

Effects of sublingual immunotherapy for *Dermatophagoides farinae* on Th17 cells and CD4⁺CD25⁺ regulatory T cells in peripheral blood of children with allergic asthma

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Background: Sublingual immunotherapy is becoming a more common treatment for allergic diseases, particularly in pediatric clinics. This type of treatment is highly effective for *Dermatophagoides farinae* allergy, but the mechanisms resulting in immune tolerance have not been investigated. We explored the effects of sublingual immunotherapy with *D. farinae* drops on populations of subsets of T immune cells, specifically Th17 cells and CD4⁺CD25⁺ regulatory T cells (Treg cells), in peripheral blood of children with allergic asthma.

Methods: We assessed immune cell populations in 60 patients allergic to *D. farinae* who were randomly divided into 2 groups: a treatment group (n = 30) and a control group (n = 30), treated with sublingual administration of *D. farinae* drops or placebo, respectively, for 48 weeks. Clinical symptoms of asthma were scored for each individual before and after treatment, and the percentages of Th17 cells and CD4⁺CD25⁺ Treg cells in the peripheral blood were evaluated by flow cytometry at 12-week intervals beginning at baseline.

Results: Both the mean daily symptom scores and percentages of Th17 cells significantly declined in the treat-

ment group throughout the study period ($p < 0.05$), and in the control group both declined but without significant differences between time points. In contrast, the percentages of Treg cells significantly increased in the treatment group throughout the study period ($p < 0.05$), but no statistical difference was observed among different sampling times.

Conclusion: Sublingual administration of *D. farinae* drops alters T immune cell profiles and reduces asthma symptoms, likely resulting in enhanced immunosuppression in children with asthma. © 2014 ARS-AAOA, LLC.

Key Words:

Dermatophagoides farinae drops; asthma; Th17 cells; CD4⁺CD25⁺ regulatory T cells; children

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Sublingual immunotherapy (SLIT) is a new, non-injection approach to treating allergic asthma and allergic rhinitis advocated by the World Health Organization (WHO).^{1,2} In SLIT, patients take a specific allergen preparation under the tongue, beginning at a low dose and gradually increasing the dose until a maintenance dose is reached. The maintenance dose is then administered to induce

immune tolerance against the allergen, thereby eliminating or lessening allergic symptoms. This approach is widely used in Europe to treat a number of allergies and is considered safe and effective in both adults and children.³

Dermatophagoides farinae produce allergens that significantly contribute to allergic disease worldwide. These dust mites induce allergic rhinitis, allergic asthma, atopic dermatitis, and other diseases; indeed, as many as 85% of patients with asthma are allergic to *D. farinae*.⁴ Several studies indicate that sublingual administration of *D. farinae*-specific drops can ameliorate symptoms of children with asthma by reducing the number of acute attacks, attack length and severity, and the dose of inhaled glucocorticoids needed to treat symptoms, as well as by improving small airways function.⁵⁻⁸ However, little is known about the direct mechanisms by which SLIT for *D. farinae* exerts an effect on immune cells.

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Recent advances in asthma biology have uncovered the importance of 2 types of T cells, which mediate cellular immunity, in its pathogenesis. A reduction in number and changes in function of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg), which are involved in peripheral tolerance, appear to be associated with asthma incidence.^{9–11} Treg cells and Th17 cells, a subset of effector CD4⁺ T cells, exhibit expression changes that can affect airway inflammation and airway reactivity in asthma in animal models.^{12,13} Studies are needed to determine the relationship between these T cells and the molecular mechanisms leading to immune desensitization following treatment with *D. farinae* drops. In this study, to begin understanding the molecular mechanisms for SLIT efficacy, flow cytometry was used to analyze the percentages of Th17 cells and CD4⁺CD25⁺Treg cells in peripheral mononuclear cells in children with asthma before and after immunotherapy with *D. farinae* drops. These results will help refine and improve clinical application of *D. farinae* preparations.

Patients and methods

Patients

Sixty children with allergic asthma, diagnosed at the Asthma Special Outpatient Center of our hospital from January to December 2011, were selected for the study. Patients were ages 4 to 18 years. Patients were divided into a treatment group and a control group in order of admission, with 30 cases in each group. These patients met the following inclusion criteria: (1) diagnosed with mild to moderate allergic asthma according to diagnostic criteria for bronchial asthma in children, and without allergic rhinitis;⁷ (2) allergic to *D. farinae*, as confirmed by skin-prick test (++) or greater and serum immunoglobulin E (sIgE) detection (>2), with species of allergen not >3; and (3) no other cardiovascular diseases, autoimmune diseases, or other underlying diseases. Guardians of all participants provided informed consent. This study was approved by the Ethics Committee of Affiliated Nanjing Children's Hospital, Nanjing Medical University, Nanjing, Jiangsu Province, China.

Treatment administration

This study applied a randomized, double-blind, placebo-controlled clinical trial approach. Participants were treated with either placebo (Starch film; Nanjing Stable Trading Co. Ltd, Nanjing, China) or SLIT over a 4-week baseline period and a 48-week observation period during which they were evaluated at the end of every 12 weeks. Children in the treatment group received sublingual *D. farinae* drops (Zhejiang Wolwo Biological Technology Co. Ltd, Deqing, Zhejiang Province, China) numbered 1–4, as follows: No. 1 (total protein concentration of *D. farinae*: 1 µg/mL) was administered in week 1; No. 2 (total protein concentration: 10 µg/mL) was administered in week 2; No. 3 (total protein concentration: 100 µg/mL) was administered in week 3; No. 4 (total protein concentration: 333 µg/mL) was administered from week 4 to week 48. Drop Nos. 1 to 3 were

dosed as 1, 2, 3, 4, 6, 8, and 10 drops from Monday to Sunday, respectively, 40 µL per drop, once per day. Drop No. 4 was dosed at 3 drops per time, 40 µL per drop, once per day. Children in the control group received placebo sublingually, with doses numbered 1 to 4 and administered in the same amounts. Appearance, smell, packaging, volume, storage conditions, and modes and methods of administration were all identical between placebo and drug.

The combination of short-acting bronchodilators and inhaled steroids (ICSs) was used for the patients with clinical symptoms scores above “4” (for definition of symptoms scores, see Standard for clinical symptoms scores below). Otherwise, the long-acting antihistamine and antileukotrienes were used alternately for patients. According to Eifan et al.,¹⁴ the medications were scored as follows: “1” for treatment with β_2 receptor agonist or antihistamines; “2” for treatment with ICSs; and “3” for treatment with 1 tablet of oral prednisone.

Reagents and equipment

PerCP mouse anti-human CD4 monoclonal antibody (mAb) IgG1, fluorescein-isothiocyanate (FITC) anti-human interleukin 17 (IL-17) mAb IgG1, and isotype control IgG1 were purchased from Becton, Dickinson and Company, (New Jersey, USA), human regulatory T cell fluorescence-labeled antibody kits (including FITC anti-human CD4mAb/APC, anti-human CD25mAb, PE anti-human Foxp3 [Forkhead box p3] mAb, isotype control antibody PE2 rat IgG2a, and Fixation/Permeabilization) were purchased from eBioscience, Inc (San Diego, CA, USA); phorbol ester (PMA), monensin, and ionomycin were purchased from Sigma (St. Louis, MO, USA); Cytofix/Cytoperm solution, lymphocyte separation medium, and RPMI-1640 medium were purchased from Gibco (USA); and the flow cytometer (FACSCalibur) was purchased from BD Biosciences (New Jersey, USA).

Standards for clinical symptoms scores

Clinical symptoms were evaluated and recorded for each patient in the morning and night daily for 7 days. The mean daily symptom scores for each patient were average values of the total scores in 1 week divided by 7 days. Daytime symptoms were scored as follows: “0” for no symptoms; “1” for mild cough, tightness in chest, and shortness of breath after significant activities; “2” for mild cough, tightness in chest, and shortness of breath after minor activities; and “3” for asthma spell, tightness in chest, and shortness of breath without any activity. Night symptoms were scored as follows: “0” for no arousals during sleep; “1” for arousal once during sleep; “2” for two arousals during sleep; and “3” for more than two arousals during sleep.

Cell isolation and culture

Peripheral venous blood samples (5 mL) were extracted from each subject, placed in sterile heparin tubes, separated by Ficoll density-gradient centrifugation at 1138×g for 25 minutes to isolate peripheral blood mononuclear

cells (PBMCs), then washed 2 times with Hank's solution (Sigma Chemical, St. Louis, MO, USA) and resuspended in RPMI-1640 medium containing 100 mL/L fetal bovine serum (FBS). Cell viability was detected by trypan blue staining and cells were counted by using a cell counting plate. PBMCs were then cultured in a 24-well plate at 1×10^9 cells/L. PMA (50 $\mu\text{g/L}$ final concentration), ionomycin (750 $\mu\text{g/L}$), and golgiosome blocker monensin (2.0 μM) were added to each well, and suspensions were mixed at 37°C. Cells were cultured in a 50 mL/L carbon dioxide incubator for 4 hours, then transferred to a 1.5-mL Eppendorf tube and centrifuged at 2000 rpm for 8 minutes. Supernatant was discarded and cells were washed twice with PBS. PBMCs (2×10^6 cells) were used in fluorescence-activated cell sorting (FACS) flow cytometry to detect Th17 cells (CD4⁺IL17⁺ cells) or Treg cells.

Flow cytometry

To identify Th17 cells, PBMCs were divided into a control tube and an experimental tube. PerCP-mouse anti-human CD4 mAb IgG (110 μL) was added to each tube, and tubes were placed at room temperature for 20 minutes to stain surface antigens. Cytofix/Cytoperm solution (200 μL) was added, and tubes were placed at 4°C for 20 minutes. Cells were permeated, fixed, and washed. Intracellular cytokine staining was achieved with 10 μL FITC anti-human IL-17 mAb IgG1. Isotype control antibody was added to control tubes, and cells were incubated at 4°C in the dark for 30 minutes. After washing twice with 1 mL Perm/Wash solution, 300 μL of washing liquid was used to resuspend cells. FACS was performed within 24 hours. CD4 histogram gating was used to distinguish Th17 cells (CD4⁺), and scatter plot of IL-17–positive cells was used to represent Th17 cells. Cellsquest software (Becton Dickinson Biosciences, Heidelberg, Germany; version 5.1) was used for analysis.

Treg cells were identified separately. Briefly, PBMC suspension (2×10^6 cells) was added into control and experimental tubes. Cells were washed and resuspended in 200 μL washing liquid. FITC anti-human CD4 mAb and APC anti-human CD25 mAb were added to suspensions, which were incubated at 4°C in the dark for 30 minutes. Cells were washed, and Fix/Perm solution was added for fixation and membrane penetration. Cells were incubated at 4°C for 30 minutes. Perm/Wash solution was used to wash repeatedly until 200 μL cell suspension remained. PE anti-human Foxp3 antibody or isotype control antibody were added to the experimental tube and control tubes, respectively, which were incubated at 4°C in the dark for 1 hour. Up-flow cytometry was used for detection. CD4,CD25 gating was used to distinguish CD4⁺, CD25⁺ cells; Foxp3⁺ cells were identified separately and represented by scatter plot.

Statistical methods

SPSS17.0 software was used for analysis; measurement data are expressed as mean $\pm$ standard deviation (SD). Anal-

ysis of variance (ANOVA) using repeated data measurements was used to compare the ratio of Th17 cells and CD4⁺CD25⁺Treg cells in PBMCs before and after treatment for each group. Independent-sample *t* test and χ^2 test were used to compare general conditions of asthmatic children between the 2 groups. Tests were 2-sided with an α level of 0.05; $p < 0.05$ was considered as statistically significant.

Results

Population characteristics

Baseline characteristics of patients were measured prior to beginning treatment (Table 1). No statistically significant differences were observed in age, gender, height, or body weight between patients in the treatment and control groups. Differences in course of the disease, skin-prick test, specific IgE grading, and allergen species between the 2 groups were also not statistically significant.

Daily symptoms scores

Mean daily symptom scores in the treatment group were 1.52 ± 0.29 , 1.03 ± 0.22 , 0.56 ± 0.19 , 0.35 ± 0.17 , and 0.16 ± 0.08 , before treatment, and 12 weeks, 24 weeks, 36 weeks, and 48 weeks after treatment, respectively (Fig. 1). These scores significantly decreased over the study period ($F = 226.305$, $p = 0.001$). Although mean daily symptom scores appeared to decrease in the control group, the changes were not statistically different ($F = 2.120$, $p = 0.083$). Intergroup comparison demonstrated that the magnitude of change in the treatment group was significantly higher than in the control group ($F = 299.879$, $p = 0.001$). The difference of mean daily symptom scores between the 2 groups became statistically significant from 12 weeks after treatment ($p < 0.05$; Fig. 1).

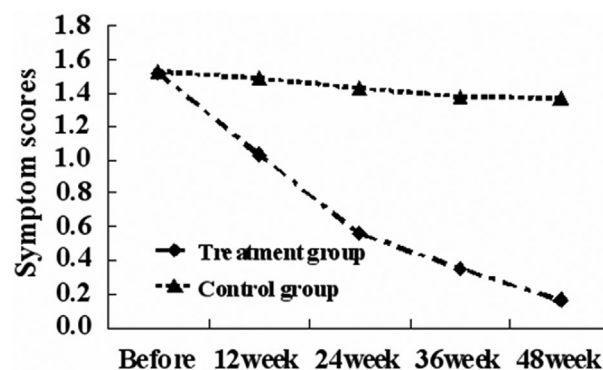
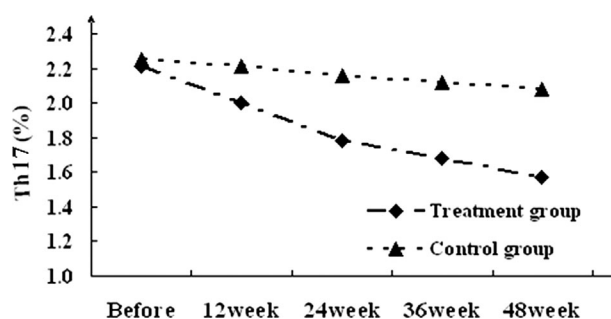
Reduction in Th17 cells following treatment

To determine whether SLIT against *D. farinae* allergens affects the percentage of Th17 cells in PBMCs, FACS was used to identify and quantify IL-17–positive cells (Fig. 2). Percentages of Th17 cells of SLIT-treated patients were 2.21 ± 0.63 , 2.00 ± 0.61 , 1.78 ± 0.58 , 1.68 ± 0.60 , and 1.57 ± 0.60 before treatment and 12 weeks, 24 weeks, 36 weeks, and 48 weeks after treatment, respectively. Thus, Th17 cell populations gradually, but significantly, declined throughout the study period in patients receiving SLIT ($F = 237.804$, $p = 0.001$). Percentages of Th17 cells of patients in the control group were 2.25 ± 0.40 , 2.21 ± 0.50 , 2.17 ± 0.39 , 2.12 ± 0.37 , and 2.08 ± 0.40 before treatment and 12 weeks, 24 weeks, 36 weeks, and 48 weeks after treatment, respectively. Although there appeared to be a slight decline in Th17 cell populations in the control group, these differences were not significant ($F = 0.908$, $p = 0.462$). The percentages of Th17 cells between the 2 groups became statistically different from 24 weeks after treatment ($p < 0.05$; Fig. 2).

TABLE 1. Baseline characteristics of patients in control and treatment groups at study onset

Group	n	Gender (M/F)	Age (years)	Body mass (kg)	Height (cm)	Course of disease (years)	Skin prick test (%)		Specific IgE grading (%)				Allergen species (%)		
							++	+++	II	III	IV	V	1	2	3
Treatment	30	14/16	11.1 ± 3.9	28.0 ± 3.4	135.3 ± 7.8	2.5 ± 1.0	16 (53.3)	14 (46.7)	4 (13.3)	14 (46.7)	8 (26.7)	4 (13.3)	5 (16.7)	15 (50.0)	10 (33.3)
Control	30	16/14	10.8 ± 3.8	27.5 ± 3.2	132.6 ± 8.3	2.6 ± 1.0	15 (50.0)	15 (50.0)	5 (16.7)	13 (43.3)	9 (30.0)	3 (10.0)	6 (20.0)	17 (56.7)	7 (23.3)
χ^2/t		0.267	0.333	0.620	1.293	0.261	0.067		0.350				0.745		
p		0.606	0.740	0.538	0.201	0.795	0.796		0.950				0.689		

IgE = immunoglobulin E.

FIGURE 1. Mean daily symptom scores at baseline and following placebo or *D. farinae* SLIT. Mean symptom scores significantly decreased in the treatment group over the study period ($F = 226.305$, $p = 0.001$); symptom scores did not differ within the control group ($F = 2.120$, $p = 0.083$). SLIT = sublingual immunotherapy.FIGURE 2. Decreases in Th17 cells following treatment. FACS was used to detect IL-17-positive PBMCs in patients with allergic asthma prior to beginning treatment with either placebo or *D. farinae*-specific sublingual immunotherapy. Analysis was repeated again at 12-week intervals to determine effect of treatment on number of Th17 cells. In the treatment group, the percentage of Th17 cells significantly declined over the study period ($F = 237.804$, $p = 0.001$); Th17 cell populations were unchanged in the control group ($F = 0.908$, $p = 0.462$). FACS = fluorescence-activated cell sorting; IL-17 = interleukin 17; PBMC = peripheral blood mononuclear cell.

Increase in CD4⁺CD25⁺Treg cells

The percentage of CD4⁺CD25⁺Treg cells was also determined by FACS over the study period (Fig. 3). In contrast to the observed decline in Th17 cells, the percentage of CD4⁺CD25⁺Treg cells significantly increased over the study period in patients in the treatment group ($F = 279.672$, $p = 0.001$), from 5.52% ± 0.73% at baseline to 6.53% ± 0.81% at 48 weeks after treatment. This cell population was statistically unchanged in patients of the control group ($F = 0.142$, $p = 0.966$). The percentage of CD4⁺CD25⁺Treg cells between the 2 groups became statistically different at 24 weeks, 36 weeks, and 48 weeks after treatment ($p < 0.05$).

Discussion

D. farinae are found commonly in human environments. The excrement, metabolites, and mites of these species have strong allergenicity and induce bronchial asthma, atopic dermatitis, allergic rhinitis, and other allergic diseases in those with allergic constitutions. Traditional treatment of

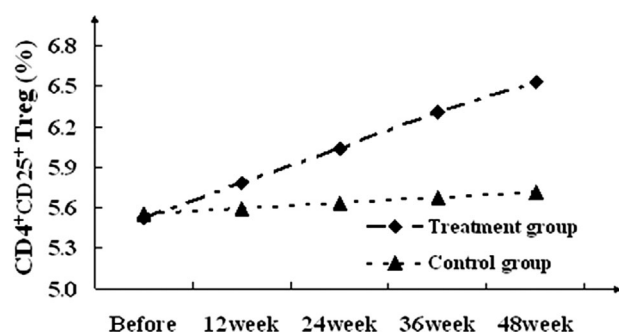


FIGURE 3. Increases in CD4⁺CD25⁺ Treg cells following treatment. FACS was used to detect CD4⁺CD25⁺ Treg cells in PBMCs in patients with allergic asthma prior to beginning treatment with either placebo or *D. farinae*-specific sublingual immunotherapy. Analysis was repeated again at 12-week intervals to determine effect of treatment on number of CD4⁺CD25⁺ Treg cells. The treatment group demonstrated significant increases in the percentage of these cells over the study period ($F = 279.672$, $p = 0.001$), but no statistical difference was observed in the control group ($F = 0.142$, $p = 0.966$). FACS = fluorescence-activated cell sorting; PBMC = peripheral blood mononuclear cell.

symptoms is through immune desensitization therapy via subcutaneous injection. Immune desensitization improves immune tolerance against specific allergens by gradually increasing doses and repeating applications of specific allergens, lessening the immune response when a patient encounters the allergen in the environment. However, injection is traumatic and is associated with poor compliance, particularly for pediatric patients. Sublingual administration of allergens is increasing in usage because it is less traumatic yet effective. Indeed, after 1 year of SLIT for *D. farinae*, clinical symptoms of allergic asthma in children significantly improve.¹⁵

Although immunogenicity of *D. farinae* allergens has been well studied, the mechanisms leading to improved

tolerance in SLIT for *D. farinae* have been little investigated. We hypothesized that subsets of T cells, which have varying roles in immune response, would be altered by SLIT. Of the immune cells, CD4⁺ T cells can differentiate into Th1, Th2, Th17, and Treg cells under different cell cytokine environments.¹⁶ Th17 cells secrete IL-17, a proinflammatory cytokine that contributes to autoimmune diseases and the body's normal defense reactions. In contrast, CD4⁺CD25⁺ Treg cells are anti-inflammatory cells that inhibit activation, proliferation, and antitoxic effects of effector T lymphocytes, thereby regulating the immune response. A reduction in number and function of CD4⁺CD25⁺ Treg cells is associated with a variety of serious hypersensitivity and autoimmune reactions.^{17,18}

We found that administration of *D. farinae*-specific SLIT altered the profiles of Th17 and CD4⁺CD25⁺ Treg cells in peripheral blood of children with allergic asthma. The treatment group exhibited significant declines in both symptoms scores and Th17 cells. A reduction in Th17 cell population would reduce the proinflammatory response in patients treated with *D. farinae* drops. In contrast, CD4⁺CD25⁺ Treg cell populations significantly increased in the treatment group. An increase in this cell population would promote anti-inflammatory responses. Thus, changes in these cell populations may be responsible for decreased symptoms following *D. farinae* SLIT. Interestingly, symptom improvement became significant at 12 weeks, whereas Th17 and CD4⁺CD25⁺ Treg cells became significant at 24 weeks; thus, T cell changes cannot be used as a marker for successful desensitization in clinical experiences. Further studies on changes in other immune cells in the peripheral blood should be performed to better understand the mechanisms of efficacy in specific immunotherapy. Ⓔ

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