10 levels were higher in SIT patients relative to controls only when CD25^{neg} and CD25^{pos} were cultured together. Furthermore, neither *FOXP3* levels nor proportions of CD25^{high} T cells were enhanced in SIT patients compared to allergic controls.

Discussion These results suggest that the Th2-suppressive capacity of allergen-stimulated CD25^{pos} Treg in vitro is not improved by SIT in spite of increased IL-10 production from T cells.

Keywords Human · CD4⁺CD25⁺ regulatory T cells · allergy · immunotherapy · suppression · IL-10

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Introduction

Specific allergen immunotherapy (SIT), the only routinely administered antigen-specific treatment for any immunological disease, is highly effective for IgE-mediated diseases, such as pollen-induced allergic rhinitis and bee venom anaphylaxis, and inhibits both early and late responses to allergens [1–4]. Although it is clear that immunologic tolerance to the allergen develops during SIT, the specific mechanisms are still a matter of debate. Increased numbers of interleukin (IL)-10 producing T cells after SIT have been a consistent observation, as were elevated levels of allergen-specific IgG4 antibodies [5–7]. These findings are connected since IL-10 inhibits IgE and increases IgG4 synthesis by IL-4-stimulated peripheral blood mononuclear cells (PBMCs) [8]. IL-10 may also have other effects such as inhibition of IgE-dependent release of histamine and pro-inflammatory cytokines by mast cells [9] and suppression of eosinophil survival and cytokine production [10]. Moreover, IL-10 inhibits the function of antigen-presenting cells (APC), including the maturation of dendritic cells [11], the expression of MHC class II and co-stimulatory molecules, and consequently their activation of Th2 and Th1 cells [12].

Several subtypes of T cells with regulatory properties have been described. Type 1T regulatory cells (Tr1) are defined by their ability to produce high levels of IL-10 and transforming growth factor beta (TGF-β), and their inhibitory effects on the proliferation of CD4⁺ T cells are mediated by these cytokines and not by direct cell-to-cell interactions [13]. Induction of such cells is most likely a contributing factor for successful SIT. Another subset of regulatory T cells, naturally occurring CD4⁺CD25⁺ T-regulatory cells (CD25⁺ Treg), constitutively possess high levels of CD25 (CD25^{high}) and express FOXP3, a key transcription factor that is required for their development, maintenance, and function [14, 15]. These cells, in

contrast to Tr1 cells, produce low amounts of cytokines, and their mechanism of suppression of T-cell proliferation and cytokine production in vitro is cell contact-dependent and is generally not mediated by IL-10 [16, 17]. Despite this, murine in vivo models of intestinal inflammation and also allergic inflammation in the lung show that IL-10 and TGF- β are clearly involved in the suppression by these cells [18–20]. The increased numbers of IL-10 producing T cells following SIT are primarily found among the CD4⁺CD25⁺ T cells [6, 21–23]. Whether these cells are CD25⁺FOXP3⁺ Treg, Tr1 cells, or activated CD25⁺ T cells that have deviated into a IL-10 secreting phenotype is still not clear.

We and others have previously shown that the Th2-suppressive capacity of putative CD25⁺ Treg from allergic individuals is lower compared with CD25⁺ Treg from non-allergic controls [24–26]. Thus, we showed that CD25^{pos} T cells from birch pollen-allergic individuals were unable to suppress the production of IL-5 and IL-13 during birch pollen season, while the capacity to suppress interferon gamma (IFN- γ) production and proliferation was retained [26]. A reduced capability of CD25^{pos} T cells to suppress IL-5 production as well as proliferation by CD25^{neg} T cells was shown in grass pollen-allergic individuals as compared to healthy controls, particularly during the grass pollen season and also to a lesser extent during the outside season [25]. In a recent study, allergen-specific CD4⁺CD25⁺FOXP3⁺ Treg that were able to suppress proliferation were shown to be present in peripheral blood of both healthy and allergic individuals [27]. Thus, other types of regulatory T cells than natural CD25⁺ Treg were suggested to be defective in allergic individuals. However, this study did not address whether these allergen-specific cells were able to inhibit the production of Th1 or Th2 cytokines.

We here tested the hypothesis that SIT might restore the Th2-suppressive capacity of CD25^{pos} T cells by comparing the function of CD25^{pos} T cells obtained from SIT patients and birch pollen-allergic controls on birch allergen-induced T-cell responses during birch pollen season. Moreover, we measured the IL-10 production from T-cell fractions from SIT patients and birch pollen-allergic controls and the capacity of IL-10 to block IFN- γ and IL-5 after allergen stimulation. Our results demonstrate that SIT does not restore the capacity of allergen-stimulated CD25^{pos} Treg to suppress Th2 responses in vitro during the first season in spite of increased IL-10 production by T cells from SIT patients.

Methods

Subjects

This study included 11 birch pollen-allergic patients aged 17 to 38 who had received SIT with birch pollen extract for

6 months and ten birch allergic patients aged 25 to 51 who served as birch pollen-allergic controls. All patients had a history of birch pollen-induced moderate or severe rhinoconjunctivitis with a duration of at least 4 years. Each patient also had a positive skin prick test result to *Betula verrucosa* (Soluprick SQ 10 HEP *B. verrucosa*, ALK-Abelló, Hørsholm, Denmark) and was positive for specific IgE to *B. verrucosa*. The SIT patients had a median-specific IgE value of 20.1 kU/L (range 3.96–100 kU/L) and the controls had a median-specific IgE value of 17.2 kU/L (range 4.6–46.6 kU/L). Specific IgE was measured by ImmunoCAP (Phadia, Uppsala, Sweden). Peripheral blood (200 mL) was obtained by venous puncture and collected into preservative-free heparin tubes. The study was approved by the Human Research Ethics Committee of the Medical Faculty, Göteborg University.

Immunotherapy Protocol

SIT was started with an induction phase constituted of increasing doses of Alutard SQ *B. verrucosa* given subcutaneously at weekly intervals. Alutard SQ is a depot extract containing water-soluble allergen protein with aluminum hydroxide as adjuvant. The starting dose was between 10 and 20 SQ of the allergen extract and the induction phase covered 6–16 weeks. When the maintenance dose of 1 mL of 100,000 SQ/mL Alutard SQ *B. verrucosa* (containing 12.3 μ g of Bet v1-major allergen of birch pollen) was reached, injections were given at intervals of 6 weeks. SIT-treated patients received on average 74 μ g of Bet v1 (range 53–140) distributed over 17–22 injections up to seasonal blood sampling. Previous studies using this protocol have shown clinical improvement of SIT patients at an early stage of the treatment [3, 28].

Isolation and Characterization of Cells and Flow Cytometry

Lymphocytes were separated by Lymphoprep™ (Nycomed, Oslo, Norway) gradient centrifugation. CD4⁺ cells were purified with Dynabead® CD4 Positive Isolation Kit (Dyna Biotech ASA, Oslo, Norway). In order to favor isolation of CD25^{pos} Tregs rather than activated CD25^{pos} effector cells, the CD4⁺ cells were incubated with suboptimal amounts of beads (3 μ L per 10⁷ cells) instead of the bead/cell ratio recommended by the manufacturer (10 μ L per 10⁷ cells) [29, 30]. CD25^{neg} and CD25^{pos} fractions were recovered using a LS-magnetic column according to manufacturer's instructions (Mileny Biotech, Bergisch Gladbach, Germany). PBMCs were depleted of CD3⁺ cells (Dynabeads®), γ -irradiated (25 Gy), and used as APC. Purity of cell populations was determined by flow cytometry using the following monoclonal antibodies (mAb) obtained from Becton Dickinson (Erembodegem, Belgium): PerCP-anti-

CD4 (SK3), APC-anti-CD3 (SK7) and PerCP-, APC-, or PE-isotype control IgG₁. PE-anti-CD25 (clone M-A251) was obtained from BD Pharmingen (San Diego, CA, USA). The purity of the CD4⁺ cells was >90%, and the purity of the CD25^{pos} cells was >94%. The proportion of CD25^{high} T cells within the CD4⁺ T-cell fraction was 2.6% (median) in SIT patients and 2.7% (median) in allergic controls, and the proportion of CD25^{high} T cells within the CD25^{pos} T-cell fraction was 33% (median) in SIT patients and 35% (median) in allergic controls.

FOXP3 Expression by Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from 10⁵ cells using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and treated with DNase-I (DNA-free, Ambion, Austin TX, USA). Complementary DNA was prepared in a random hexamer-primed Superscript RT reaction (Invitrogen, Stockholm, Sweden) according to the manufacturer's protocol. *FOXP3* messenger RNA (mRNA) levels were measured in duplicate using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany), with reagents from the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) and the primers: 5'-CAG CAC ATT CCC AGA GTT CCT-3' (forward) and 5' GCG TGT GAA CCA GTG GTA GAT-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous reference gene for relative quantification and detected by the primers: 5'-GGC TGC TTT TAA CTC TGG-3' (forward) and 5'-GGA GGG ATC TCG CTC C-3' (reverse). Primers were ordered from TIB MOLBIOL (Berlin, Germany) and designed not to amplify genomic DNA. Polymerase chain reaction cycling conditions were: 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 65°C for 7 s, and 72°C for 10 s. Data were collected using the LightCycler Data Analysis Software, and this software was used for calibrator-normalized relative quantification.

Cell Culture

The suppressive capacity and the IL-10 production by different cell fractions were determined by culturing 50×10³ CD4⁺CD25⁻ T cells with 50×10³ CD4⁺CD25⁺ T cells and 200×10³ APC (irradiated CD3-depleted PBMC) for 6 days in round-bottomed 96-well plates in X-Vivo15 medium (Bio Whittaker, Walkersville, MD, USA). CD4⁺CD25⁻ T cells (100×10³ and 50×10³) and CD4⁺CD25⁺ (50×10³) were also cultured separately in the presence of APC. Culture with 100×10³ CD4⁺CD25⁻ T cells (double negative CD25⁻:CD25⁻ co-culture) served as control for the co-cultures with 50×10³ CD4⁺CD25⁻ and

50×10³ CD4⁺CD25⁺ T cells, as it contained the same amount of cells. Cultures were stimulated with 50 µg/ml of birch pollen extract (Greer Laboratories, Lenoir, NC, USA). The suppressive role of IL-10 was analyzed by blocking the binding of IL-10 with 5 µg/mL anti-IL-10R (clone 37607.11, R&D Systems, Minneapolis, MN, USA) using 5 µg/mL IgG₁ as isotype control (clone 11711.11, R&D Systems). At the end of the culture period, supernatants were collected and the medium was added back to the cells prior to pulsing overnight with 1 µCi ³H-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK). Plates were harvested with a Filtermate 196 cell harvester (Packard) and ³H-thymidine incorporation was measured with a Matrix 96 Direct Beta Counter (Packard).

Enzyme-Linked Immunosorbent Assay

Supernatants were collected at the last day of culture and stored at -20°C until analysis. A standard enzyme-linked immunosorbent assay (ELISA) protocol was performed as described elsewhere [31]. All antibodies and standards were purchased from BD Pharmingen. Costar plates (Invitrogen, San Diego, CA, USA) were coated with the following anti-human capture mAb: IL-5 (TRFK5), IFN-γ (NIB42), and IL-10 (JES3-9D7). Standard curves were generated using recombinant human IL-5, IFN-γ, and IL-10, respectively. The following biotinylated mAbs were used: IL-5 (JES1-5A10), IFN-γ (4S.B3), and IL-10 (JES3-12G8). Samples, standards, biotinylated antibodies, and streptavidin-horseradish peroxidase were diluted in high performance ELISA dilution buffer (Sanquin, Amsterdam, Netherlands).

Statistics

Data were analyzed by two-tailed Mann–Whitney *U* test and Wilcoxon-signed rank test using GraphPad Prism 3.00 (GraphPad, San Diego, CA, USA). *P* values of ≤0.05 were regarded as statistically significant: **P*<0.05, ***P*<0.01, and ****P*<0.001.

Results

Proportions of FOXP3⁺ and CD25^{pos} T Cells in Peripheral Blood from SIT Patients and Birch Pollen-Allergic Controls During Birch Pollen Season

First, we wanted to explore if the frequencies of circulating CD25^{pos} regulatory T cells were increased in birch pollen-allergic patients after 6 months of SIT compared to allergic controls. To address this, we first analyzed if SIT patients and allergic controls differed in their expression level of *FOXP3* mRNA. Cells in the CD25^{pos} fraction expressed

14-fold higher ratios of *FOXP3* mRNA compared to cells in the CD25^{neg} fraction, but there was no difference in *FOXP3* expression level between SIT patients and controls neither in the CD25^{pos} nor in the CD25^{neg} cell fraction (Fig. 1a). We also examined if the percentage of CD25^{pos} or CD25^{high} T cells (putative CD25⁺ Treg) differed between SIT patients and controls. Similar proportions of CD25^{pos} and CD25^{high} T cells were observed within freshly isolated CD4⁺ T cells from SIT patients and allergic controls (Fig. 1b). In addition, no differences in mean fluorescence intensity were detected between the groups (data not shown). Altogether, these results indicate that SIT does not elevate the proportions of total CD25^{pos} Treg in peripheral blood.

Capacity of CD25^{pos} T Cells to Suppress Birch Pollen-Induced Proliferation and Cytokine Production in Birch Pollen-Allergic SIT Patients and Controls

To address whether SIT might be able to restore the suppressive ability of CD25^{pos} T cells on Th2 effector cells, we collected blood from SIT patients and birch pollen-allergic controls during birch pollen season and compared the capacity of the CD25^{pos} T cells from the two groups of patients to inhibit the proliferation and the IFN- γ and IL-5 production. The CD25^{pos} T cells from both SIT patients and controls significantly suppressed the proliferation of CD25^{neg} T cells (Fig. 2a). CD25^{pos} T cells from both groups also significantly suppressed the production of IFN- γ (Fig. 2b). However, CD25^{pos} T cells from neither SIT patients nor birch pollen-allergic controls were able to suppress the production of IL-5 (Fig. 2c). In fact, the capacity of the putative CD25⁺ Treg to suppress IL-5 seems to be reduced after SIT since they failed to suppress even the contaminating effector T cells that were present within

the CD25^{pos} T-cell culture. Thus, the suppression of Th2 cytokines by CD4⁺CD25⁺ regulatory T cells was not restored during the first birch pollen season after the initiation of SIT.

Birch Allergen-Induced IL-10 Production Is Increased only in Co-cultures of CD25^{pos} and CD25^{neg} T Cells from SIT Patients

A highly consistent finding after SIT is detection of IL-10 producing T cells. These T cells have previously been shown to be contained within the CD4⁺CD25⁺ T-cell fraction [6, 21–23]. However, conventional CD25^{neg} T cells upregulate CD25 on their surface after in vitro stimulation. Therefore, we separated the CD25^{pos} cells from the CD25^{neg} cells prior to stimulation to identify the T-cell fraction that produced IL-10. In accordance with previous results, we observed significantly increased levels of IL-10 in SIT patients as compared to birch pollen-allergic controls. However, the rise in IL-10 production after birch allergen stimulation was only observed when CD25^{neg} and CD25^{pos} T cells were cultured together and not when these cells were cultured separately or when CD25^{neg} T cells as a control were co-cultured with CD25^{neg} T cells (Fig. 3). Thus, IL-10 was produced in higher levels from T cells of birch pollen-allergic patients following SIT compared to controls and only from the cultures in which both CD25^{neg} and CD25^{high} T cells were present.

IFN- γ and IL-5 Production After Blocking the Effects of IL-10 in the T-Cell Cultures from SIT Patients and Controls

To further evaluate the role of IL-10, we added antibodies against IL-10 receptor (IL-10R) or isotype control to the

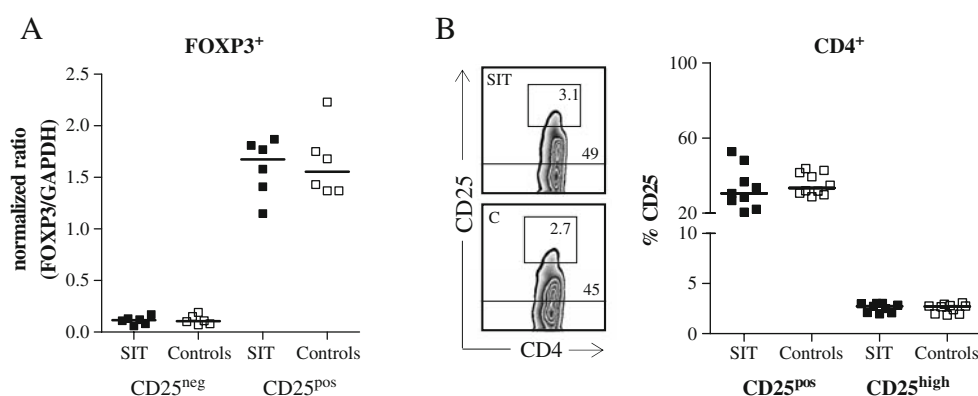


Fig. 1 FOXP3 expression and proportion of CD25^{pos} T cells in SIT patients and birch pollen-allergic controls. **a** Normalized ratios of FOXP3/GAPDH in the CD25^{neg} and the CD25^{pos} T-cell fractions in SIT patients and allergic controls. **b** Representative zebra plots show

the gates for CD25^{pos} and CD25^{high} and a summarizing graph show the proportions of CD25^{pos} cells and CD25^{high} T cells within the CD4⁺ T-cell fraction isolated from peripheral blood from SIT patients and allergic controls. Median values are depicted as a horizontal line

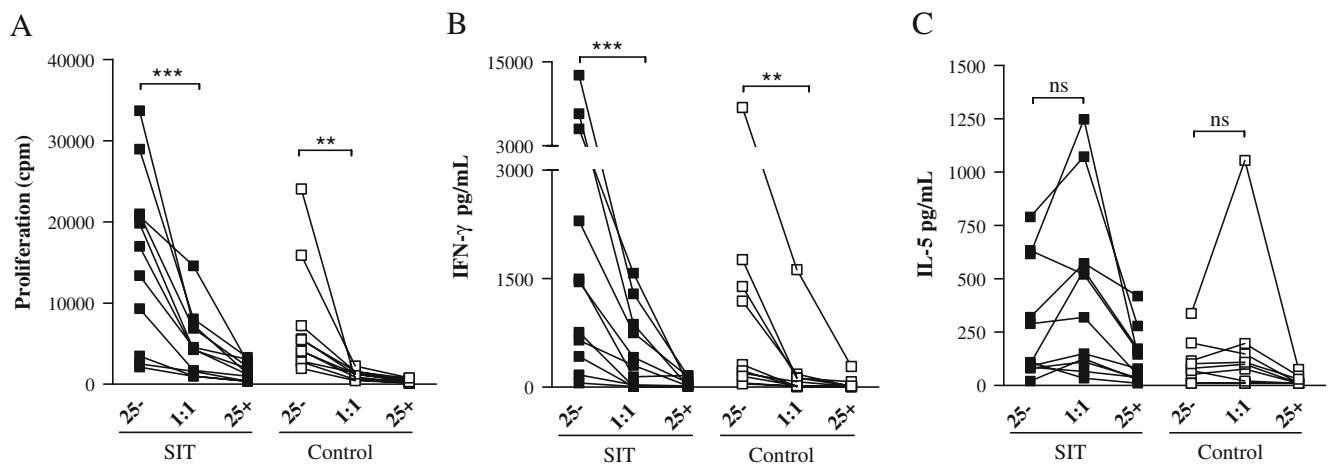


Fig. 2 Capacity of CD25^{pos} T cells to suppress proliferation and production of IFN- γ and IL-5 in birch pollen-allergic SIT patients and controls. Individual proliferation counts (a), IFN- γ production (b), and IL-5 production (c) of CD25^{neg} cells alone, CD25^{neg} cells in a co-culture with CD25^{pos} (ratio 1:1), and CD25^{pos} cells alone in SIT

patients (filled squares), and allergic controls (open squares) after stimulation with birch allergen extract for 6 days. Values from each individual are connected with a line and statistical differences between cell cultures were calculated using the Wilcoxon-signed rank test. ** $P < 0.01$, *** $P < 0.001$

birch allergen-stimulated cultures of T cells from SIT patients and controls. We found that blocking the effects of IL-10 significantly increased the levels of IFN- γ in the CD25^{neg}/CD25^{pos} co-cultures from SIT patients but not from allergic controls, showing that IL-10 had a T-cell inhibitory function (Fig. 4a). Of note, the production of IFN- γ by cells in the CD25^{pos} T-cell fraction from SIT patients, but not from allergic controls, was also increased after the addition of anti-IL-10R. In contrast, neither the production of IL-5 (Fig. 4b) nor the proliferative response (data not shown) was affected by the presence of anti-IL-10R. In summary, IL-10 appears to have a role in the suppression of IFN- γ production in SIT patients but not in controls, and it failed to suppress IL-5 production from both patient groups.

Clinical Evaluation During the Third Pollen Season

This study was conducted during the first birch pollen season after the start of immunotherapy. Previous studies have shown clinical improvement of SIT patients at that early stage [3, 29]. During the third pollen season after the start of immunotherapy, we recorded allergic symptoms such as runny/blocked nose or sneezing. The symptoms were scored as follows: 0, none; 1, slight; 2, moderate; and 3, severe. During 7 days in the week with the highest birch pollen counts, the daily symptom score was recorded and calculated as a weekly mean for each patient. From the means of all patients, a median symptom score was calculated for the two groups. The median symptom score in the SIT and birch-allergic patient groups were 1 (0–

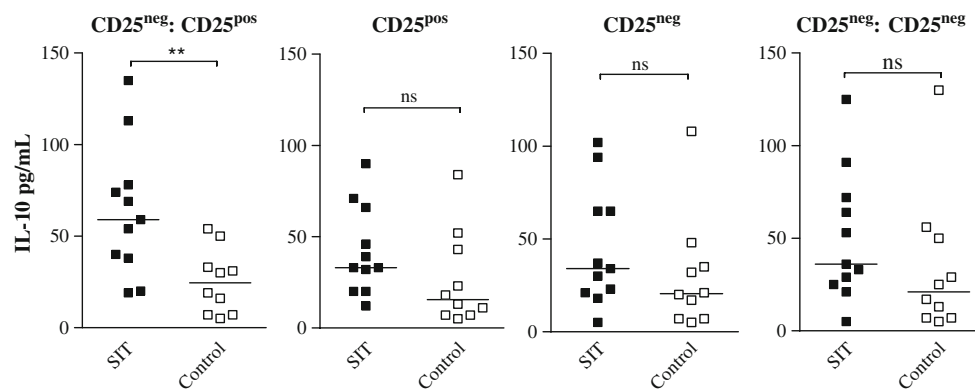


Fig. 3 Increased IL-10 production in SIT patients but not in controls in the co-culture. Individual levels of IL-10 in the cell fractions CD25^{neg}/CD25^{pos} (ratio 1:1), CD25^{pos}, CD25^{neg}, and CD25^{neg}/CD25^{neg} (ratio 1:1) from SIT patients (filled squares) and allergic

control patients (open squares) after stimulation with birch allergen extract for 6 days. Median values are shown as a line and statistical differences between SIT patients and controls were calculated using the Mann-Whitney U test. ** $P < 0.01$

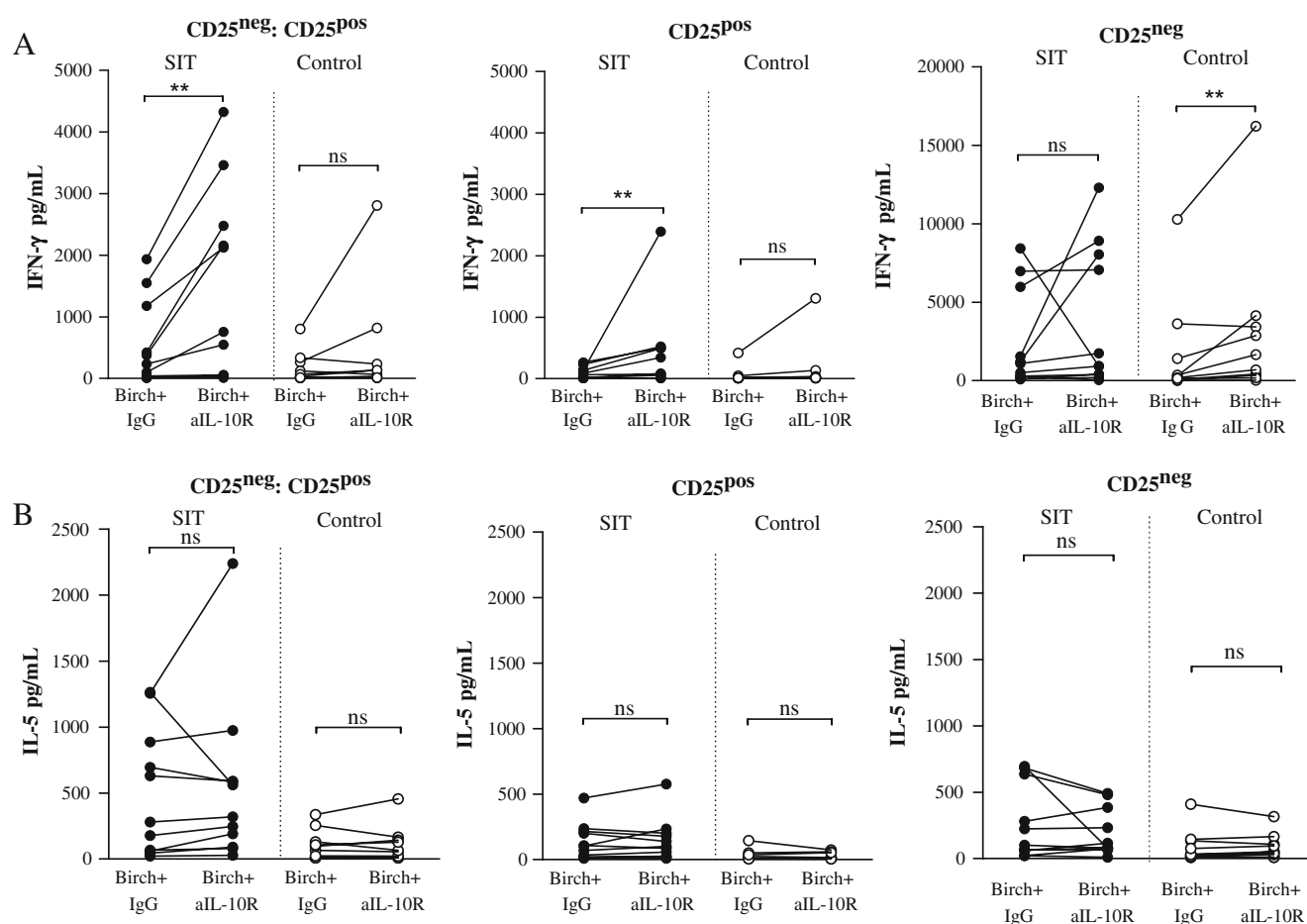


Fig. 4 Blocking IL-10R increased the levels of IFN- γ but not IL-5. Absolute individual levels of IFN- γ (a) and IL-5 (b) produced by CD25^{neg}/CD25^{pos} (ratio 1:1), CD25^{pos}, or CD25^{neg} T cells alone from SIT patients (filled circles) and allergic controls (open circles) after

stimulation with birch allergen extract and anti-IL-10 receptor Ab or IgG control Ab for 6 days. Values from each individual are connected with a line and statistical differences between cell cultures were calculated using the Wilcoxon-signed rank test. ** $P < 0.01$

1.29) and 1.57 (0.57–3), respectively, and the difference between groups was significant ($P = 0.015$). Thus, SIT patients showed milder symptoms of birch pollen-induced rhinoconjunctivitis compared to allergic controls, while there were no differences between the groups with respect to medication.

Discussion

IL-10-producing regulatory T cells have been suggested to play an important role in the mechanism of SIT, but it is not known whether the suppressive capacity of CD25⁺ Treg from allergic individuals is increased after treatment with SIT. In this work, we show for the first time that the suppressive capacity of allergen-stimulated CD25^{pos} Treg in vitro is not improved by SIT during the first season after initiation of the treatment in spite of an increased IL-10 production from T cells compared to controls. A second novel finding is the observation that IL-10 was produced in higher levels by SIT

patients than controls only when CD25^{neg} and CD25^{pos} T cells were cultured together and that IL-10 did not control IL-5 production in contrast to IFN- γ production. Moreover, we found that SIT patients and birch pollen-allergic controls have similar proportions of total CD25⁺ Treg in the circulation during the birch pollen season.

We observed similar levels of *FOXP3* mRNA in fresh magnetic bead-isolated CD25^{pos} T cells from SIT patients and allergic controls indicating that 6 months of SIT does not lead to a measurably increased peripheral induction of total CD25⁺ Treg. Moreover, we observed no difference between birch pollen-allergic SIT patients and allergic controls regarding the proportions of CD25^{pos} or CD25^{high} T cells. In accordance with our results, a recent study also showed no significant changes in the levels of FOXP3⁺ T cells before as compared to after 1 year of SIT with house dust mite allergen [32]. However, nasal mucosa of grass pollen-allergic patients who have received SIT for 2 years contained significantly elevated numbers of FOXP3⁺ cells compared to allergic controls patients and healthy controls

[33]. Thus, it is possible that the absence of increased numbers of FOXP3⁺ T cells in the circulation might be due to homing of FOXP3⁺ T cells into the inflammatory site. In a mouse model of Th2-type airway inflammation, it was shown that activated allergen-specific Treg cells upregulated CCR4 and migrated into the inflammatory site where they suppressed the proliferation and cytokine production by polarized Th2 cells [34]. In summary, we conclude that SIT does not result in increased proportions of CD25⁺ Treg in the circulation of birch pollen-allergic patients. However, we cannot exclude that the CD25⁺ Treg in the circulation after SIT may contain a higher frequency of allergen-specific cells compared to untreated patients.

We and others have previously shown that putative CD25⁺ Treg from allergic individuals are less effective than cells from healthy controls in suppressing Th2 but not Th1 cytokine production in response to allergen, especially during the pollen season [24–26]. To explore whether SIT treatment would restore the capacity of CD25^{pos} T cells to suppress the production of Th2 cytokines, we herein for the first time compared the ability of CD25^{pos} T cells to suppress cytokine production of CD25^{neg} T cells from SIT patients and birch pollen-allergic controls after stimulation with birch pollen extract. We show that SIT during 6 months did not restore the suppression of IL-5 by the CD25^{pos} T cells and that the suppression of IFN- γ remained. However, Th2 responses are probably harder to suppress than Th1 responses not only by CD25⁺ Treg from allergic individuals but also by CD25⁺ Treg from non-allergic individuals. For example, it was found that birch allergen-induced production of IL-4 and IL-5 was not suppressed by CD25^{pos} T cells from neither birch pollen-allergic patients nor healthy controls [35]. Also, Th2 clones have been suggested to be less susceptible than Th1 clones to suppression by human thymus-derived CD4⁺CD25⁺ T-cell clones [36]. In a mouse model of autoimmune gastritis, co-transfer of CD4⁺CD25⁺ Treg was able to suppress the Th1-mediated disease while they were more resistant to suppress the Th2-induced disease and were not effective in suppressing Th17-mediated disease [37]. Consistent with the findings indicating that Th2 responses are harder to suppress than Th1 responses, we observed a selective suppressive effect of IL-10 on the production of IFN- γ , while neither effect on the production of IL-5 nor the proliferative response was observed. One explanation for this finding could be that in the early phase of SIT activation of Th2 cells might dominate over activation of Th1 cells and that more strongly activated T cells would be harder to suppress than less activated T cells not only by CD25⁺ Treg but also by IL-10.

A consistent immunological finding after SIT in allergic patients is increased IL-10 production from T cells. In a mouse model of SIT, in which the treatment was effective to suppress allergen-induced asthma, the beneficial effects of SIT were to

a large extent abrogated after blocking the IL-10 receptors [38]. Several studies have analyzed the levels of intracellular IL-10 in PBMC before and during the first year of SIT following stimulation with allergen and found that IL-10 is mainly present in the CD4⁺CD25⁺ T-cell subset [6, 21–23]. However, conventional CD25^{neg} T cells express CD25 on the surface after in vitro stimulation, and therefore, we separated the CD25^{pos} cells from the CD25^{neg} cells prior to stimulation in order to identify the T-cell fraction that produced IL-10. In accordance with previous studies, we observed higher levels of IL-10 in cultures from SIT patients relative to allergic controls. However, the levels were significantly higher only when CD25^{neg} T cells were cultured together with CD25^{pos} T cells and not when the CD25^{neg} or CD25^{pos} T cells were cultured separately. In a study comparing a patient deficient in CD25 with a patient deficient in FOXP3, it was shown that the CD4⁺ T cells of the FOXP3^{neg} patient were able to express IL-10, whereas the FOXP3^{pos}CD25^{neg} patient failed to express IL-10 [39]. This indicates that stimulation of the IL-2 receptor α (CD25) by IL-2 produced by CD4⁺ T cells is important for production of IL-10 by CD25^{pos} T cells. Consequently, it is possible that the CD25^{pos} T cells produce IL-10 in vivo after SIT since IL-2 producing T cells are always present. Another possibility would be that the CD25^{pos} cells in SIT patients might induce the CD25^{neg} cells to become IL-10-producing Tr1 cells. This explanation is supported by data from a study of murine airway inflammation in which transfer of allergen-specific CD25⁺ Treg cells reduced airway hyperreactivity and recruitment of eosinophils and Th2 cells in the lung while increasing the levels of pulmonary IL-10 produced by CD4⁺ T cells but not from the CD25⁺ Treg themselves [20]. Also, human CD25⁺ Treg have been reported to be able to transfer suppressive properties to CD25^{neg} T cells in a contact-dependent manner by inducing IL-10 or TGF- β [40, 41].

In summary, this paper demonstrates that the Th2-suppressive capacity of allergen-stimulated CD25^{pos} Treg in vitro is not improved during the first season after the initiation of SIT. Our data also indicate that IL-10 is produced at higher levels in SIT patients relative to controls and that both CD25^{pos} T cells and CD25^{neg} T cells are important and need to be present for this production to occur. However, further studies are needed to explore the interplay between CD25⁺ Treg and IL-10-producing Tr1 cells and their exact role in SIT.

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