

Effect of house dust mite immunotherapy on transforming growth factor β 1-producing T cells in asthmatic children

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Background: Recent evidence suggests that regulatory T cells (Treg cells) and immunosuppressive cytokines, such as transforming growth factor β 1 (TGF- β 1) and interleukin 10 (IL-10), may have a role in clinically effective allergen specific immunotherapy (SIT).

Objective: To evaluate the effect of SIT on the induction of Treg cells in house dust mite-allergic children and on the expression of specific Treg cell markers (cytotoxic T-lymphocyte-associated protein 4 [CTLA-4], IL-10, and TGF- β 1).

Methods: In this uncontrolled open-label study, the percentage of peripheral blood CD4⁺ Treg cells (CD69⁻CD45RO⁺CTLA-4⁺ and CD3⁺CD4⁺CD25⁺FOXP3⁺) and the expression of molecules associated with their functions (CTLA-4, TGF- β 1, and IL-10) were analyzed using flow cytometry in 16 children allergic to house dust mites before and at 3 and 12 months of subcutaneous SIT. Clinical variables, such as symptom score, medication requirements, forced expiratory volume in 1 second, peak expiratory flow rate, and serum IgE levels, were also determined. Ten healthy children were included as controls.

Results: All the clinical variables improved during immunotherapy. The percentage of CD4⁺CD25⁺CD69⁻CD45RO⁺ Treg cells remained unchanged. The percentage of CTLA-4⁺-expressing Treg cells transiently increased after 3 months of immunotherapy, whereas the percentage of FOXP3⁺ Treg cells did not change after 1 year of immunotherapy. Levels of IL-10⁺ cells transiently decreased after 3 months of immunotherapy. Four children who required inhaled fluticasone propionate administration for significant symptom worsening had no statistically significant increase in TGF- β 1-secreting T cells at 12 months of SIT, in contrast to 12 children without inhaled corticosteroid treatment.

Conclusions: The increase in TGF- β 1-positive T cells only in children without significant symptom worsening requiring inhaled corticosteroid treatment limits the usefulness of TGF- β 1 in monitoring response to allergen immunotherapy.

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INTRODUCTION

Unbalanced, T_H2-like immune reactions against allergens such as house dust mites (HDMs) in the lungs led to an impaired ability to regulate inflammation after exposure to the allergen.¹ Specific immunotherapy (SIT) is an effective prophylactic treatment first described by Curtis and now widely used as an effective treatment for pollen, mite, animal, and insect venom allergy.^{2,3} During immunotherapy, numerous immunologic changes have been described, such as modest reductions in serum allergen specific IgE levels and increases in allergen specific IgG levels, particularly of the IgG4 isotype.^{4,5} Because interleukin 10 (IL-10) stimulates the production of IgG4, IL-10-producing T cells may be involved in the induction of tolerance by SIT.⁶ However, an accurate immunologic marker of the efficacy of this treatment method has not yet been found.

In the past few years, a great deal of interest has been focused on recently described regulatory T cells (Treg cells) that seem to control the development of autoimmune disease, transplant rejection, and even allergy.⁷ At least 4 major types of CD4⁺ Treg cells have been described: naturally occurring Treg cells,⁷ T-helper type 3 (T_H3) cells,⁸ type 1 Treg cells,⁹ and natural killer T cells.¹⁰ Naturally occurring CD4⁺CD25⁺ Treg cells were first identified in mice, but a population with similar properties was subsequently described in humans.^{11–14} Their suppressive activity is related to the 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 the involvement of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-mediated signals.¹⁵ A possible role could also be associated with their ability to secrete or to induce other cells to secrete immunosuppressive cytokines, such as IL-10 and transforming growth factor β (TGF- β).^{6–9,16–18} Because CD25 is considered to be an activation marker of T lymphocytes, not all CD4⁺CD25⁺ cells have regulatory properties because they could represent recently activated T cells. Therefore, additional surface and intracellular molecules (such as HLA-DR, CD122, CD45RO, CD62, CTLA-4, GITR, PD-1, Notch, FOXP3, and CD127) are being investigated for further phenotypic characterization of these

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cells. The aim of this study was to determine whether SIT could differentially alter the expression of molecules associated with functions of Treg cells (CTLA-4, IL-10, and TGF- β 1) and whether effective SIT results in the induction of Treg cells in HDM-allergic children.

METHODS

Patients and Study Design

This uncontrolled open-label study included 16 children (8 boys and 8 girls) aged 5 to 12 years (mean age, 7 years) with mild to moderate persistent asthma recruited from the Out-patient Clinic, Department of Pediatrics, Josip Bencevic General Hospital in Slavonski Brod, Croatia. All the children met the following criteria: (1) history of bronchoconstriction with wheeze and chest tightness; (2) decrease in forced expiratory volume in 1 second (FEV₁) of 20% or more; (3) positive skin test reaction to *Dermatophagoides pteronyssinus* (Der p); (4) elevated total IgE levels (>150 kU/L); (5) radioallergosorbent test (RAST) to Der p at least class 3; (6) no history of immunotherapy; and (7) no treatment with topical or systemic corticosteroids in the 4 weeks before the start of the study (Table 1). Children with concomitant sensitization to perennial allergens, such as cat and dog (if animal in home), and to seasonal pollen allergens that induce allergic symptoms lasting longer than 4 months per year were excluded from the study (based on a clearcut clinical history, positive skin test reactions, and specific IgE RAST results greater than or equal to class 2). Total and specific IgE levels were determined using paper radioimmunosorbent test and RAST kits (Institute of Immunology, Zagreb, Croatia).

Table 1. Demographic Characteristics of the Study and Control Groups

Group and characteristic	Values
Study group	
Sex, M/F, No.	8/8
Age, mean (range), y	7 (5–12)
Duration of asthma, mean $\pm$ SD, y	3.03 $\pm$ 2.75
FEV ₁ , mean $\pm$ SD, % of predicted ^a	72.6 $\pm$ 7.8
Morning PEFR, mean $\pm$ SD, L/min ^a	180 $\pm$ 102
Peripheral blood eosinophils, mean $\pm$ SD, % ^a	10.6 $\pm$ 6.5
Total IgE, mean $\pm$ SD, kU/L ^a	1,004 $\pm$ 1,259
RAST to Der p, No. ^a	
Class 3	1
Class 4	3
Class 5	3
Class 6	9
Skin prick test reaction to HDM, mean $\pm$ SD wheal diameter, mm ^a	8.2 $\pm$ 3.3
Control group	
Sex, M/F, No.	6/4
Age, mean (range), y	9 (8–12)

Abbreviations: FEV₁, forced expiratory volume in 1 second; HDM, house dust mite; PEFR, peak expiratory flow rate; RAST, radioallergosorbent test.

^a Before inclusion in the study.

Physical examination and routine laboratory analyses (C-reactive protein, erythrocyte sedimentation rate, and leukocyte count) were performed to exclude other disorders. Samples of heparinized blood and serum were obtained before and at 3 months and 1 year of immunotherapy. In addition, symptom score, medication requirements, lung function FEV₁ and peak expiratory flow rate [PEFR]), specific and nonspecific IgE levels (RAST and radioimmunosorbent test), and eosinophil count in peripheral blood were also determined at these time points. A symptom diary and a medication chart were kept daily for 2 weeks before SIT and before the 3- and 12-month visits by recording respiratory symptoms and medication use. Asthma symptom scores (0 = no symptoms, 1 = mild symptoms that did not interfere with daily activities, 2 = moderate symptoms that interfered with some activities, and 3 = severe symptoms that interfered with many activities) were determined by the physician before SIT initiation and at 12 months of SIT. Scores for each symptom were then added together to determine the total symptom score.¹⁹

The control group consisted of 10 children (6 boys and 4 girls) aged 8 to 12 years (mean age, 9 years) free of manifest allergic disorders and with a negative family history of atopy. The difference between the mean ages of allergic children and the control group was not significant. Samples were also obtained from 7 adult controls and used in preliminary experiments for optimization of protocols. Patients and controls were included in the study on having obtained written informed consent from their parents. The study was approved by the medical ethics committee of Josip Bencevic General Hospital.

Allergen Extract and Immunotherapy Protocol

Lyophilized and standardized Der p extract (Institute of Immunology),²⁰ which serves as a Croatian national standard for the manufacture of Der p allergen extracts, was used for immunotherapy and tests. Skin prick tests were performed on the volar aspect of the child's forearm, and wheal size was measured after 15 minutes. A histamine chloride solution (1 mg/mL) was used as a positive control and 50% glycerol and 50% buffered saline as a negative control. The longest diameter and its perpendicular diameter were identified and measured. A positive skin prick test reaction corresponded to a wheal with a mean diameter (half the value of the horizontal plus the vertical diameter) exceeding the negative control by at least 3 mm. Taking skin reactivity to histamine into account, we also defined a positive skin prick test reaction as an average diameter corresponding to at least half of that caused by histamine. The relative potency of the allergen extract, which was determined and compared with the World Health Organization International Standard for Der p by means of RAST inhibition assay, was 6.5 $\times$ 10⁵ IU, and 0.049 mg protein/mL contained 10,000 Bodansky units (BU), or 1 ampoule of the tested extract contained 65,300 BU. For SIT, patients were given injections of Der p once a week according to the standard protocol beginning at a dose of 30 BU/mL

during the induction period and then once a month during the maintenance period (Table 2).

Monoclonal Antibodies

For phenotypic analysis using the flow cytometer, we used fluorescein isothiocyanate (FITC)–conjugated monoclonal antibodies (mAbs) to CD4; phycoerythrin (PE)–conjugated mAbs to CTLA-4, CD45RO, CD69, and HLA-DR; CyChrome-conjugated CD25 mAb (all from BD Biosciences, Heidelberg, Germany); PE-conjugated anti-FOXP3 (eBioscience, San Diego, California); allophycocyanine (APC)–conjugated anti-CD3 (Serotec, Oxford, England), PE-conjugated mAbs to IL-10 (BD Biosciences); and TGF- β 1 (IQ Products, Groningen, the Netherlands). Fluorochrome-labeled normal murine immunoglobulins of the same isotype as the corresponding mAbs were used for the preparation of control suspensions.

Phenotype of Treg Cells

Samples of heparinized peripheral blood obtained from patients and controls were used to isolate peripheral blood mononuclear cells (PBMCs) by means of centrifugation over Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The standard procedure of 4-color direct immunofluorescence staining of PBMCs was performed according to the manufacturer's protocol. The following combinations of mAbs were used: CD4 FITC/CTLA-4 PE/CD25 CyChrome/CD3 APC, CD4 FITC/CD69 PE/CD25 CyChrome/CD3 APC, CD4 FITC/CD45RO PE/CD25 CyChrome/CD3 APC, and CD4 FITC/HLA-DR PE/CD25 CyChrome/CD3 APC. For CTLA-4 determination, cells were stained with anti-CD3, anti-CD4, and anti-CD25; fixed with 4% formaldehyde in phosphate-buffered saline (PBS); then permeabilized with 0.1% saponin (Sigma-Aldrich Corp, St Louis, Missouri) in PBS before the addition of PE-conjugated anti-CTLA-4. For FOXP3⁺ analysis, permeabilization was performed with 150 μ L of ice cold 99.9% methanol. CD3⁺CD4⁺ helper T lymphocytes (gated from scatter graph based on size and coexpression of CD3 and CD4) double positive for CD4 and CD25^{hi} were designated as Treg cells as described previously (Fig 1).¹⁴ For each sample, at least 5,000 CD4⁺CD25^{hi} events were collected and

analyzed for the co-expression of CD69, CD45RO, HLA-DR, CTLA-4, or FOXP3 staining using a dual-laser, 4-color FACSCalibur flow cytometer and CellQuest software or a BD LSR II flow cytometer using FACS Diva software (BD Biosciences, San Jose, California).

Intracellular Cytokine Staining

For cytokine detection, PBMCs were cultured in RPMI 1640 supplemented with 10% heat-inactivated AB serum and antibiotics alone or in the presence of 50 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Corp) and 0.75 μ g/mL of ionomycin (Sigma-Aldrich Corp) at 37°C and 5% carbon dioxide, as described previously.²¹ Based on preliminary experiments on 4 healthy adult volunteers, the earliest time point for intracellular cytokine staining of TGF- β 1 and IL-10 was 24 hours of in vitro culture (data not shown). Brefeldin A (Sigma-Aldrich Corp) (10 μ g/mL) was added for the last 6 hours of incubation. For the last 15 minutes, 20-mmol/L EDTA was added. The PBMCs were stained with CD3-FITC and CD8-PerCP, washed, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.1% saponin in PBS, and then PE-conjugated anti-IL-10 or anti-TGF- β 1 was added and incubated for a further 30 minutes at 4°C. After washing in PBS containing 0.1% saponin to wash out unbound antibody, cells were resuspended in PBS and analyzed immediately using a flow cytometer. CD3⁺CD8[−] cells were considered to be CD4⁺ cells because PMA and ionomycin stimulation results in internalization of CD4, leading to less accurate analysis of cytokine-positive CD4 T cells.²²

Control suspensions were prepared using the same procedure, with fluorochrome-labeled normal murine immunoglobulins of the same isotype as the corresponding mAbs. Samples from patients and their age- and sex-matched controls were analyzed after 24 hours of cell culture (see the "Results" section).

Statistical Analysis

Because of the non-Gaussian distribution and the small sample size, the nonparametric Mann-Whitney test was used for the study and control group comparison. Friedman analysis of variance followed by multiple comparisons using Wilcoxon tests were used to detect changes within study groups during immunotherapy. The relationship between variables was evaluated by means of the Spearman rank-order coefficient. We used a statistical software package (Statistica v 6.0; Statsoft Inc, Tulsa, Oklahoma) and considered the probability significant at $P < .05$.

RESULTS

Clinical Effectiveness of D pteronyssinus Immunotherapy

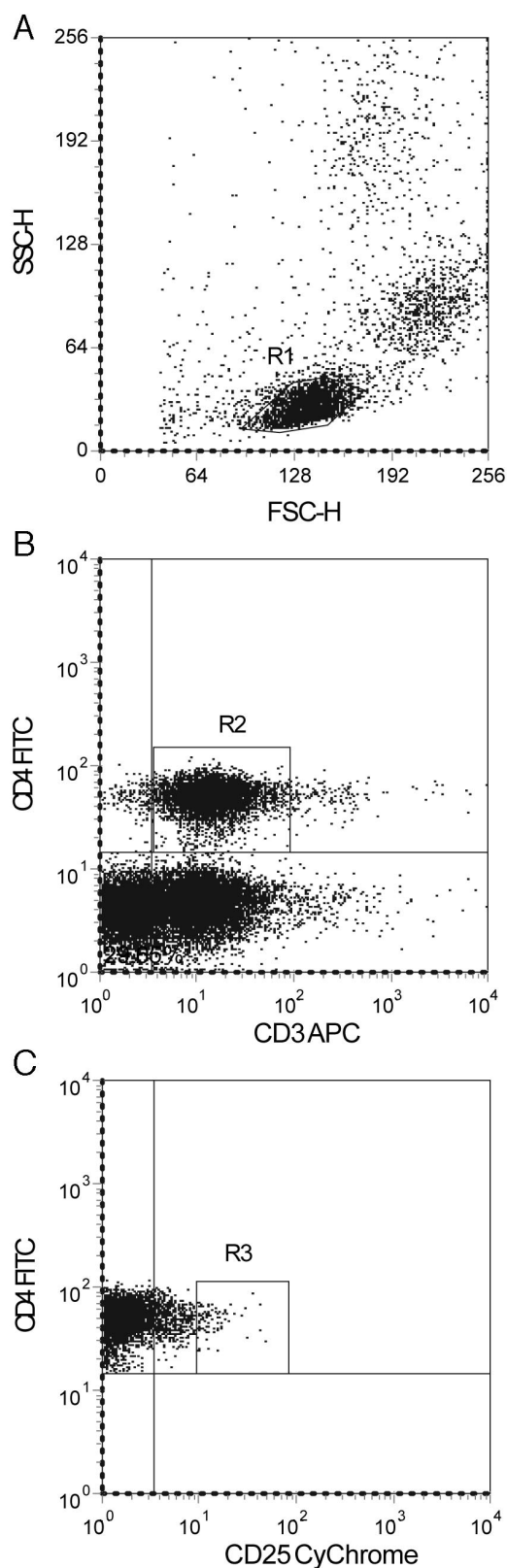
All 16 patients received immunotherapy following the schedule depicted in Table 2. At 12 months of treatment, all SIT-treated patients showed reduced symptom scores compared with baseline levels. The mean (SD) total symptom score in patients undergoing SIT was 1.77 (1.10) at baseline, and this score significantly improved after 12 months of

Table 2. Schedule of Immunotherapy With Standardized Der p1 Extract^a

Injection No	Vial (dilution)	Biological activity, BU/mL	Volume, mL
1–5	A (1:10,000)	30	0.1–0.5
6–10	B (1:5,000)	60	0.1–0.5
11–15	C (1:1,000)	300	0.1–0.5
16–20	D (1:500)	600	0.1–0.5

Abbreviation: BU, Bodansky unit.

^a Increasing doses of allergen were administered once a week following the standard protocol. After injection 20, identical doses were given once a month.



immunotherapy (0.54 [0.97]; $P = .005$). Two children received montelukast for 1 month, and 4 children received fluticasone propionate (125 μ g twice a day) for 4 weeks (2 children), 2 months (1 child), and 3 months (1 child) because of symptom worsening during the maintenance phase of SIT. Exacerbation of asthma symptoms was observed only in a subgroup of children who had a history of grass pollen sensitization before inclusion in the study (clinical history, positive skin test reaction, and positive specific IgE level) and took place during the pollen season. Lung function variables (FEV₁ and PEF_R) improved significantly during immunotherapy ($P < .05$) (Fig 2A and B). In addition, eosinophil count decreased with immunotherapy ($P < .05$) (Fig 2C). There was no significant change in IgE level at 12 months in the SIT group (Fig 2D).

Effect of D pteronyssinus Immunotherapy on the Frequency of Treg Cells in Peripheral Blood

The frequency of CD4⁺CD25⁺CD45RO⁺CD69⁻ Treg cells was low in the peripheral blood of controls and allergic children before SIT and showed no between-group differences (Fig 3A). No changes in the percentage of Treg cells (CD4⁺CD25⁺CD45RO⁺CD69⁻) were observed in patients at either immunotherapy time point (Fig 3A). The percentage of CTLA-4⁺ cells expressing CD4⁺CD25⁺ increased significantly at 3 months of SIT compared with before SIT ($P < .05$) (Fig 3B) but decreased significantly at 12 months of SIT ($P < .05$) compared with before SIT and at 3 months of SIT. Compared with healthy children, at 3 months of immunotherapy the expression of CTLA-4 was significantly higher ($P = .001$). Because of the limited quantity of blood samples, we measured the frequency of FOXP3⁺ Treg cells in 7 patients at 2 time points (before and at 12 months of SIT) and in 7 controls. We found that SIT did not modify the frequency of CD25⁺FOXP3⁺ cells in children administered SIT. However, the frequency of these cells was significantly lower in allergic children at 12 months of SIT compared with controls ($P = .01$) (Fig 4).

Cytokine Production During SIT

The effect of SIT on the frequency of TGF- β 1⁺ T cells was observed at 12 months of SIT only ($P = .01$) (Fig 5A). There was a trend toward increased proportions of TGF- β 1-secreting T cells at 12 months of SIT compared with the control group; however, statistical significance was not reached. The percentage of IL-10⁺CD3⁺CD8⁻ T cells significantly de-

Figure 1. Representative examples of flow cytometry plots showing CD4⁺CD25^{hi} T cells. A, Region 1 (R1) was selected to set the lymphocyte gate according to forward (FSC) and side (SSC) light scatter properties. B, The region 2 (R2) gate was selected to separate the CD4⁺ T cells from the rest of the CD3⁺ lymphocytes. C, Region 3 (R3) was used to set the third gate to select CD4⁺CD25^{hi} T cells from rest of the CD4⁺ T cells. The quadrants were drawn on the basis of control cells stained with isotypic antibody of irrelevant specificity. Region 3 was used to analyze CD4⁺CD25^{hi} T cell expression. APC indicates allophycocyanine; FITC, fluorescein isothiocyanate.

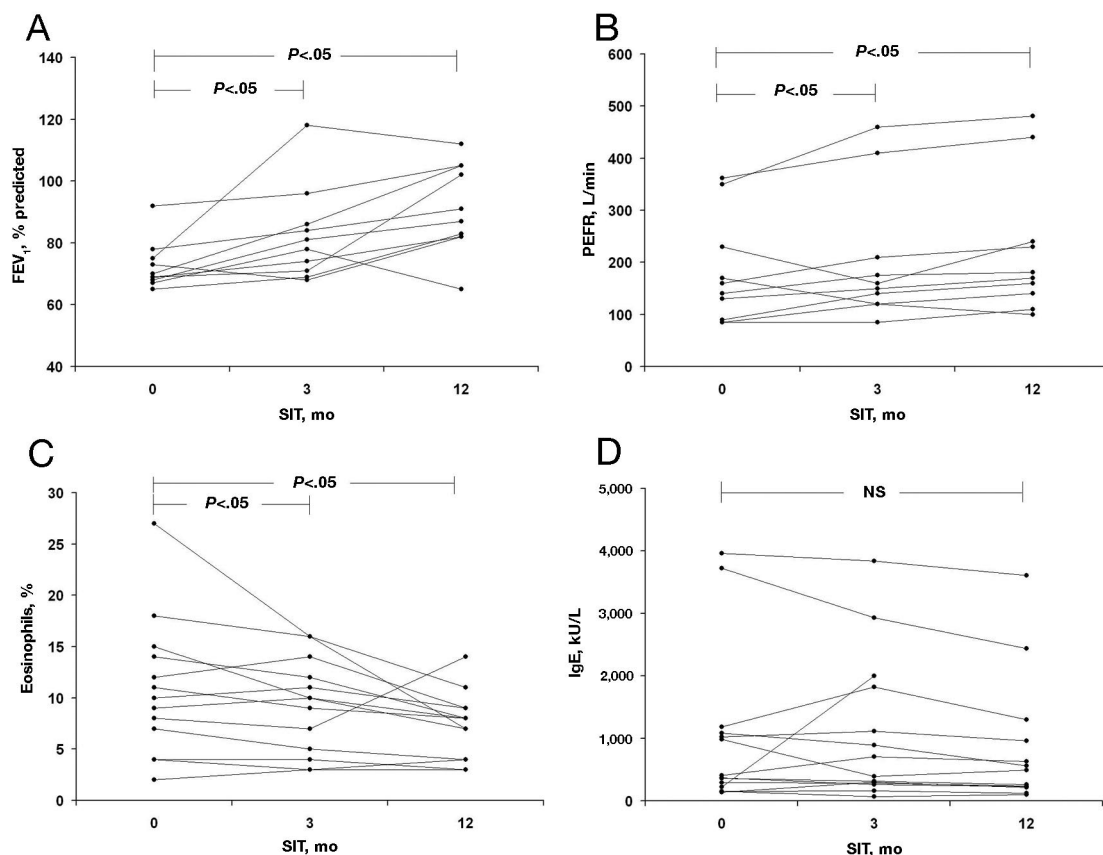


Figure 2. Clinical variables of Der p specific immunotherapy (SIT). Forced expiratory volume in 1 second (FEV₁) (A), peak expiratory flow rate (PEFR) (B), percentage of eosinophils (C), and serum level of IgE (D) were determined before and at 3 and 12 months of SIT in 16 children with asthma. Each symbol represents 1 participant; NS, nonsignificant.

creased at 3 months of SIT. At 12 months of SIT, the proportion of IL-10-secreting T cells was high in only 2 of 16 patients, but generally there was no significant difference between the values before and after 12 months of SIT (Fig 5B).

Correlation Between Changes in Clinical and Immunologic Variables

No significant linear correlation between the cumulative score of symptom intensity and the percentage of either of CTLA-4- or FOXP3-expressing or IL-10- or TGF- β 1-secreting CD4⁺ T cells at any of the time points tested was found. We also performed subgroup analyses to determine tentatively whether a subgroup of patients who require therapy owing to the worsening of symptoms during SIT differ from patients who received no therapy. Only a percentage of TGF- β 1-secreting CD4⁺ T cells in children who did not require inhaled corticosteroid treatment owing to significant symptom worsening ($n = 12$) approached statistical significance compared with values before SIT ($P < .05$) and after SIT ($P < .005$) in children who were treated with inhaled corticosteroids ($n = 4$) (Fig 6).

DISCUSSION

The aim of this study was to investigate the possible effects of SIT on the secretion of IL-10 and TGF- β 1 and on the frequency of Treg cells in asthmatic children allergic to HDMs after 1 year of HDM immunotherapy. Consistent with standardized SIT for other allergens, Der p SIT was efficacious, with significant improvement in symptom scores in all children, accompanied by improvements in lung function and eosinophil percentages.

Studies investigating the frequency of Treg cells in allergic children are scarce, especially covering the periods of 1 year or longer, and have shown that the percentage of CD4⁺CD25⁺ T cells is similar to the percentage measured in children who have outgrown cow's milk allergy or those who received sublingual immunotherapy with grass pollen.^{23,24} However, the possibility that these CD25-expressing T cells represent recently activated T cells rather than Treg cells has only recently been questioned by Shi et al.²⁵ To critically address this possibility, we used 4-color flow cytometry and determined additional Treg cell-associated markers, such as memory T-cell marker, CD45RO, and activation marker,

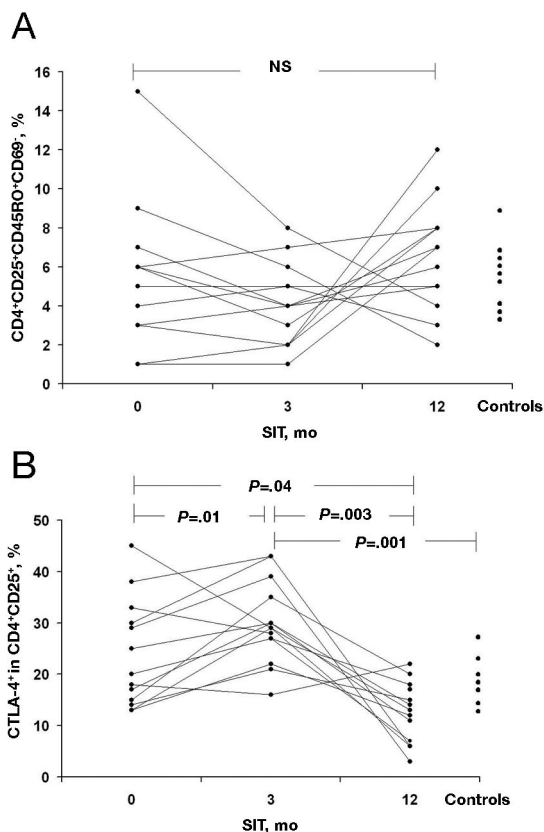


Figure 3. Percentage of $CD4^+CD25^+CD45RO^+CD69^-$ and $CD4^+CD25^+CTLA-4^+$ cells in healthy children and children allergic to house dust mites undergoing specific immunotherapy (SIT). Sixteen patients were analyzed before and at 3 and 12 months of SIT. For each sample, at least 5,000 $CD3^+CD4^+CD25^+$ events (gated from a scatter graph based on size and expression of CD3 and double positive for CD4 and CD25) were collected and analyzed for the coexpression of CD45RO (A) or intracellular coexpression of CTLA-4 staining (B). No significant changes in the percentage of $CD4^+CD25^+CD45RO^+CD69^-$ cells were detected at either time point, and there was no significant between-group difference. A significant increase in $CD4^+CD25^+CTLA-4^+$ cells was detected after 3 months of SIT compared with before SIT and decrease at 12 months of SIT compared with before SIT and at 3 months of SIT. We also detected a significant increase in $CD4^+CD25^+CTLA-4^+$ cells at 3 months of SIT compared with healthy children ($n = 10$). Each symbol represents 1 participant; NS, nonsignificant.

CD69, in addition to CD25 on CD4 T cells. In this study, the proportion of $CD4^+CD25^+CD69^-CD45RO^+$ Treg cells remained unchanged, which might indicate that these cells seem to be a spontaneously arising population of cells with suppressive functions rather than a subset of recently activated cells.

The expression of CTLA-4 was determined by intracellular staining in $CD3^+CD4^+CD25^+$ cells because this molecule has also been described as one of the constitutive markers of Treg cells.^{14,26} The present study showed that the percentage of CTLA-4-expressing $CD4^+CD25^+$ cells transiently in-

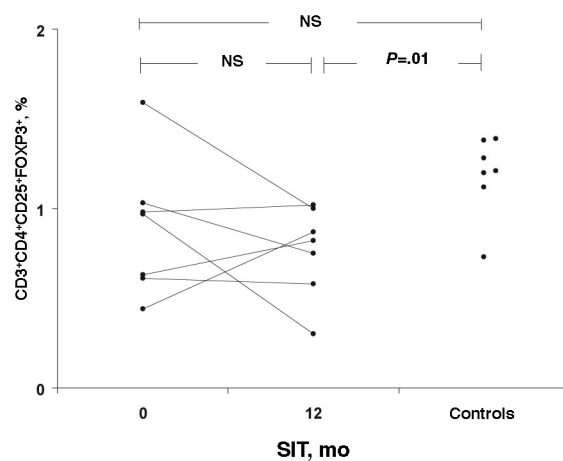


Figure 4. Comparison of the percentage of $CD3^+CD4^+CD25^+FOXP3^+$ cells before and at 12 months of specific immunotherapy (SIT) between allergic and healthy children. For each sample, at least 20,000 $CD3^+CD4^+$ events (gated from a scatter graph based on size and granularity and double positive for CD3 and CD4) were collected and analyzed for the coexpression of surface CD25 and intracellular FOXP3 staining as described in the "Methods" section. There was no significant change in FOXP3⁺ cells, but the level of FOXP3⁺ cells significantly decreased in allergic children ($n = 7$) at 12 months of SIT compared with healthy children ($n = 7$). Each symbol represents 1 participant; NS, nonsignificant.

creased at 3 months of SIT (when the allergen dose used for immunotherapy reached the maintenance level) compared with before SIT and the control group. One possible explanation could refer to the gradual increase in the dose of the allergen administered. A previous attempt did not detect changes in CTLA-4 expression in skin biopsy samples obtained at 4 weeks of therapy in patients allergic to cat allergen (Fel d 1) undergoing specific peptide immunotherapy with a constant dose of peptide mixture.²⁷ The observed transitory increase in CTLA-4-expressing $CD4^+$ T cells in children undergoing SIT is supported by recent findings in mice treated with anti-CTLA-4 mAb and exposed to aerosolized allergen.²⁸ This animal study shows that in vivo blockade of CTLA-4 modulates the initial immune response to a protein antigen, allowing the development of allergen-induced airway inflammation in naive mice. However, once inhalation tolerance is established, it may not be disrupted by blockade of CTLA-4.

We also investigated expression of the FOXP3 molecule, considered to be a master control gene for the development and function of natural Treg cells.²⁹ We recorded no significant change in the levels of FOXP3 before and at 1 year of SIT, although there was a significantly lower frequency of $CD25^+FOXP3^+$ cells at the second time point compared with the control group. To our knowledge, this is the first attempt to follow the pattern of expression of $CD25^+FOXP3^+$ cells, by means of flow cytometry, in allergic children undergoing SIT. The mechanism of the observed effect of SIT is not clear. Lower levels could result from either specific homing

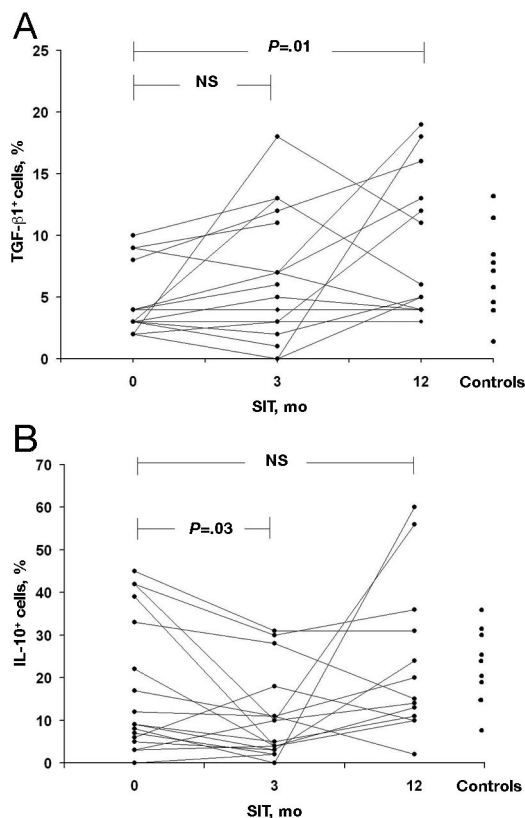


Figure 5. Percentage of CD3⁺CD8⁺TGF-β1⁺ and CD3⁺CD8⁺IL-10⁺ cells before and at 3 and 12 months of specific immunotherapy (SIT). Blood samples were cultured with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 24 hours. Brefeldin A was added for the last 6 hours of incubation. Levels of PMA- and ionomycin-induced transforming growth factor β1 (TGF-β1) and interleukin 10 (IL-10) were determined by means of intracellular cytokine staining, as described in the "Methods" section, in 10 controls and in 16 children undergoing SIT. A significant increase in CD3⁺CD8⁺TGF-β1⁺ cells was detected at 12 months of SIT (A). The percentage of CD3⁺CD8⁺IL-10⁺ cells significantly decreased at 3 months of SIT, but there was no significant change between the values before and at 12 months of SIT (B). Each symbol represents 1 participant; NS, nonsignificant.

of FOXP3⁺ Treg cells into the lungs³⁰ or decreased IL-4 secretion as a consequence of successful SIT. The latter assumption is supported by recent findings that IL-4 and IL-13 induce FOXP3-expressing CD25⁺CD4⁺ Treg cells from CD25⁺CD4⁺ precursors.³¹ The decreased frequency of circulating FOXP3⁺ cells in the present patients was an unexpected finding because at the same time the percentage of TGF-β1-secreting T cells was increased; TGF-β1 is considered to be one of the most important factors regulating the size of the CD4⁺CD25⁺ Treg cell pool in vivo³² and the suppression of allergic processes during immunotherapy.³³ It remains unclear whether TGF-β1-mediated suppression of the specific immune response to allergens ameliorates chronic inflammation or whether TGF-β1-mediated tissue

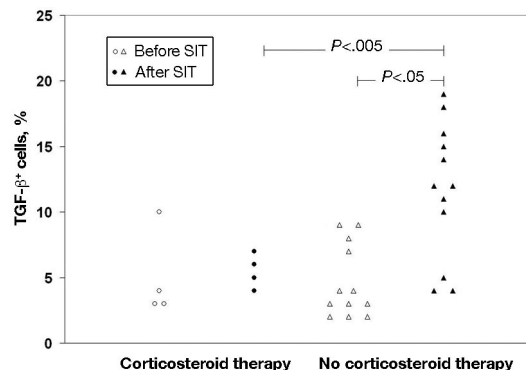


Figure 6. Comparison of CD3⁺CD8⁺TGF-β1⁺ cells between 4 allergic children who received inhaled corticosteroid therapy owing to significant symptom worsening and 12 children who received no such therapy. A statistically significant change in TGF-β1-secreting cells in children who did not receive corticosteroids was observed compared with children who received fluticasone propionate. Each symbol represents 1 participant.

remodeling aggravates disease more than it helps control immune reactions.³⁴

Recently, several research groups^{18,35} suggested that allergen immunotherapy induces a CD25⁺ Treg cell subset producing IL-10, an immunomodulatory cytokine that could down-regulate the production of T_H1 and T_H2 cytokines. Although IL-10-expressing cells detected after allergen immunotherapy also expressed CD25⁺, it is unclear whether these are naturally occurring Treg cells or activated effector cells that up-regulate CD25 and acquire an IL-10-secreting phenotype or a third population that is distinct from either of these, as suggested elsewhere.³⁶ These findings have been challenged by a recent study³³ in which cat allergen peptide immunotherapy altered T-cell response to allergen through mechanisms other than changes in CD4⁺CD25⁺ T-cell suppression.

In the present study, TGF-β1 and IL-10 cytokine production induced after HDM SIT injections was enhanced for flow cytometric detection with additional PMA and ionomycin stimulation of PBMCs to induce preprogrammed cytokine production.^{37,38} However, even if SIT is considered to be specific for HDM allergy, we observed an effect that clearly was not limited to HDM-specific cells because the number of T cells was far higher than the number of antigen-specific T lymphocytes, as expected. This finding might be a result of so-called infectious tolerance, associated with a nonspecific switch of the immune system toward allergens, as already observed in HDM SIT.³⁹

Functional experiments and the ability to test the immunosuppressive activity of Treg cells after SIT in vitro have to be analyzed to better understand the changes observed in this study. In preliminary experiments, we successfully purified CD4⁺CD25⁺ T cells from peripheral blood using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in healthy adults (data not shown). However, the amount of peripheral blood

required was too high to be obtained in children owing to ethical restraints on blood sampling in children. Therefore, modified or even new approaches should be used to test the possible functional changes in these cells.

The overall lack of correlation with clinical variables in children undergoing 1 year of SIT is, however, not an unexpected finding because, to our knowledge, a variety of cells and mediators can contribute to the control of inflammatory processes in asthma. However, when additional subgroup analysis of children undergoing SIT was performed, we observed that 4 children who required inhaled fluticasone propionate administration due to significant symptom worsening had no statistically significant increase in TGF- β 1-secreting T cells at 12 months of SIT. The exacerbation of asthma symptoms was observed only in a subgroup of children who had a history of grass pollen sensitization before inclusion in the study (clinical history, positive skin test reactions, and positive specific IgE levels) and took place during the pollen season. At present, it is not clear whether the difference among subgroups of children and lower-achieved TGF- β 1 is associated with the administration of inhaled corticosteroids or with at least partial failure of SIT. Previously, it has been shown that SIT for a single predominant allergen is less likely to change the course of disease in polysensitized asthmatic patients.⁴⁰ Fluticasone propionate enhances IL-10 synthesis in allergen-stimulated cultures in a concentration-dependent manner,⁴¹ but its effect on TGF- β 1 in similar culture conditions has not been published. A significant decrease in TGFB1 messenger RNA expression was observed in untreated asthmatic patients in relation to healthy individuals and compared with glucocorticoid-treated asthmatic patients.⁴²

Therefore, the present results allow us to conclude that changes in Treg cells and the immunosuppressive cytokines TGF- β 1 and IL-10 might play a significant role in the mechanism of antigen SIT. However, further studies with larger numbers of patients are necessary to investigate whether the increased number of TGF- β 1⁺ T cells only in children without significant symptom worsening requiring inhaled corticosteroid treatment qualify the determination of TGF- β 1 as an important marker of SIT efficacy.

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REFERENCES

- Janeway CA, Travers P, Walport M, Shlomchik MJ, eds. *Immunobiology: The Immune System in Health and Disease*. 6th ed. New York, NY: Garland Science Publishing; 2005.
- Francis JN, Larche M. Peptide-based vaccination: where do we stand? *Curr Opin Allergy Clin Immunol*. 2005;5:537–543.
- Curtis HH. The immunizing cure for hay fever. *N Y Med J*. 1900;77:16–19.
- Van Ree R, Van Leeuwen WA, Dieges PH, et al. Measurement of IgE antibodies against purified grass pollen allergens (Lol p1, 2, 3 and 5) during immunotherapy. *Clin Exp Allergy*. 1997;27:68–74.
- Devey ME, Wilson DV, Wheeler AW. The IgG subclasses of antibodies to grass pollen allergens produced in hay fever patients during hypo-sensitization. *Clin Allergy*. 1976;6:227–236.
- Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest*. 1998;102:98–106.
- McHugh RS, Shevach EM. The role of suppressor T cells in regulation of immune responses. *J Allergy Clin Immunol*. 2002;110:693–702.
- Weiner HL. Induction and mechanism of action of transforming growth factor- β -secreting Th3 regulatory cells. *Immunol Rev*. 2001;182:207–214.
- Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. *Immunol Rev*. 2001;182:68–79.
- Wilson SB, Delovitch TL. Janus-like role of regulatory iNKT cells in autoimmune disease and tumour immunity. *Nat Rev Immunol*. 2003;3:211–222.
- Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+) CD25(+) T cells with regulatory properties from human blood. *J Exp Med*. 2001;193:1303–1310.
- Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+) CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med*. 2001;193:1285–1294.
- Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+) CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol*. 2001;31:1122–1131.
- Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4⁺CD25^{high} regulatory cells in human peripheral blood. *J Immunol*. 2001;167:1245–1253.
- Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4⁺CD25⁺ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 2000;12:431–440.
- Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev*. 2001;182:18–32.
- Shevach EM, McHugh RS, Piccirillo CA, Thornton AM. Control of T-cell activation by CD4⁺ CD25⁺ suppressor T cells. *Immunol Rev*. 2001;182:58–67.
- Jutel M, Akdis M, Budak F, et al. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol*. 2003;33:1205–1214.
- Roberts G, Hurley C, Turcanu V, Lack G. Grass pollen immunotherapy as an effective therapy for childhood seasonal allergic asthma. *J Allergy Clin Immunol*. 2006;117:263–268.
- Trescec A, Kolevska T, Cvorisec B, et al. Characterization and partial purification of the Croatian national standard *Dermatophagoides pteronyssinus* allergen extract. *Allergy*. 1993;48:454–459.
- Bendelja K, Gagro A, Baće A, et al. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. *Clin Exp Immunol*. 2000;121:332–338.
- DiSanto JP, Klein JS, Flomenberg N. Phosphorylation and down-regulation of CD4 and CD8 in human CTLs and mouse L cells. *Immunogenetics*. 1989;30:494–501.
- Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4⁺CD25⁺ regulatory T cells in children who have outgrown cow's milk allergy. *J Exp Med*. 2004;199:1679–1688.
- Rolinck-Werninghaus C, Kopp M, Liebke C, Lange J, Wahn U, Niggemann B. Lack of detectable alterations in immune responses during sublingual immunotherapy in children with seasonal allergic rhinoconjunctivitis to grass pollen. *Int Arch Allergy Immunol*. 2005;136:134–141.
- Shi HZ, Li S, Xie ZF, Qin XJ, Qin X, Zhong XN. Regulatory CD4⁺CD25⁺ T lymphocytes in peripheral blood from patients with atopic asthma. *Clin Immunol*. 2004;113:172–178.
- Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25⁺CD4⁺ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*. 2000;192:

- 303–310.
27. Alexander C, Ying S, B Kay A, Larche M. Fel d 1–derived T cell peptide therapy induces recruitment of CD4⁺CD25⁺; CD4⁺ interferon- γ ⁺ T helper type 1 cells to sites of allergen-induced late-phase skin reactions in cat-allergic subjects. *Clin Exp Allergy*. 2005;35:52–58.
 28. Alenmyr L, Matheu V, Uller L, et al. Blockade of CTLA-4 promotes airway inflammation in naive mice exposed to aerosolized allergen but fails to prevent inhalation tolerance. *Scand J Immunol*. 2005;62:437–444.
 29. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057–1061.
 30. Hori S, Carvalho TL, Demengeot J. CD25⁺CD4⁺ regulatory T cells suppress CD4⁺ T cell–mediated pulmonary hyperinflammation driven by *Pneumocystis carinii* in immunodeficient mice. *Eur J Immunol*. 2002;32:1282–1291.
 31. Skapenko A, Kalden JR, Lipsky PE, Schulze-Koops H. The IL-4 receptor α -chain–binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25⁺CD4⁺ regulatory T cells from CD25⁺CD4⁺ precursors. *J Immunol*. 2005;175:6107–6116.
 32. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF- β regulates *in vivo* expansion of Foxp3-expressing CD4⁺CD25⁺ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A*. 2004;101:4572–4577.
 33. Smith TR, Alexander C, Kay AB, Larche M, Robinson DS. Cat allergen peptide immunotherapy reduces CD4⁺ T cell responses to cat allergen but does not alter suppression by CD4⁺ CD25⁺ T cells: a double-blind placebo-controlled study. *Allergy*. 2004;59:1097–1101.
 34. Schmidt-Weber CB, Blaser K. The role of TGF- β in allergic inflammation. *Immunol Allergy Clin North Am*. 2006;26:233–244.
 35. Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol*. 2003;111:1255–1261.
 36. Hawrylowicz CM, O’Garra A. Potential role of interleukin-10 secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol*. 2005;5:271–283.
 37. Richter A, Lohning M, Radbruch A. Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J Exp Med*. 1999;190:1439–1450.
 38. Mamessier E, Birnbaum J, Dupuy P, Vervloet D, Magnan A. Ultra-rush venom immunotherapy induces differential T cell activation and regulatory patterns according to the severity of allergy. *Clin Exp Allergy*. 2006;36:704–713.
 39. Pajno GB, Morabito L, Barberio G, Parmiani S. Clinical and immunologic effects of long-term sublingual immunotherapy in asthmatic children sensitized to mites: a double-blind, placebo-controlled study. *Allergy*. 2000;55:842–849.
 40. Bousquet J, Hejjaoui A, Clauzel AM, et al. Specific immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract, II: prediction of efficacy of immunotherapy. *J Allergy Clin Immunol*. 1988;82:971–977.
 41. Peek EJ, Richards DF, Faith A, et al. Interleukin-10-secreting “regulatory” T cells induced by glucocorticoids and β_2 -agonists. *Am J Respir Cell Mol Biol*. 2005;33:105–111.
 42. Karagiannidis C, Akdis M, Holopainen P, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol*. 2004;114:1425–1433.

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