

Induction of CD4⁺CD25⁺Foxp3⁺IL-10⁺ T Cells in HDM-Allergic Asthmatic Children with or without SIT

Wang Wei Yongge Liu Yonghong Wang Yuhong Zhao Jianxin He Xiang Li
Kunling Shen

Asthma Center, Beijing Children's Hospital, Capital Medical University, Beijing, PR China

Key Words

CD4⁺CD25⁺Foxp3⁺ · Children · House dust mite · IFN- γ · IL-4 · IL-10 · Regulatory T cells · Specific immunotherapy · TGF- β_1

Abstract

Background: Regulatory T cells and immunosuppressive cytokines, such as IL-10 and TGF- β_1 , may have a role in clinically effective allergen-specific immunotherapy. IL-10-secreting regulatory T cells have emerged as potential mediators of immune tolerance in numerous murine models of immunopathology. The aim of this study was to evaluate the frequency and function of regulatory T cells in the response to house dust mite (HDM) immunotherapy. **Methods:** PBMCs were isolated from 27 HDM-allergic asthmatic children who underwent immunotherapy for 1.5–2 years (SIT group) and from 27 matched treated asthmatic children allergic to HDM (asthma group). After 48 h of in vitro stimulation with HDM extracts, regulatory T cells were measured by flow cytometry. Production of IL-4, IFN- γ and TGF- β_1 in supernatants from allergen-stimulated cultures and the PBMC proliferations were measured by ELISA. Sera were tested for allergen-specific IgE using the ImmunoCAP 100 assay. **Results:** Patients undergoing immunotherapy produced significantly more IL-10 and showed a significant reduction in prolifera-

tion induced by HDM extract compared with the asthma group. In cultures stimulated with HDM extract, the amounts of IL-4 and TGF- β were lower and the amounts of IFN- γ were higher in the SIT group compared with the asthma group. **Conclusion:** There is a functional, but quantitative, insufficiency of Treg cells in allergic asthmatic children, which was reversed in SIT-treated children. SIT can up-regulate the function of CD4⁺CD25⁺Foxp3⁺ Treg cells.

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Introduction

The interaction of environmental and genetic factors can lead to the development of atopic disorders in some individuals, but not in others, following allergen exposure. The generation of allergen-specific CD4⁺ T helper (Th) cells provides the initial event responsible for the development of allergic diseases. The current view is that IL-4-influenced naive T cells differentiate into Th2 cells when activated by antigen-presenting cells [1]. These effector Th2 cells produce IL-4, IL-5 and IL-13, which mediate several regulatory and effector functions. These functions include allergen-specific IgE production by B cells, eosinophil development and recruitment, mucus production and smooth muscle contraction, as well as tis-

Table 1. Subject characteristics

	Asthma subjects	Patients undergoing immunotherapy
Subjects, n	27	27
Sex, m/f	19/8	19/8
Age, years	8.50 ± 2.47	9.79 ± 2.14
Duration, years	3.83 ± 1.89	4.46 ± 2.19
FEV ₁ , %	102.94 ± 16.41	102.50 ± 13.72
HDM-specific IgE (class)	4.52 ± 1.50	4.37 ± 1.04
HDM-specific IgE, kU/ml	122.16 ± 144.79	81.68 ± 107.65

Data are expressed as means ± SEs.

sue homing of Th2 cells [2, 3]. Th1 cells, on the other hand, may contribute to chronicity and the effector phase in allergic disease [4, 5].

A further subset of T cells, Treg cells, have an immunosuppressive function and cytokine profile distinct from that of either Th1 or Th2 cells [6]. Treg cells are able to inhibit the development of allergic Th2 cell responses, as well as effector Th1 cell responses, and therefore play an important role in allergen-specific immunotherapy [7]. The suppressive activity of Treg cells is related to their ability to inhibit IL-2 production and to promote cell cycle arrest in CD4⁺ and CD8⁺ T cells via mechanisms that probably require cell-to-cell contact and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-mediated signals. Another possible role is associated with their ability to secrete or to induce other cells to secrete immunosuppressive cytokines, such as IL-10 and TGF-β₁ [8].

The precise mechanism of house dust mite (HDM)-specific immunotherapy (SIT) is not well understood. The change in frequency and function of Treg cells in asthmatic children undergoing HDM-SIT is still not clear. Therefore, the aim of this study was to determine whether effective SIT could up-regulate the frequency and function of CD4⁺CD25⁺ forkhead box P3 (Foxp3)⁺ Treg cells in HDM-allergic children, providing an insight into the possible mechanism of HDM-SIT.

Methods

Patient Characterization

Twenty-seven children with mild asthma caused by HDM allergy, and 27 HDM-allergic asthmatic children who underwent HDM immunotherapy for 1.5–2 years were recruited for this study. The definition of asthma was based on the Global Initiative for Asthma guidelines. Subjects undergoing immunotherapy had

received subcutaneous injections of standardized HDM allergen extract (Alutard-SQ; ALK-Abello, Denmark) for 1.5–2 years and were well matched to the asthma group for age, sex, duration, pathogenic conditions, symptom score, medication requirements, lung function test and HDM-specific IgE levels. The asthmatic group was also matched to the SIT group prior to SIT for age, sex, duration, pathogenic conditions, lung function tests and HDM-specific IgE levels. All of the children received small-to-medium-dose inhaled glucocorticoids (Pulmicort Turbuhaler). When necessary, β₂ agonists were also given. Children with concomitant sensitization to perennial allergens, such as cat and dog, and to seasonal pollen allergens that induce allergic symptoms lasting longer than 4 months per year were excluded from the study (based on a clear-cut clinical history and positive skin test reactions greater than or equal to class 2). The HDM-specific IgE levels were determined using the fluoroenzyme immunoassay, Immuno CAP 100.

Physical examination and routine laboratory analyses (C-reactive protein, erythrocyte sedimentation rate and leukocyte counts) were performed to exclude other disorders. Studies were performed with the approval of the Trust Ethics Committee of the Beijing children's hospital affiliated to Capital Medical University. Written informed consent was obtained from the parents of all patients included in the study. The study subjects' clinical characteristics are summarized in table 1.

Antigens

Freeze-dried HDM, *Dermatophagoides pteronyssinus*, kindly provided by ALK-Abello, Denmark, was prepared as an aqueous extract in PBS and sterilized. The concentration of the extract was determined by the dry weight of freeze-dried HDM per volume of PBS. Endotoxin (LPS) levels in the HDM extract were below detectable (3 U/ml).

Standardized HDM Allergen Extract and Immunotherapy Protocol

Standardized Der p extract (Alutard-SQ, ALK-Abello, Denmark) was used for immunotherapy. During the induction period, children were given subcutaneous injections of Der p according to the standard protocol, beginning at a dose of 20 SQ-U once a week, increasing weekly until being changed to a maintenance dose of 100,000 SQ-U by week 15. At the beginning of the maintenance period, children were injected every 2–4 weeks, and then every 8 weeks for 1.5–2 years.

Clinical Evaluation

Children were evaluated for a change in respiratory symptoms by using asthma symptom scores (0 = no symptoms, 1 = mild symptoms that did not interfere with some activities, and 3 = severe symptoms that interfered with many activities). Total symptom score was the sum of each individual symptom.

SIT-treated asthmatic children were evaluated at baseline (before SIT) and after 1.5–2 years (after SIT) during clinical examination. Allergic asthma children were evaluated after recruitment.

Culture of T Cells from Peripheral Blood

The heparinized blood samples were collected from the control group (asthma children allergic to HDM) and the SIT group (asthmatic children that underwent HDM immunotherapy for

1.5–2 years). PBMCs were isolated from heparinized blood by Ficoll-Hypaque Premium centrifugation (Company Tianjin TBD) gradient within 1 h of collection. Briefly, blood samples diluted with an equal volume of PBS were layered over the Ficoll-Hypaque Premium and centrifuged at 400 g for 30 min. PBMCs were collected from the interface, then washed twice with PBS and resuspended at 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% calf serum, 100 U/ml penicillin-streptomycin, and 2 mmol/l L-glutamine (Nanjing KeyGen Biotech.). Cell viabilities were more than 95% as examined by trypan blue exclusion assays. Cells were incubated with 20 µg/ml HDM extract in a final volume of 1 ml/well for 48 h at 37°C in a 5% CO₂ humidified incubator. Controls wells contained PBMCs in the absence of antigen (negative control) or PHA (positive control).

PBMC Proliferation Assays

Proliferation of PBMCs was measured by the cell counting kit-8 (Beyotime Institute of Biotechnology, China). PBMCs (5×10^4 cells/well) from 48-hour cultures were incubated in 96-well flat-bottom plates in triplicates in a final volume of 50 µl/well. Blank control wells in triplicates contained medium alone in a volume of 50 µl/well. Then, 5 µl of the cell-counting kit-8 reagent was added to each well. After culture for 2 h at 37°C in a 5% CO₂ humidified incubator, increase in cell number was measured by increase in the production of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] by absorbance at 450 nm using a Full-auto microplate EIA analyzer. The OD values were read automatically and the average value of each sample manually calculated. Finally, the stimulation index of the PBMCs was calculated as: $SI = (OD_{\text{HDM-stimulated}} - OD_{\text{blank control}}) / (OD_{\text{unstimulated}} - OD_{\text{blank control}})$.

Flow Cytometry Assay

To determine the percentage of CD4⁺CD25⁺Foxp3⁺ Treg cells and IL-10-secreting CD4⁺CD25⁺Foxp3⁺ Treg cells, cultured PBMCs were harvested 48 h later for flow cytometry assays. Brefeldin A (Alexis) (10 µg/ml) was added for the last 4 h of incubation. Cells were stained with a human Treg cell-staining kit according to the manufacturer's protocols. Briefly, cells were incubated with a cocktail of FITC anti-human CD4 (Beckman Coulter) and PerCPCy5.5 CD25 (eBioscience) in the dark at 4°C for 30 min and washed with staining buffer for further intra-nuclear staining of Foxp3 and IL-10. Cells were then fixed and permeabilized with fixation/permeabilization buffer in the dark at 4°C for 1 h, washed twice with permeabilization buffer and then blocked with normal rat serum (2% final volume) for 15 min. They were then incubated with a cocktail of PE anti-human Foxp3 (eBioscience) and Alexa 647 anti-human IL-10 (eBioscience) in the dark at 4°C for 45 min, and then washed twice with permeabilization buffer. Control suspensions were prepared using the same procedure, with fluorochrome-labeled normal murine immunoglobulins of the same isotype as the corresponding monoclonal antibody. All the labeled cells were resuspended in 200 µl staining buffer and analyzed by flow cytometry using a FACS-Calibur instrument (Becton Dickinson) and the Cell Quest software package (Becton Dickinson).

Enzyme Linked Immunosorbent Assay

Concentrations of IL-4, IFN-γ and TGF-β₁ (Bender MedSystems) in culture supernatants were determined by ELISA kits ac-

cording to the manufacturer's protocols. Culture supernatants were harvested at 48 h and stored at -70°C until analysis.

Immunological Tests

Serum allergen-specific IgE levels were measured by the Immuno CAP 100 assay (Phadia AB, Uppsala, Sweden).

Statistical Analysis

Statistical analysis was performed with the SPSS 16.0 software. Data from proliferations, flow cytometry and cytokine concentrations were analyzed using the Wilcoxon matched-pairs signed-rank test for paired data or the Mann-Whitney U test for unpaired data. The data are presented as means ± standard deviations.

Results

Effect of HDM-Specific Immunotherapy on the Frequency and Function of Treg Cells in PBMCs

First we determined the effect of HDM extract on the proliferation of PBMCs in the study groups. We found that the HDM-induced PBMC proliferation in the SIT group (1.03 ± 0.18) was significantly lower than that in the asthma group (1.51 ± 0.45 ; fig. 1).

We next investigated if there were any changes in the frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells in these cultures of PBMCs following HDM stimulation. We found that levels of CD4⁺CD25⁺FOXP3⁺ cells were unchanged, irrespective of HDM stimulation, in both the SIT and asthma groups (SIT group: unstimulated $7.67 \pm 1.68\%$, HDM-stimulated $7.59 \pm 1.54\%$; control group: unstimulated $7.92 \pm 1.69\%$, HDM-stimulated $8.07 \pm 1.69\%$). However, we detected an increase in the percentage of IL-10⁺CD4⁺CD25⁺Foxp3⁺ Treg cells in cultured PMBCs without HDM stimulation in the SIT group ($1.65 \pm 1.22\%$) compared with the asthma group ($0.97 \pm 0.91\%$, $p = 0.18$). This difference was even more striking in the HDM-stimulated cultures (SIT group $2.27 \pm 1.49\%$, control group $0.97 \pm 1.05\%$). The percentage of IL-10⁺CD4⁺CD25⁺Foxp3⁺ Treg cells in the SIT group was significantly increased after HDM stimulation, while it was not affected in the asthma group.

Cytokine Levels in Culture Supernatants

The levels of IL-4, IFN-γ and TGF-β₁ were measured from the culture supernatants collected from the above experiments. The baseline concentration of IL-4 was lower in supernatants from the SIT group (1.13 ± 0.44 pg/ml) than the asthma group (1.38 ± 0.47 pg/ml). After stimulation with the HDM extract, the concentration of IL-4 increased in both groups (SIT group 1.30 ± 0.68 pg/ml, asthma group 1.70 ± 0.82 pg/ml), but no statisti-

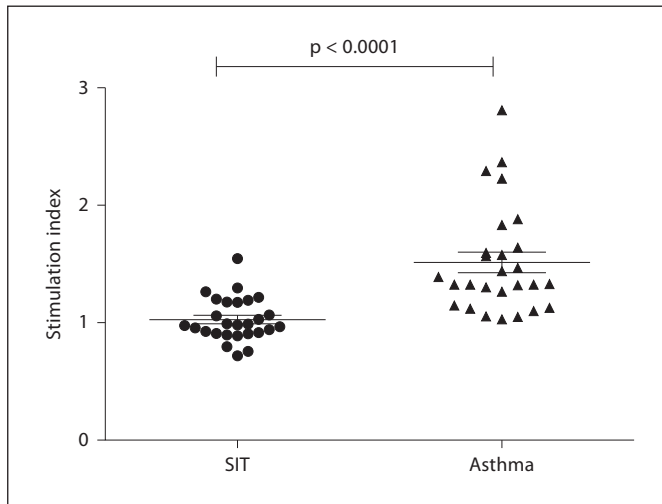


Fig. 1. Effects of HDM-specific immunotherapy on the proliferative response.

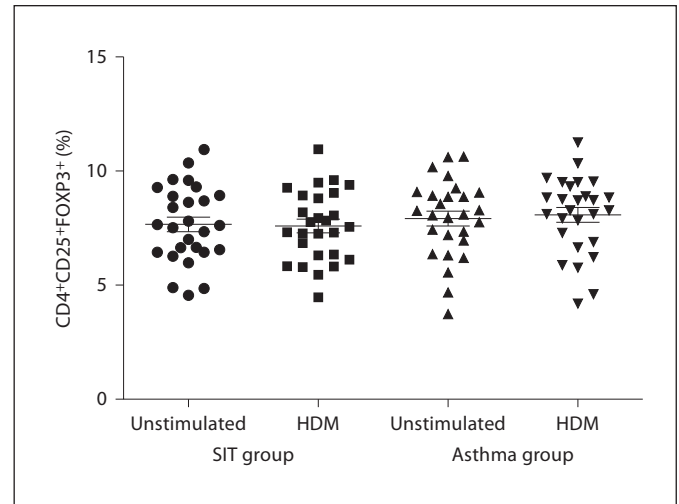


Fig. 2. Percentages of CD4⁺CD25⁺Foxp3⁺ T cells in cultured PBMCs derived from the SIT and asthma groups.

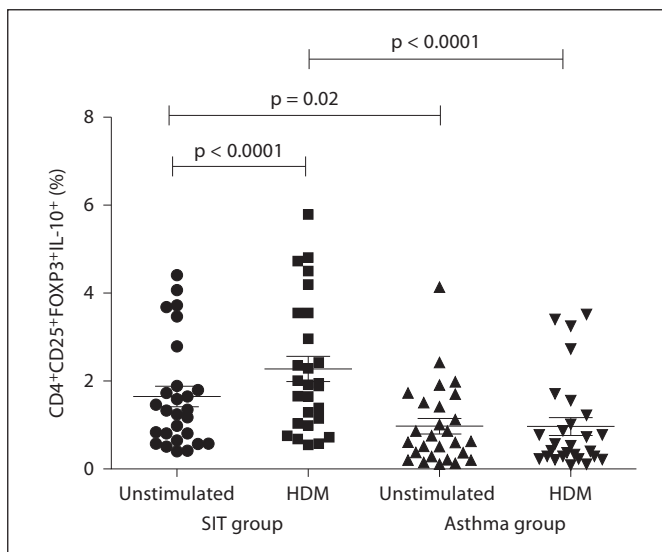


Fig. 3. Percentages of CD4⁺CD25⁺Foxp3⁺IL-10⁺ T cells in cultured PBMCs derived from the SIT and asthma groups.

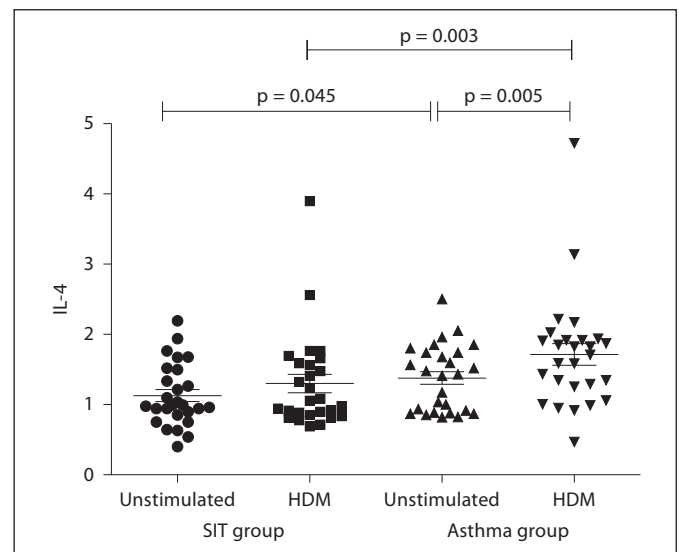


Fig. 4. Concentration of IL-4 in supernatants of cultured PBMCs derived from the SIT and asthma groups.

cal difference was found in stimulated conditions compared with baseline levels in the SIT group.

The baseline level of IFN- γ in supernatants from the SIT group (86.98 ± 81.89 pg/ml) was higher than those from the asthma group (45.92 ± 63.86 pg/ml). Stimulation with HDM extract caused the concentration of IFN- γ to increase in both the asthma group (66.84 ± 93.41 pg/ml) and the SIT group (123.27 ± 106.77 pg/ml).

The concentration of TGF- β_1 in supernatants from the SIT group (9.12 ± 6.21 ng/ml) was lower than in the asthma group (12.28 ± 5.30 ng/ml). Stimulation with HDM extract did not affect the levels of TGF- β_1 in the SIT group (8.57 ± 6.14 ng/ml), but it was significantly increased in the asthma group (14.79 ± 6.54 ng/ml).

Therefore, in baseline cultures, supernatants from the SIT group displayed lower concentrations of IL-4 and

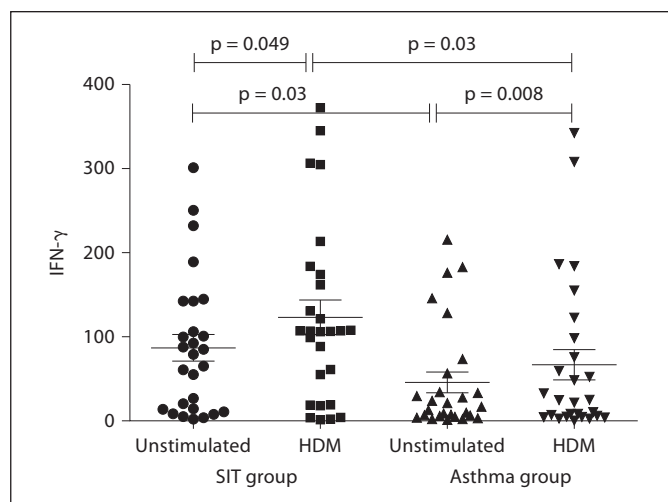


Fig. 5. Concentration of IFN- γ in supernatants of cultured PBMCs derived from the SIT and asthma groups.

TGF- β_1 but higher concentrations of IFN- γ than those from the asthma group. These differences were even more striking in HDM-stimulated cultures.

Discussion

SIT has been used for almost a century as a desensitizing therapy for allergic diseases and represents the only curative and specific method of treatment [9]. The balance between allergen-specific Treg and Th2 cells appears to be decisive in the development of allergic and healthy immune responses against allergens. The induction of a tolerant state in peripheral T cells represents an essential step in allergen-SIT. Peripheral T cell tolerance is characterized mainly by the generation of allergen-specific Treg cells leading to suppressed T cell proliferation and Th1 and Th2 cytokine responses against the allergen [10, 11]. Currently, the precise mechanism of immune tolerance induced by HDM-specific immunotherapy is not clear.

The aim of this study was to determine whether effective SIT could up-regulate the frequency and function of CD4⁺CD25⁺Foxp3⁺ Treg cells in HDM-allergic children. Our results demonstrated that the frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells was not different in HDM-stimulated compared with unstimulated cultures of PBMCs in both the asthma and SIT group. However, an increased percentage of IL-10-secreting CD4⁺CD25⁺Foxp3⁺ Treg cells was observed in cultured PMBCs without HDM

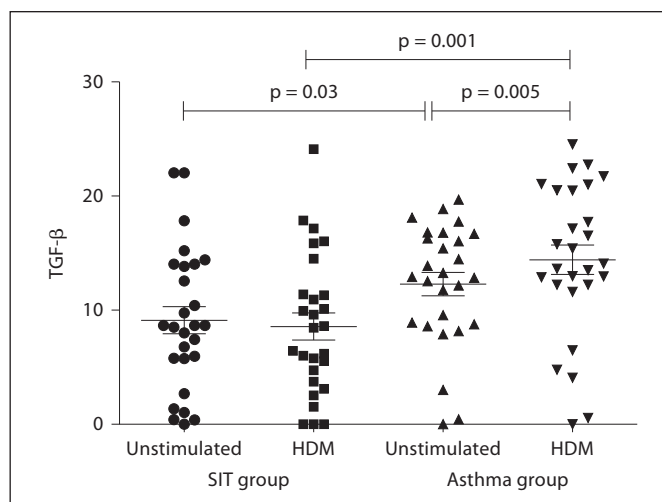
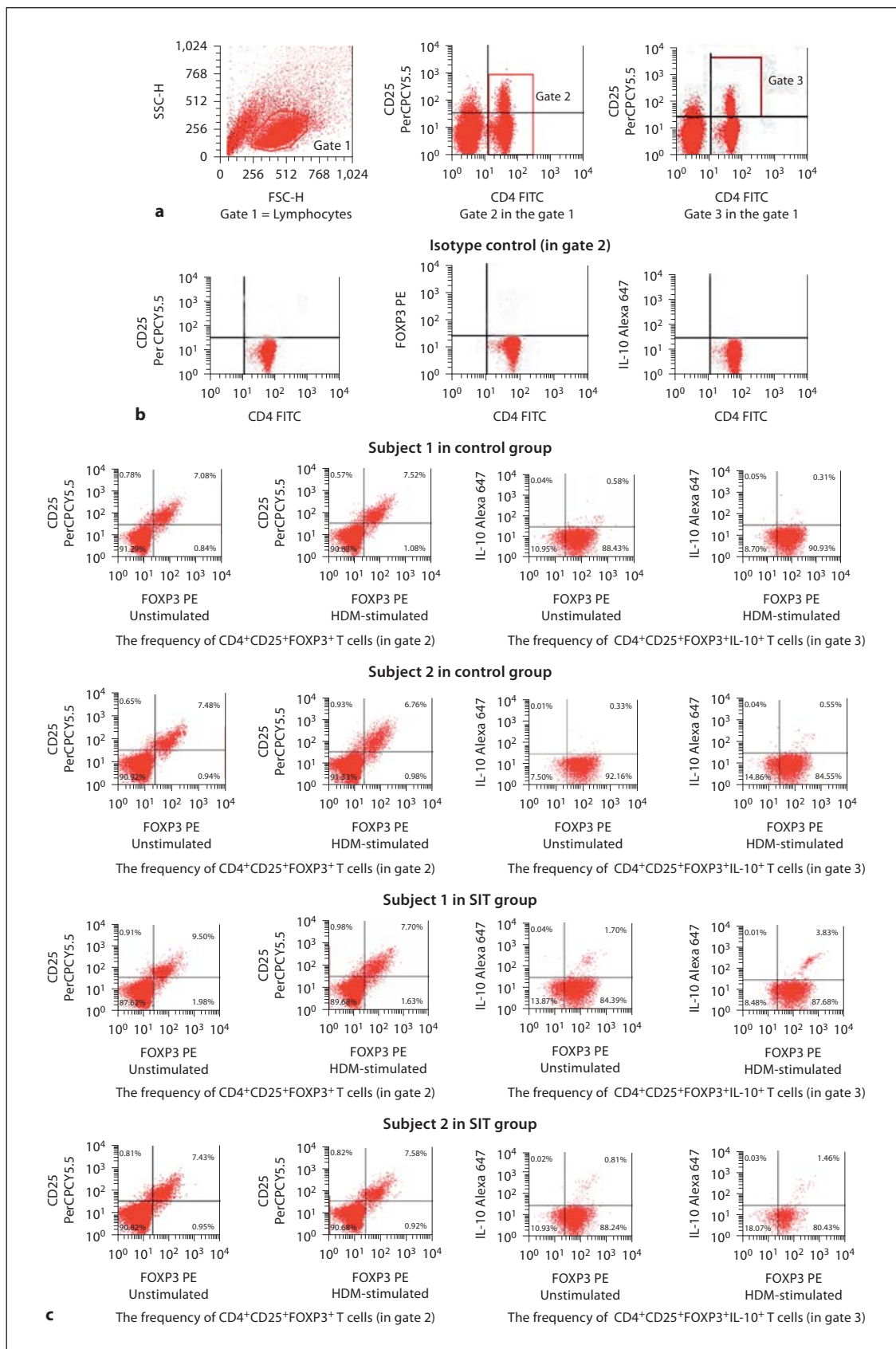


Fig. 6. Concentration of TGF- β_1 in supernatants of cultured PBMCs derived from the SIT and asthma groups.

stimulation in the SIT group compared with the asthma group, which was even more striking in HDM-stimulated cultures. Therefore, we concluded that there is a functional, but quantitative, insufficiency of natural (n)Treg cells in allergic asthmatic children. Furthermore, this impaired activity was reversed in SIT-treated children. It is known that SIT can up-regulate the frequency of IL-10- and TGF- β_1 -secreting nTreg cells inducing T cell immune tolerance. The suppression by these cells could be partially blocked by the use of neutralizing antibodies against secreted or membrane-bound IL-10 and TGF- β_1 .

Some studies have found that the frequency of CD4⁺CD25⁺ T cells is increased in asthmatic children receiving pollen-SIT or HDM-SIT [12, 13]. However, now we know that CD25 is an activation marker of T lymphocytes and not a specific marker of Treg cells. Additional surface and intracellular markers [such as the transcription factor Foxp3, CTLA-4, and IL-7 receptor- α (CD127)] are being investigated for further phenotypic characterization of these cells. It is now accepted that Foxp3 plays a key role in the development and function of nTreg cells and represents a specific marker for them [14, 15].

Two articles addressing the changes in CD4⁺CD25⁺Foxp3⁺ Treg cells in asthmatic children undergoing HDM-specific immunotherapy were published in 2008, with contradictory conclusions. Ajduk et al. [16] have demonstrated that the percentage of Foxp3⁺ Treg cells did not change after 1 year of immunotherapy. But Lin et al. [17] have demonstrated that HDM-specific im-



munotherapy can up-regulate the expression of Foxp3 and the functional activity of nTreg cells. Overall, the function of Treg cells in HDM-specific immunotherapy has not been clearly elucidated.

Antigen-specific T cell suppression by IL-10, a cytokine known to inhibit T cell proliferation and cytokine production, is essential in peripheral tolerance to allergens, autoantigens, transplantation antigens and tumor antigens. The inhibitory effect of IL-10 plays a key role in inducing anergy, and hence has great importance in allergen-SIT [18]. The presence of peripheral blood T cells that produce IL-10 in response to allergen stimulation after immunotherapy has emerged as a consistent finding from studies of venom immunotherapy [19, 20]. This phenomenon was not always observed in HDM-specific immunotherapy and pollen immunotherapy. Ajduk et al. [16] found that the percentage of IL-10⁺ T cells significantly decreased after 3 months of HDM-SIT in asthmatic children, while after 12 months of SIT the proportion of IL-10-secreting T cells was high in only 2 of 16 patients. For the first time, we detected the frequency of IL-10-secreting CD4⁺CD25⁺Foxp3⁺ T cells in asthmatic children receiving HDM-specific immunotherapy by 4-color flow cytometry, so we were able to determine the function of nTreg cells.

Unbalanced, Th2-like immune reactions against allergens such as HDM in the lungs lead to an impaired ability to regulate inflammation after exposure to allergens. SIT is an effective prophylactic treatment which can cause the deviation from a Th2 response to a Th1 response. Most studies that have addressed the effects of immunotherapy on T cell responses have used readouts based on the responses of T cells isolated from peripheral blood to allergen extracts in vitro. A number of studies of patients treated with venom or pollen immunother-

apy reported a reduction in the proliferation of peripheral blood T cells to allergen [21, 22]. Superimposed on this reduced reactivity was a shift away from Th2 towards Th1 responses after treatment. Nevertheless, in addition to the findings presented here, other studies have failed to observe similar changes after conventional grass pollen immunotherapy and HDM-SIT [16, 17, 23]. Overall, these findings suggest that peripheral blood T cell responses after immunotherapy are variable and that the induction of Th2/Th1 immune deviation is not a necessary feature of clinically successful immunotherapy. Our results have shown that HDM-specific immunotherapy can down-regulate the function of Th2 cells and up-regulate the function of Th1 cells. Consequently, the levels of IL-4 in supernatants were decreased and the levels of IFN- γ in supernatants were increased.

TGF- β_1 is an intercellular signaling molecule that demonstrates a plethora of biologic effects in both in vitro and in vivo contexts. TGF- β_1 is an important suppressive cytokine secreted by Treg cells and is essential for the maintenance of immunological self-tolerance in the CD4⁺ T cell compartment [24]. However, the evidence for a role of TGF- β_1 in maintaining immune homeostasis in the lung is more complex, as this cytokine exhibits both regulatory and pro-fibrotic actions. TGF- β_1 is produced by many different cells and inhibits a wide range of immune cells and functions. But on the other hand, TGF- β_1 may be important in the repair of asthmatic airways, but it also has the capacity to induce fibrosis and structural remodeling [25]. Expression of TGF- β_1 is altered in asthma and the current weight of evidence suggests that TGF- β_1 is up-regulated in human and animal asthmatic airways. The change of TGF- β_1 in patients receiving SIT is also controversial. Some studies have found that the TGF- β_1 was increased after SIT, while others have not. For example, Francis et al. [26] reported that no allergen-induced TGF- β_1 production was detected in the supernatants of PBMCs from patients receiving grass pollen immunotherapy. Our study found that the expression of TGF- β_1 in supernatants from allergen-stimulated cultures from the asthma group was increased compared with unstimulated cultures. But the change in TGF- β_1 was reversed in the SIT group, contrary to our hypothesis. One possible reason for this is that the level of TGF- β_1 in the supernatants may not represent the function of TGF- β_1 -secreting Treg cells, but only the function of TGF- β_1 -secreting PBMCs. It is known that TGF- β_1 is widely expressed throughout the body and that every resident structural and immune cell, as well as every inflammatory cell mobilized to the

Fig. 7. a Initially, a density plot of FSC vs. SSC allows identification of lymphocytes (gate 1) and then a density plot of CD4 vs. CD25 allows identification of CD4⁺ T cells (gate 2) and CD4⁺CD25⁺ T cells (gate 3). The CD25⁺FOXP3⁺ T cells in gate 2 are the CD4⁺CD25⁺FOXP3⁺ T cells and the FOXP3⁺IL-10⁺ T cells in gate 3 are the CD4⁺CD25⁺FOXP3⁺IL-10⁺ T cells. **b** Rat IgG2a isotype controls of CD25 PerCPCY5.5, FOXP3 PE and IL-10 Alexa 647 are shown respectively (after analysis using gate 2). These were used to confirm the positive and negative cell groups. **c** The proportion of CD4⁺CD25⁺Foxp3⁺ T cells and CD4⁺CD25⁺-Foxp3⁺IL-10⁺ T cells was determined by flow cytometry in unstimulated and HDM-stimulated PBMCs from the SIT and asthma groups. Representative plots are shown from 2 subjects per group. Data from all subjects are summarized in figures 2 and 3.

airways during asthma exacerbations is able to express and secrete TGF- β_1 . TGF- β_1 is also produced by Th2 cells, so we hypothesized that the decrease in TGF- β_1 in supernatants from allergen-stimulated cultures of the SIT group may be interpreted as the down-regulation of Th2 cells. Overall, the changes in TGF- β_1 in asthmatics receiving SIT are complex and more investigations are needed to define their exact contribution. In future research, detection of the frequency of TGF- β_1 -secreting

CD4⁺CD25⁺Foxp3⁺ T cells should be by 4-color flow cytometry, which will precisely determine the function of Treg cells.

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