

Original Article

Changes in T cell subpopulations after specific sublingual immunotherapy against *Dermatophagoides farinae*

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Received October 22, 2015; Accepted January 16, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: The mechanisms by which allergen-specific immunotherapy induce clinical improvement in allergic asthma remain incompletely understood. Here, the changes in T cell subpopulations, CD4⁺ CD25⁺ Foxp3⁺ Treg cells and Th1/Th2 cells, were determined following sublingual specific immunotherapy against the house dust mite species *Dermatophagoides farinae* in pediatric asthma patients. The study cohort comprised 25 children with asthma who had received standardized dust mite-specific sublingual immunotherapy for one year (observation group), 27 healthy children (control group), 24 children with medication-uncontrolled asthma (uncontrolled group), and 21 children with medication-controlled asthma (controlled group). Peripheral blood mononuclear cells were isolated from all participants and were stimulated with dust mite extracts for 72 h. The proportions of nTreg and Th1/Th2 cells were measured by flow cytometry. After in vitro dust mite extract stimulation, the percentages of nTreg (nTreg% CD4⁺T) cells were significantly increased ($P<0.05$) in each group compared to before stimulation; however, there were no significant differences between groups. The ratio of Th1/Th2 cells in each group increased after stimulation, significantly so in the controlled group and observation group ($P<0.05$), with the ratio of Th1/Th2 cells in the healthy control group higher than that of the other three groups. Thus, a decreased ratio of Th1/Th2 cells exists in children with asthma. Dust mite-specific sublingual immunotherapy may work through reducing the number of Th2 cells and increasing the ratio of Th1/Th2 cells. Further, the ratio of Th1/Th2 cells in peripheral blood could be measured, which may predict the efficacy of dust mite-specific sublingual immunotherapy.

Keywords: Sublingual, specific immunotherapy, dust mite, nTreg cells, Th1/Th2 cells

Introduction

Bronchial asthma is an allergic airway disease involving multiple types of cells and cellular components. In China, *Dermatophagoides farinae* and *D. pteronyssinus* are the most common allergens inducing asthma [1]. Currently, the main treatments for allergic asthma are oral leukotriene receptor antagonists, antihistamines, inhaled glucocorticoids, β -receptor agonists, and allergen-specific immunotherapy (ASIT). ASIT is the only treatment that targets the cause of disease and changes the natural course of disease [2]. In ASIT, the improvement of symptoms in a population with IgE-mediated allergic disease is mediated by a gradually increased administration of allergen. Many studies have found that ASIT for bronchial asthma

can reduce the symptom score in patients, decrease the needs for drug administration, relieve nonspecific bronchial hyperresponsiveness, and also prevent new allergies [3-5].

Although its effectiveness in treating allergic asthma has been confirmed, the mechanism of action of ASIT remains unknown, leading to a lack of objective indices for assessing its therapeutic effects. Current hypotheses suggest that ASIT produces clinical response by inducing peripheral T-cell tolerance [6, 7]. Asthma is mediated through type 2 T helper (Th2) cells, which secrete inflammatory factors such as IL-5, IL-9, and IL-13 and induce lymphocytes to generate specific IgE, while the Th2 cells migrate toward diseased regions [8, 9]. In addition, patients with bronchial asthma exhibit

Table 1. Baseline *D. farinae* allergy characteristics

	Control group (27 cases)	Observation group (25 cases)	Treatment with drugs	
			Controlled group (21 cases)	Uncontrolled group (24 cases)
Total IgE (KU/L)	46.78±29.44	341.72±289.43 ^a	365.12±296.13 ^a	498.25±411.56 ^a
Eosinophil count (×10 ⁹ /L)	0.17±0.07	0.49±0.24 ^a	0.45±0.19 ^a	0.46±0.18 ^a
Skin prick test-wheal diameter				
Der p (mm)	0	9.18±4.46 ^a	10.02±4.53 ^a	8.72±4.96 ^a
Der f (mm)	0	9.67±4.13 ^a	7.23±3.10 ^a	6.89±3.22 ^a
Serum specific-IgE				
Der p (grading)	0	3.47±1.65 ^a	4.02±1.70 ^a	3.96±1.18 ^a
Der f (grading)	0	4.68±1.29 ^a	4.11±1.63 ^a	4.21±1.73 ^a

Note: ^a*P*<0.05 denotes that a difference between the control group and other group was statistically significant.

insufficient function and numbers of regulatory T helper cells, which suppress the immune response of other cells, acting in an anti-inflammatory capacity. ASIT alters the immune response mediated mainly by Th2 cells to one mediated mainly by Th1 cells, and Treg cells are induced to appear [10].

The current study sought to determine the mechanism by which ASIT improves asthma by observing the changes in proportions of natural regulatory T cells (nTreg), or those positive for CD4+, CD25, and FOXP3 expression, and type 1 and 2 helper T cells (Th1/Th2 cells) after specific sublingual immunotherapy with *Dermatophagoides farinae* drops in pediatric patients with asthma.

Methods

Study population

Twenty-five pediatric patients with bronchial asthma, all of whom received specific sublingual immunotherapy with *Dermatophagoides farinae* drops, were selected as an observation group. These patients were treated in the allergy clinic of our hospital between April 2012 and March 2014. In addition, three other groups that did not receive sublingual therapy with the *D. farinae* drops were established for comparison. Twenty-seven healthy children were selected as a healthy control group; 24 pediatric patients whose bronchial asthma was not controlled after treatment with drugs served as an uncontrolled group; and 21 pediatric patients whose bronchial asthma was controlled after treatment with drugs served as a controlled group. Each group underwent pulmonary func-

tion tests, allergen skin tests, bronchial challenge tests, and blood tests to check peripheral eosinophil counts and detect *D. farinae*-specific IgE and total IgE (Data in **Table 1**). In addition, venous blood was collected and cultured, and flow cytometry was used to determine the percentages of nTreg cells and Th1/Th2 cells in CD4⁺T cells (hereafter referred to Th1/Th2% CD4⁺T and nTreg% CD4⁺T, respectively). Inclusion criteria for pediatric asthma patients were as follows: all had mild-moderate allergic asthma accompanied or unaccompanied by allergic rhinitis, and consistent with the diagnostic criteria for bronchial asthma in children [11]; allergies to dust mites were confirmed by skin prick tests and specific IgE detection, and results of skin prick tests were greater than (++), with dust mite-specific IgE levels of less than grade 2, and with not more than 3 types of allergens; and, finally, no other underlying diseases, including cardiovascular and autoimmune diseases, were present. All healthy children from the control group also underwent allergen skin tests and detection for *D. farinae* and *D. pteronyssinus*-specific IgE and total IgE, and allergy was ruled out. The differences among the four groups regarding their sex and age were not statistically significant. The guardians of all participants provided written informed consent.

Therapeutic methods

The observation group received No. 1-4 Sublingual *Dermatophagoides farinae* Drops (40 µl/drop; Wolwo Bio-Pharmaceutical, Zhejiang, China). During week 1, No. 1 drops (total protein concentration 1 µg/mL) were administered; During week 2, no. 2 drops (total protein concentration 10 µg/mL) were administered;

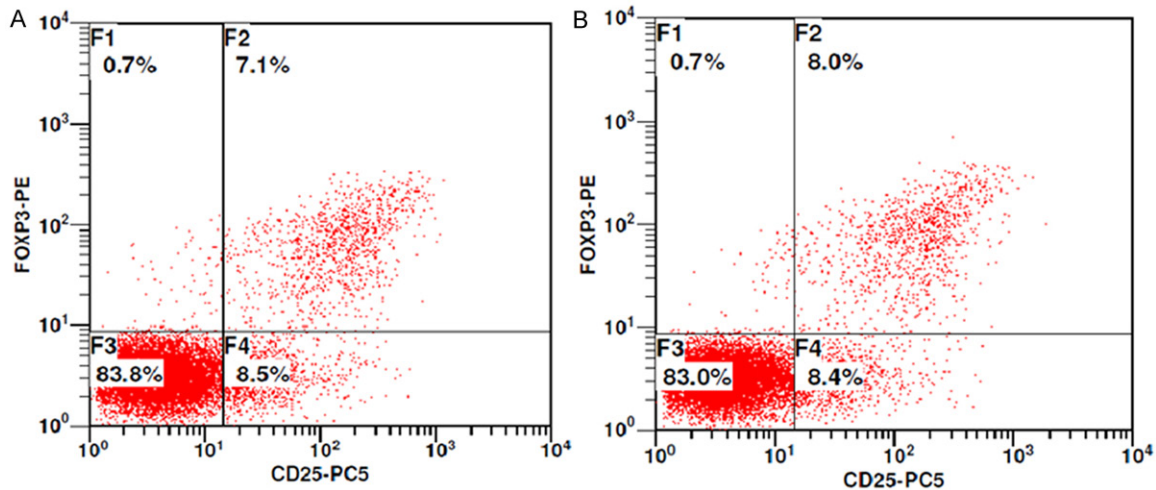


Figure 1. Results of nTreg measured by flow cytometry. A. Before stimulation; B. After stimulation.

During week 3, no. 3 drops (total protein concentration 100 µg/mL) were administered; each of these were administered in gradually increasing amounts throughout the week: 1 (day 1), 2, 3, 4, 6, 8, and 10 drops (day 7), once daily. During the 4th-48th weeks, No. 4 drops (total protein concentration 333 µg/mL) were administered as 3 drops, once daily. Both the controlled group and the uncontrolled group received inhalation therapy with Glucocorticoids-Pulmicort 200 µg/d in 2 divided doses for 8 weeks. The healthy control group received treatment with No. 1-4 Placebo (Starch Tablets) for 8 weeks; the placebo tablets matched in description, odor, package, volume, storage, and route of administration to the sublingual *D. farinae* drops.

Peripheral blood mononuclear cell culture

To isolate peripheral blood mononuclear cells (PBMCs), 7 ml of venous blood were drawn from each subject. PBMCs were immediately isolated by Ficoll density-gradient centrifugation at 2000 rpm for 25 min and transferred to a centrifuge tube. Next, 8 ml of RPMI 1640 (Gibco, USA) were added to the tube, which was centrifuged at 250×g for 10 min in a horizontal centrifuge. Supernatant was removed and sample was centrifuged again. PBMCs were re-suspended in cell culture medium (RPMI 1640, GIBCO, UK). 10 µL of cell suspension were diluted to a concentration of 2×10⁶ cells/ml for counting. Cells from each participant were then divided into two groups and cultured in a 6-well

plate; one sample was not treated with any stimulus (i.e., the concentration of stimulus was 0), while the matched sample was treated with 2 mg/L *Dermatophagoides D. farinae* extracts as a stimulus, and the mixture reached a concentration of 25 µg/mL. The homogeneous mixture was placed in a 5% CO₂ incubator at 37°C for 72 h. Then, the cell culture supernatant was extracted, put into a separate container and labeled, and stored in a refrigerator at 4°C.

Flow cytometric detection of nTreg cells

PBMCs were cultured according to the methods in 1.2.2. Cells were collected into a centrifuge tube and washed, then, cell stain buffer was added to the tube to resuspend the cells at a volume equivalent to that in culture. 5 µL of anti-CD25-PE-cy5 (12-0259-42, eBioscience, USA) and 10 µL of anti-CD4-FITC (A07750, IMMUNOTECH S.A.S, France) were added, and the mixture was incubated at room temperature away from light for 15 min. Then, according to the manufacturers' instructions, 500 µL of fixation/lysis buffer were added and the tube was incubated at 4°C away from light for 35 min. Each tube was washed with 2 mL of lysis buffer and centrifuged at 2100×g for 5 min. Supernatant was discarded, and the wash and centrifugation was repeated once. Five µL of anti-FOXP3-PE (BA2032, eBioscience, USA) were added and the tube was incubated at 4°C away from light for 30 min. After antibody staining, each tube was washed with 2 mL of lysis

Table 2. Comparison of the nTreg% CD4⁺T among four groups

	Control group (27 cases)	Observation group (25 cases)	Treatment with drugs	
			Controlled group (21 cases)	Uncontrolled group (24 cases)
nTreg% CD4 ⁺ T before stimulation	8.18±2.44	7.72±2.43	7.10±2.11	7.95±2.56
nTreg% CD4 ⁺ T after stimulation	8.78±1.07 ^a	9.13±1.24 ^b	8.21±1.19 ^c	8.56±1.18 ^d

Note: a (P=0.012), b (P<0.001), c (P=0.006), and d (P=0.008) respectively denote that a difference between the result before stimulation and the one after stimulation was statistically significant in the control group, observation group, controlled group, and uncontrolled group.

buffer and centrifuged at 2100×g for 5 min. After, the supernatant was discarded, 500 µL of 1% paraformaldehyde were added to each tube.

An isotype control was designed for each tube so as to rule out interference caused by non-specific binding, and all incubated cells were analyzed using the flow cytometer.

Flow cytometric detection of Th1/Th2 cells

Cultured PBMCs were placed in a 6-well plate; the cells were then stimulated for 4 h with 1 µg/mL ionomycin (Wuhan Yitai Science and Technology, Shanghai, China), 20 ng/mL phorbol 12-myristate 13-acetate (PMA, Ascent Scientific, Britain) and 10 µg/mL Brefeldin A (BFA, Chembest Research Laboratories Limited, Shanghai, China). Then the cells were placed in centrifuge tubes and washed and centrifuged. Cell stain buffer, containing calf serum as a carrier protein to reduce the non-specific binding of antibodies and fluorescent reagents to target cells and sodium azide as a metabolic inhibitor of the repair and sealing of cell surface antigens, was added to the tube to resuspend the cells. Samples were centrifuged, stained, and fixed as in 1.2.3, but antibodies anti-IFN-γ-FITC (Sigma, USA) and anti-IL-4-PE (Sigma, USA) were used to detect Th1/Th2 cells.

Statistical analysis

SPSS17.0 was used to perform statistical analysis of the data. The normally distributed quantitative data are expressed as mean ± standard deviation, while those not normally distributed are expressed as the median. The comparison among various groups was performed using one-way analysis of variance (ANOVA) for homogeneity of variances, and Kruskal Wallis Test for heterogeneity of variances. Comparison bet-

ween results before stimulation and those after stimulation was performed using paired sample t-test to analyze the normally distributed quantitative data, or the signed-rank test to analyze those not normally distributed. P<0.05 denotes that a difference was statistically significant.

Results

General conditions

Baseline characteristics for hypersensitivity to *D. farinae* were determined in all groups. The observation group, controlled group, and uncontrolled group were not statistically different in the degree of allergy to *D. farinae* (the wheal diameter shown by the skin prick test as well as the serum specific-IgE level), the total IgE level, or the absolute eosinophil count in the peripheral blood (**Table 1**). However, the total serum IgE levels and the eosinophil concentrations in the peripheral blood in all three groups were significantly higher than those in the control group (P<0.05).

nTreg cells

nTreg cells were detected by flow cytometry as the proportion of PBMCs that were CD4⁺ CD25⁺ FOXP3⁺ (**Figure 1**). The left panel shows the results before stimulation, and the right shows the results after stimulation. In the figures, the third quadrant represents the percentage of nTreg CD4⁺T. *D. farinae* extracts from each group were stimulated and cultured. Compared with the result before stimulation, the nTreg% CD4⁺T in each group was significantly elevated after stimulation (P <0.05; **Table 2**). Regarding the nTreg% CD4⁺T before and after stimulation, the differences among four groups were not statistically significant (P>0.05). After stimulation, the nTreg% CD4⁺T rose by (0.59±0.51)% in the control group, (0.78±0.65)% in the observa-

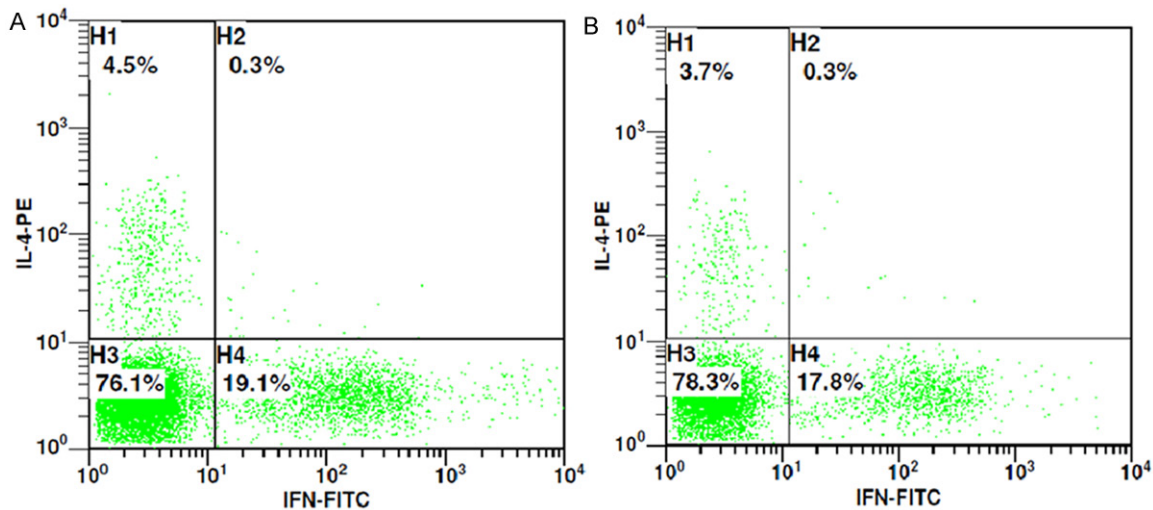


Figure 2. Results of Th1/Th2 cells measured by flow cytometry. A. Before stimulation; B. After stimulation.

Table 3. Comparison of Th1/Th2 ratios among four groups

	Control group (27 cases)	Observation group (25 cases)	Treatment with drugs	
			Controlled group (21 cases)	Uncontrolled group (24 cases)
Th1 cells before stimulation	14.73±5.62	13.68±6.04	11.98±3.64	14.36±4.59
Th1 cells after stimulation	13.83±5.67	13.14±4.56	11.67±3.08	14.50±6.57
Th2 cells before stimulation	3.20±1.60	4.56±1.63 ^a	3.49±1.28	4.45±1.34 ^a
Th2 cells after stimulation	2.98±1.34	3.30±1.08 ^b	3.12±1.44	4.01±1.01 ^a
Th1/Th2 ratio before stimulation	5.18±2.51	3.42±1.45 ^a	4.01±1.74 ^a	3.95±2.06 ^a
Th1/Th2 ratio after stimulation	5.57±1.46 ^a	4.11±1.25	4.80±1.32 ^b	4.06±2.19

Note: "a" denotes that a difference between the control group and other group was statistically significant ($P < 0.05$); "b" denotes that a difference between the result before stimulation and the one after stimulation in the same group was statistically significant ($P < 0.05$).

tion group, (0.60±0.71)% in the uncontrolled group, and (1.09±0.98)% in the controlled group, respectively; the differences among these percentages were not statistically significant ($P > 0.05$).

Th1/Th2 ratios

The proportion of Th1/Th2 cells among PBMCs was also determined. In **Figure 2**, the left panel shows the results before stimulation, and the right figure shows the results after stimulation. In the panels, the fourth quadrant represents Th1% CD4⁺T; while the first quadrant represents Th2% CD4⁺T. The differences in Th1% CD4⁺T before and after stimulation among the four groups were not statistically significant ($P > 0.05$); in contrast, the Th2% CD4⁺T in the observation group and uncontrolled group were significantly higher than those in the control group and controlled group. The Th2%

CD4⁺T after stimulation dropped significantly ($P < 0.05$). *D. farinae* extracts from each group were stimulated and cultured. Compared with the result before stimulation, the Th1/Th2 ratios in all groups were elevated to various levels, but only significantly so in the controlled group and observation group ($P < 0.05$; **Table 3**). Before stimulation, the Th1/Th2 ratio in the control group was higher than that in the observation group ($P = 0.004$), controlled group ($P = 0.026$), or uncontrolled group ($P = 0.043$); after stimulation, the differences among the four groups were not statistically significant ($P > 0.05$).

Discussion

Although there are both environmental and genetic factors contributing to the pathogenesis of bronchial asthma, environmental allergens, particularly those produced by *Der-*

matophagoides farinae and *Dermatophagoides pteronyssinus* house dust mite species, are the most common causes in China. After exposure to allergens, complexes of antigens with MHC class II molecules are formed and presented to CD4⁺T cells via dendritic cells. The CD4⁺T cells are activated, proliferate, and develop into Th2 cells to mediate the immune response. Th2 cells submit many inflammatory factors, such as IL-5, IL-9, and IL-13, which induce lymphocytes to generate specific IgE and promote the production, maturation, and accumulation of eosinophils; these phenomena promote airway hyperresponsiveness and mucus production and stimulate Th2 cells to migrate toward diseased regions [9, 10]. In addition, patients with bronchial asthma have insufficient Treg function and counts, thereby preventing them from suppressing the immune response. Most of the treatments for bronchial asthma, i.e., oral leukotriene receptor antagonists, antihistamines, inhaled glucocorticoids, and β -receptor agonists [11, 12], target symptoms; in contrast, allergen-specific immunotherapy is the only treatment that targets the Th2-caused disease and changes the natural progression of the disease. Indeed, following allergen-specific immunotherapy, the immune response mediated by Th2 cells is converted into one mediated mainly by Th1 cells and Treg cells are induced to appear [13].

In this analysis of T cell populations in pediatric asthma patients, Th1 populations were unchanged in response to *D. farinae* allergen. In contrast, the Th2 population in the allergen-specific immunotherapy group and the group with uncontrolled asthma was significantly larger than those in the healthy control group and the controlled asthma group. The higher Th2% in the observation group might be associated with the degree of allergy to *D. farinae* in pediatric patients [14]. After stimulation with allergen, the Th2% dropped significantly in the observation group, but not in the controlled group and uncontrolled group. Thus, allergen-specific immunotherapy may reduce Th2 responses to *D. farinae*. Before stimulation, the Th1/Th2 ratio in the control group was higher than that in the other three groups, which suggests that there was a drop in Th1/Th2 ratios in the pediatric patients with asthma, and the low Th1/Th2 ratio in the observation group might be associated with a high Th2 cell count. After stimulation, Th1/Th2 ratios showed a signifi-

cant rise in both the observation group and the controlled group, and the Th1 cell count before and after stimulation was not significantly different, which suggests that allergen-specific immunotherapy might reduce Th1 and Th2 responses to *D. farinae* and more markedly inhibit Th2 cells in the observation group. The nTreg counts in the controlled group and uncontrolled group were not markedly lower than those in the observation group and control group, which might be because the glucocorticoids increased CD25⁺ Treg cells in pediatric patients with asthma, i.e., glucocorticoids elevated the expression level of FOXP3 mRNA and contributed to an increase in the number of nonspecific nTreg cells. Therefore, a further study on the allergen-specificity of nTreg cells may achieve a more marked difference. In addition, the determined percentage of nTreg cells in CD4⁺T cells is a relative number; usually, Treg cells do not directly respond to the original stimulation but target effector cells to regulate their immune responses [15-17]. Thus, the role of Treg cells may be mainly manifested not as a change in their own number but as a decrease in the number of their effector cells. In this study, after stimulation, Th2 cells showed a significant difference only in the observation group, which was consistent with the high Th2 cell count after stimulation by *D. farinae* in this group.

In summary, there was a drop in the Th1/Th2 ratio in pediatric patients with bronchial asthma; allergen-specific sublingual immunotherapy for patients with bronchial asthma caused by *Dermatophagoides farinae* allergens could reduce the Th2 cell count and increase the Th1/Th2 ratio, thereby producing clinical effects. The determination of the Th1/Th2 ratio in the peripheral blood and the capacity of Th1/Th2 responses to allergens can predict the effectiveness of specific sublingual immunotherapy with *D. farinae* drops. A 1-year specific sublingual immunotherapy with *D. farinae* drops would make the nTreg cell count approach that in the control group; with the continuation of treatment, the nTreg count may continue to rise, but this needs further observation.

Acknowledgements

This work was supported by National Sciences Foundation of China (NSFC 81302552) and Nanjing Science and Technology Bureau (Ykk10050).

Disclosure of conflict of interest

None.

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