

Local Nasal Immunotherapy: Efficacy of *Dermatophagoides farinae*-Chitosan Vaccine in Murine Asthma

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Key Words

Intranasal immunotherapy • *Dermatophagoides farinae*-chitosan nano-vaccine • Asthma

Abstract

Background: Local nasal immunotherapy has been reported to be effective for airway allergic diseases. A biodegradable material, chitosan (CS), has been reported to be safe and effective in allergen delivery. In this study, we tested immunotherapeutic efficiency by intranasal administration of Der f entrapped in CS microparticles to sensitized mice.

Methods: BALB/c mice were sensitized intraperitoneally with Der f extract absorbed to alum, followed by intranasal treatment with PBS, CS, Der f or Der f-CS nano-vaccine for 6 weeks. The mice were subsequently challenged intranasally with Der f extract for 1 week, and we analyzed their clinical symptoms, antibody expression levels, cytokine levels, T cell proliferation and regulatory T cell numbers. **Results:** Mice treated with intranasal Der f-CS nano-vaccine prior to challenge displayed an alleviated spectrum of symptoms including airway hyper-reactivity, lung inflammation and mucus production and had fewer eosinophilic cells in bronchoalveolar lavage fluid (BALF). Interestingly, the cytokine levels in Der f-specific IgE were reduced, but IgA in serum and BALF

was increased. We also observed that IL-4 was reduced and IFN- γ and IL-10 were increased among splenocytes and in BALF, which inhibits Der f-specific T-cell proliferation in splenocytes and increases regulatory T cells in the spleen. However, the mice challenged without intranasal Der f or Der f-CS vaccine treatment developed allergic asthma. **Conclusion:** Our results illustrate that intranasal administration of Der f-CS nano-vaccine plays roles in immunologic protection in murine allergic asthma by inducing regulatory T cells and Th1-type reaction.

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Introduction

Asthma is a chronic respiratory disease that affects millions of people worldwide, and recent scientific studies have shown that the prevalence of asthma is increasing [1, 2]. Dust mites have been shown to be important sources of indoor allergens associated with asthma and other allergic conditions [3, 4]. Chitosan (CS) is a biocompatible natural polysaccharide that can promote the transmucosal absorption of peptides and proteins and has been demonstrated to be safe and effective in allergen delivery [5, 6]. The protein trapped in it can be released slowly and effec-

tively, so that antigen-presenting cells can absorb it easily. Thus CS is an ideal vaccine adjuvant for immunotherapy.

It has long been recognized that local nasal immunotherapy (LNIT) is capable of controlling the symptoms of seasonal allergic rhinitis in a dose-dependent manner [7–13]. But whether LNIT is effective for asthma is not known, and the therapeutic effectiveness and the mechanism of nasal immunotherapy still remain largely unclear.

In this study, we investigated the effectiveness and mechanisms of intranasally administered Der f-CS microparticle vaccine in Der f allergen induced asthma.

Materials and Methods

Der f allergen was provided by the Allergy and Immunology Institute, Shenzhen University School of Medicine, PR China. Horseradish peroxidase-conjugated anti-mouse IgG_{2a}, IgA and IgE antibodies were purchased from Southern Biotech (Birmingham, Ala., USA). MTT [3-(4,5)-dimethyl thiazol-2-yl-2,5-diphenyl tetrazolium bromide] was purchased from Biomol (Plymouth Meeting, Pa., USA). Methacholine and chitosan were obtained from Sigma-Aldrich (St. Louis, Mo., USA). CD4⁺, CD25⁺ and Foxp3⁺ antibodies were obtained from Biolegend (San Diego, Calif., USA). BALB/c mice were obtained from the Guangzhou Center for Experimental Animals, PR China.

Preparation of Der f-CS Nano-Vaccine and Empty CS Microparticle

CS was dissolved in acetic aqueous solution at a concentration of 2 mg/ml. Tripolyphosphate was dissolved in distilled water at a concentration of 0.84 mg/ml. Then, 1.2 ml of tripolyphosphate solution was added to 3 ml of the CS solution, an opalescent suspension was formed spontaneously under magnetic stirring at 4°C, which was further examined as microparticle [14]. Der f was added to the microparticle solution, which was then centrifuged at 10,000 g for 20 min at 4°C. The suspension was discarded and the pellet was resuspended with distilled water, then Der f-CS microparticles were formed.

Immunization Procedure

Thirty BALB/c mice (all female, 4–6 weeks old and weighing 16–22 g) were randomly divided into 5 groups (n = 6 in each group). The normal control group (group A) was treated with saline. The other 4 groups were sensitized intraperitoneally with 50 µg Der f extract absorbed to 2 mg alum on days 0, 7 and 14. They were then intranasally treated with PBS (group B: asthma model), Der f-CS microparticles (group C), Der f allergen (group D) or CS microparticles (group E). The treatment dosage was 400 µg/mouse, which was delivered every 2 days from day 28 on (8 administrations in total). Seven days after the final immunization, mice were intranasally challenged with 50 µg Der f allergen for 7 days. 24 h after the last challenge, airway hyperreactivity (AHR) was measured and 24 h later the mice were bled from the retro-orbital venous plexus, bronchoalveolar lavage was performed, and the lungs and spleens were removed for use after sacrifice. The procedure is shown in figure 1.

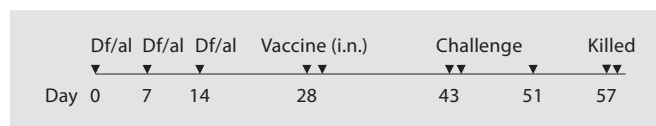


Fig. 1. Immunization scheme for local nasal treatment of allergen-induced asthma. Df/al = 50 µg Der f extract absorbed to 2 mg alum.

Measurement of AHR

AHR was measured using unrestrained whole body plethysmography, as previously described [15], with a 2-chamber system (Buxco, Wilmington, N.C., USA). 24 h after the last challenge, mice were put into the chamber and their breathing was monitored for 10 min. When acclimated, their baseline responses were measured for 5 min. Then the mice were subjected to 0.1 ml of aerosolized PBS, followed by progressively increasing doses of methacholine (1, 10, 30, 50 and 100 mg/ml). Responses were recorded for 5 min for each case with a short interval between to allow return to baseline Penh (enhanced expiratory pause).

Determination of Der f-Specific Antibody Levels in Sera

24 h after AHR assay, blood was collected from the retro-orbital venous plexus of each mouse. Der f-specific IgG_{2a}, IgA and IgE were determined by ELISA as follows: 96-well plates were coated with 100 µl Der f or serial dilutions of murine antibodies for calculation of absolute amounts overnight at 4°C. The allergen-coated plates were washed 5 times with PBST (0.5% Tween-20 in PBS) and blocked with PBST/1%BSA. Sera collected from mice blood were diluted (1:100 for IgA, IgG_{2a} or 1:10 for IgE) in PBST/0.1% BSA and incubated overnight at 4°C. Plates were washed 5 times with PBST and incubated with horseradish peroxidase-conjugated anti-mouse IgG_{2a}, IgA and IgE for 1 h at 37°C. The reaction was developed by adding TMB and stopped with 2 M H₂SO₄. Optical density (OD) was then analyzed by ELISA-Reader (Thermo, Sweden) at 450 nm.

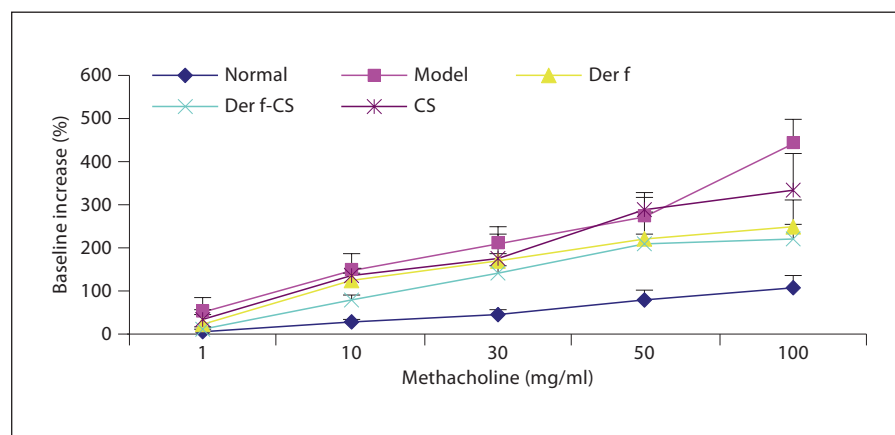
Bronchoalveolar Lavage and Nasal Lavage

To investigate the cellular infiltrate in the airways, the trachea was exposed and cannulated and lungs were gently instilled 3 times with 500 µl of cold PBS. The lavage fluid was centrifuged at 500 g for 10 min at 4°C. After washing, the cells were resuspended in 1 ml PBS and total cell counts were determined by counting in a hemocytometer. Cytoцентрифугed preparations were stained with Liu's stain [16] for differential cell counts. A minimum of 200 cells of total cells were counted and classified as macrophages, lymphocytes, neutrophils and eosinophils. Bronchoalveolar lavage fluids (BALFs) were lyophilized and stored at –20°C until they were used for the cytokine assay.

Allergen-Specific IL-4, IL-10 and IFN-γ Produced by Splenocytes and in BALF

Spleens were removed from mice and single cell suspensions were obtained by mincing the spleen and gently pressing the fragments through a 200-µm mesh stainless steel screen. After being washed twice with Hanks' balanced salt solution, red blood cells were depleted using EDTA-NH₄Cl lysing buffer from the cell sus-

Fig. 2. AHR assay for mice. Normal = Healthy control mice; model = untreated asthmatic mice; Der f = asthmatic mice treated with Der f allergen; Der f-CS = asthmatic mice treated with Der f-CS microparticles; CS = asthmatic mice treated with CS microparticles.



pensions. Then splenocytes were resuspended in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin. The viability of the cells (>98%) was assessed by trypan blue dye exclusion. Cell concentration was adjusted to 1×10^6 /ml using RPMI 1640 medium and the cells were then cultured with or without Der f (20 μ g) for 72 h. The culture supernatants were collected and centrifuged at 400 g at 4°C. The cell-free supernatants were lyophilized and stored at -20°C for subsequent cytokine assays.

The levels of IL-4, IL-10 and IFN- γ in the culture supernatants of splenocytes and BALFs were evaluated by sandwich-ELISA according to the manufacturer's instructions. Briefly, the diluted supernatants or BALFs were added into ELISA plates which were pre-coated with monoclonal rat anti-mouse IL-4, IL-10 or IFN- γ and incubated for 2 h at 25°C. Plates were washed and conjugate was added and incubated for 2 h at 25°C. After the wells were washed, substrate was added and incubated for 30 min. The absorbance at 450 nm was measured on an ELISA reader.

Assessment of Spleen Lymphocyte Proliferation Responses

Splenocytes were placed onto 96-well tissue culture plates (1×10^6 /ml $\times$ 100 μ l), then cocultured with Der f (5 μ g/ml) or concanavalin A (5 μ g/ml) or without stimulation in 96-well microtiter plate at 37°C for 68 h, following treatment with MTT (5 g/l, 10 μ l) to cultured cells. Four hours later, 100 μ l SDS (10% SDS 10 ml and 100 IU 1 N HCL) was added and incubated overnight. Then cells were harvested and assayed by ELISA-Reader (Thermo) at 570 nm. The stimulation indices were calculated from the OD of the specially stimulated cells compared with the OD of cells without stimulation.

Analysis of Regulatory T Cell in Splenocytes

Lymphocytes were separated from splenocytes and stained according to the instructions of the manufacturer (Biolegend). Briefly, cells were first incubated with antigen-presenting cell-labeled anti-CD4 and PE-labeled anti-CD25 mAbs. After washing, these cells were fixed and stained with Alexa Fluor 488-labeled anti-mouse Foxp3 mAb or negative control mAb. Cells were washed 3 times and resuspended in FCM buffer (PBS with 0.1% BSA and 0.1% NaN_3). At least 10,000 cells were assayed using a FASCalibur flow cytometry (Becton Dickinson, Franklin Lakes, N.J., USA)

and data were analyzed with Cell Quest Software. Firstly, unstained cell samples were used to set the FSC/SSC gate for separating lymphocytes. The CD4/CD25 gate was set to separate CD4⁺CD25⁺ T cells. The percentage of CD4⁺CD25⁺ T cells stained with Foxp3 was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs.

Statistics

Data are expressed as mean $\pm$ SE. Statistical analysis was performed using ANOVA and significant level was defined as a $p < 0.05$. The data were analyzed by SPSS statistical software.

Results

Characteristics of Der f-CS Microparticle and Effect of Der f-CS Nano-Vaccine on AHR

We determined the size of the Der f-CS microparticle as 789.34 nm. Der f-CS microparticles loaded 15.6% w/w of Der f allergen. The AHR assay was performed at 24 h after the last challenge. As figure 2 shows, AHR was significantly reduced in the Der f-CS intranasal treatment group compared to the asthma model group with methacholine doses starting from 10 mg/ml, and the difference continued at doses of 30, 50 and 100 mg/ml ($p < 0.05$). AHR in the Der f intranasal treatment group was only significantly reduced when doses reached 50 mg/ml and 100 mg/ml ($p < 0.05$). As expected, the AHR in the CS treatment group remained similar to that of the asthma model group.

Determining Der f-Specific IgG_{2a}, IgA and IgE in Sera

The evaluation of the efficacy of immune response from Der f-CS microparticle vaccine immunization was performed by measuring the serum levels of Der f-spe-

Table 1. The number of total cells and eosinophils in BALF ($x \pm s$)

Cells ($\times 10^4/\text{ml}$)	Healthy controls	Untreated asthma	Der f-CS	Der f	CS
Total cells	43.26 ± 6.01	159.60 ± 15.08	$79.30 \pm 4.95^{**}$	$101.00 \pm 5.32^*$	121.32 ± 4.37
Eosinophils	0	33.20 ± 3.76	$17.20 \pm 1.56^{**}$	$25.40 \pm 2.14^*$	28.35 ± 2.52

* $p < 0.05$; ** $p < 0.01$, vs. untreated asthma group. There were statistically significant differences ($p < 0.05$) when the Der f-CS, Der f and CS groups were compared with each other. Number of mice in each group = 6.

Table 2. MTT assay for splenocyte proliferation

	Healthy controls	Untreated asthma	Der f-CS	Der f	CS
Concanavalin A	0.16 ± 0.02	0.20 ± 0.06	0.35 ± 0.01	0.24 ± 0.03	0.40 ± 0.01
Der f	0.01 ± 0.01	0.35 ± 0.03	$0.18 \pm 0.02^*$	$0.25 \pm 0.03^*$	0.52 ± 0.02

* $p < 0.01$, vs. untreated asthma group. There were statistically significant differences ($p < 0.05$) when the Der f-CS, Der f and CS groups were compared with each other. Number of mice in each group = 6.

cific antibodies in all groups by ELISA assay (fig. 3). We observed a strong IgE response in the asthma model group compared to the normal control group ($p < 0.01$) after using the Der f-CS, Der f and CS intranasal treatments. Among these, the Der f-CS intranasal treatment elicited the strongest response. It was interesting that Der f-specific IgA and IgG_{2a} in the asthma model group showed reductive responses compared to the normal control group ($p < 0.01$) after being promoted with Der f-CS, Der f and CS intranasal treatment, respectively, and the promoting effect was strongest in the Der f-CS intranasally treated group.

LNIT of Nano-Vaccine Inhibits Airway Inflammation and Mucus Hypersecretion

To examine whether Der f-CS microparticle vaccine immunization could alleviate the airway inflammation and mucin production induced by allergen, total lymphocytes and different lymphocyte cell numbers in BALF were counted. Our data showed that the total number of lymphocyte cells and eosinophils were significantly decreased compared to the asthma model group after Der f-CS intranasal treatment. However, the numbers of total lymphocytes and eosinophils in the CS microparticle or Der f allergen treated groups remained the same as in the asthma model group (table 1).

LNIT of Nano-Vaccine Could Modulate the Balance of T Cell Cytokines

To examine whether Der f-CS microparticle intranasal vaccine immunization had effects through cytokines produced by splenocytes and BALF in asthma mice, levels of IL-4, IL-10 and IFN- γ from cultured splenocytes and BALF were determined by ELISA assay (fig. 4, 5). The splenocyte level of IL-4 in BALF of the Der f-CS microparticle intranasal treatment group was significantly decreased compared to the asthma model group ($p < 0.01$). Further, the levels of IFN- γ and IL-10 in BALF of the Der f-CS microparticle intranasal treatment group were significantly increased compared to the control group ($p < 0.01$).

Microparticle Vaccine Can Inhibit Der f-Specific Cell Proliferation and LNIT of Nano-Vaccine Induces Regulatory T Cell Response in Spleen

To investigate the cellular immune responses of intranasal Der f-CS nano-vaccine, T cell proliferation assays were performed in all 5 groups by stimulation with concanavalin A and Der f, and the results were assayed by MTT. As shown in table 2, splenocytes that were sensitized and challenged with Der f extract specifically responded to the Der f allergen, but not to concanavalin A. In addition, the mice intranasally treated with Der f and

Fig. 3. Der f-specific antibodies in sera. Normal = Healthy control mice; model = untreated asthmatic mice; Der f = asthmatic mice treated with Der f allergen; Der f-CS = asthmatic mice treated with Der f-CS microparticles; CS = asthmatic mice treated with CS microparticles; A450 = absorbance at 450 nm.

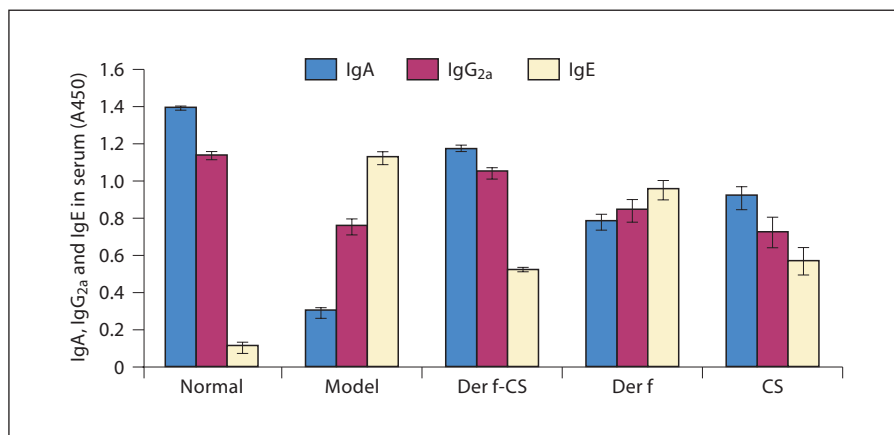


Fig. 4. Cytokines variation in splenocytes. Normal = Healthy control mice; model = untreated asthmatic mice; Der f = asthmatic mice treated with Der f allergen; Der f-CS = asthmatic mice treated with Der f-CS microparticles; CS = asthmatic mice treated with CS microparticles.

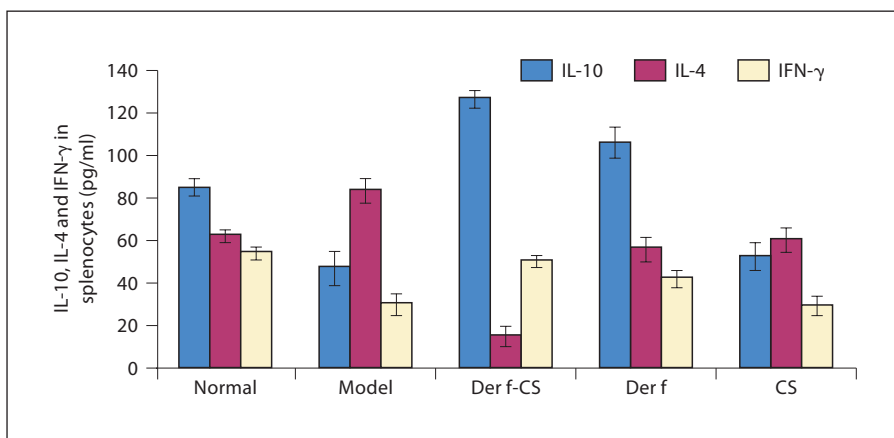
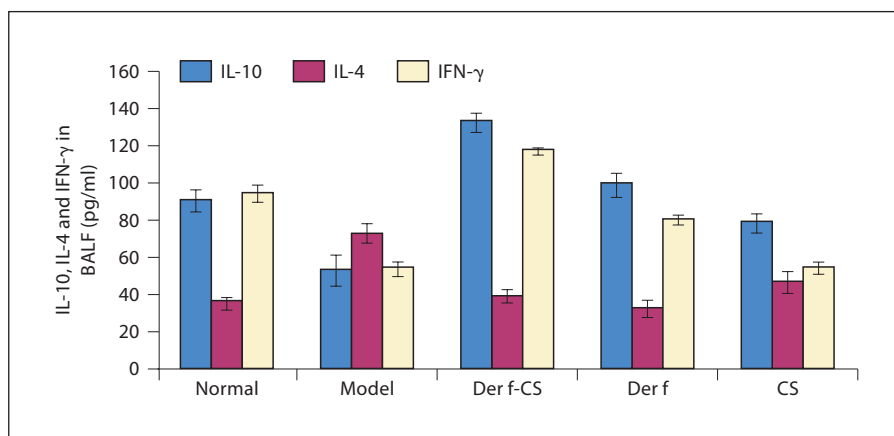


Fig. 5. Cytokines Variation in BALF. Normal = Healthy control mice; model = untreated asthmatic mice; Der f = asthmatic mice treated with Der f allergen; Der f-CS = asthmatic mice treated with Der f-CS microparticles; CS = asthmatic mice treated with CS microparticles.



Der f-CS vaccine had fewer T cells than the model control mice ($p < 0.01$).

To determine if the alteration of CD4⁺CD25⁺Foxp3⁺ T reg cells occurred in the Der f-CS intranasal nano-vaccine group, we compared CD4⁺CD25⁺Foxp3⁺ T reg cells

in different groups using FACS analysis. Our result highlighted that the percentage of CD4⁺CD25⁺Foxp3⁺ T reg cells in the CD4⁺CD25⁺ T cell population harvested from splenocytes treated with Der f-CS intranasal nano-vaccine was much higher than that of the asthma model

Fig. 6. **a** Level of CD4⁺CD25⁺ T reg cells in untreated asthmatic mice. **b** Level of Foxp3⁺ T reg cells in untreated asthmatic mice. **c** Level of CD4⁺CD25⁺ T reg cells in asthmatic mice treated with Der f-CS microparticles. **d** Level of Foxp3⁺ T reg cells in asthmatic mice treated with Der f-CS microparticles.

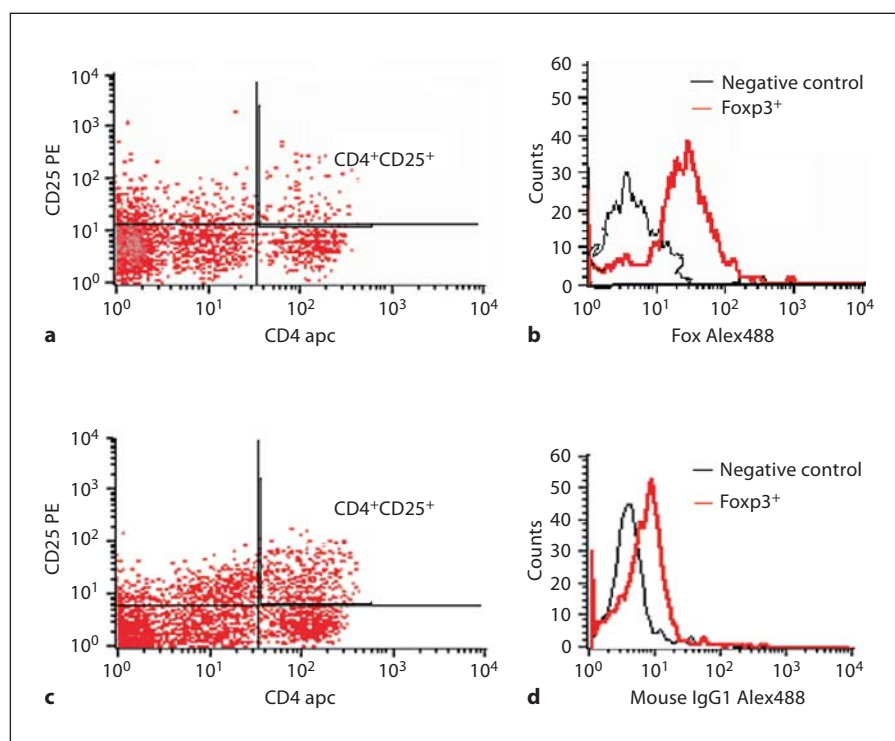


Table 3. The percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in CD4⁺CD25⁺ T cells

	Healthy controls	Untreated asthma	Der f-CS	Der f	CS
CD4 ⁺ CD25 ⁺ Foxp3 ⁺ , %	36.45 ± 4.55*	14.65 ± 5.35*	39.12 ± 3.27**	33.41 ± 4.42*	19.63 ± 4.57*

* $p < 0.05$; ** $p < 0.01$, vs. model group. There were statistically significant differences ($p < 0.05$) when the Der f-CS, Der f and CS groups were compared with each other.

group (fig. 6, table 3, $p < 0.01$). This indicates that Der f-CS vaccine induces T reg cells and modulates T regulatory cell functions.

Discussion

The prevalence of allergic diseases is increasing around the world [17]. Asthma, one of the major allergic diseases, is a pulmonary disorder marked by AHR, airway inflammation and mucus hypersecretion [18]. CD4⁺ T cells have been suggested to play a pivotal role in these pathological changes. Recent advances have shown that Th1 and Th2 paradigms, especially T reg cells and TGF- β signaling together with IL-10, play critical roles in the pathogenesis

of asthma and allergy. However, the mechanism of immunotherapy mediated by T reg cells is still unknown in terms of how T reg cells directly or indirectly suppress effector cells in allergic inflammation [19].

Specific immunotherapy, including subcutaneous, sublingual and nasal immunotherapy, has been introduced as an effective treatment for allergic diseases. It restores normal immunity against allergens and is the only treatment which leads to a lifelong tolerance against previously disease-causing allergens. Classic specific immunotherapy has been reported to have many side effects, such as lower efficacy in certain populations, and there are also considerable concerns over its safety. Local nasal immunotherapy still attracts the attention of many researchers, and there are many studies focusing on na-

sal-associated lymphoid tissue [20]. One of the main results from previous studies indicated that CS is an adjuvant which induces systemic and mucosal immune reactions against allergenic antigens such as pertussis toxin; however, the mechanisms of this process have remained largely unknown.

In our study, a higher level of AHR was observed in the asthma group than in the normal group and there were significantly increased levels of IL-4 and IgE, and significantly decreased levels of IL-10, IFN- γ and CD4⁺CD25⁺Foxp3⁺ T reg cells. These results showed that in the Der f asthma model Th2 responses were predominant and Th1 cell cytokines were not significantly reduced. However, mice treated with Der f-CS nasal vaccine showed low AHR, decreased levels of IL-4 and IgE and Der f-specific lymphoproliferation, and significantly increased levels of IL-10 and CD4⁺CD25⁺Foxp3⁺ T reg cells were observed when compared with model control. Meanwhile, mice treated with Der f-CS nasal vaccine showed significantly higher IgA levels in nasal lavage and sera than those of the asthma model. These results showed that nasal administration of CS microparticles entrapped with Der f could efficiently inhibit the airway inflammation and mucus hypersecretion induced by allergen, so Der f-CS nasal vaccine could have benefits in asthma.

In any case, tolerance to environmental allergens encountered at mucosal surfaces, such as the lungs, nose or intestinal tract, could be mediated by the development of

regulatory T cells, or alternatively by clonal deletion or anergy of allergen-specific cells. Some studies showed that subjects with a lack of CD25⁺ regulatory T cells (Foxp3 mutations) develop severe eczema, elevated IgE levels, eosinophilia and food allergy. It is very likely that some forms of regulatory T cells control the development of allergic disease and asthma [21]. On the other hand, some other studies have shown that those regulatory T cells secreting TGF- β and IL-10 could inhibit airway inflammation and AHR [22, 23]. Our study has verified these hypotheses. These observations indicate that cells other than Th1 cells are very effective in reducing pulmonary inflammation in asthma, and that responses involving TGF- β and IL-10 are very effective in downregulating asthma [24]. IL-10, T reg cells, mucosal immune response and a Th2 to Th1 shift were thought to be involved in this event. The underlying mechanisms should be further investigated.

Acknowledgments

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