

Augmentation of regulatory T cells in allergic individuals by recombinant Der f 2 peptide with fungal immunomodulatory peptide fve

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Background: The suppressive function of regulatory T (Treg) cells is compromised in allergic individuals, and the augmentation of Treg cells has been demonstrated after successful allergen immunotherapy.

Objective: To evaluate the effect of *Dermatophagoides farinae* fragments (Der f 2 N-peptides) that do not bind specific IgE in conjunction with the fungal immunomodulatory peptide fve (FIP-fve) on Treg cells derived from individuals with allergic rhinitis.

Methods: CD4⁺CD25⁺ T cells were isolated from peripheral blood mononuclear cells of 11 patients with allergic rhinitis and 7 nonallergic individuals using immunomagnetic beads. Cells were cultured with medium, Der f 2, FIP-fve, FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides for 6 days in the presence of antigen-presenting cells. The percentages and function of Foxp3⁺CD4⁺CD25⁺ Treg cells, interleukin (IL) 10⁺, and transforming growth factor β (TGF- β)⁺ Treg cells were measured.

Results: The percentage of Foxp3⁺ Treg cells in CD4⁺CD25⁺ T cells was significantly increased in *D farinae* allergic patients by Der f 2 N-peptides in conjunction with FIP-fve. Both IL-10⁺ and TGF- β ⁺ Treg cells were significantly increased in the presence of Der f 2 N-peptides and FIP-fve compared with other groups. The function of Treg cells induced by Der f 2 N-peptides and FIP-fve could be demonstrated by the inhibition of bromodeoxyuridine uptake by peripheral blood mononuclear cells.

Conclusion: The percentage of IL-10⁺ and TGF- β ⁺ cells in Foxp3⁺CD4⁺CD25⁺ T cells can be up-regulated by Der f 2 N-peptides in conjunction with FIP-fve only in *D farinae* allergic individuals. These results indicate that non-IgE-mediated fragments of allergen in conjunction with FIP-fve might have therapeutic effects on Treg cells derived from allergic individuals.

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INTRODUCTION

Allergic rhinitis, 1 of the most common chronic diseases, usually begins in childhood and is characterized by a T_H2 immune response.^{1,2} Because of the increase in T_H2-associated allergic diseases, medical scientists have hypothesized that allergic diseases might result from functional or quantitative deficiencies of regulatory T (Treg) cells that control T_H2 immune responses.³

Treg cells are a subgroup of T lymphocytes with immunosuppressive properties that act on effector T cells.^{4,5} Treg cells are found within the CD4⁺CD25⁺ population, with selective expression of the fork head/winged-helix transcription factor Foxp3 (Forkhead box P3).^{5,6} Although naturally occurring CD4⁺ Treg cells are derived centrally in the thymus and exert suppressive effects by cell contact, a subgroup of Treg cells can also be generated in the peripheral circulation

under specific conditions.^{7,8} Peripheral blood-derived Treg cells from nonatopic individuals and from most patients with allergic rhinitis are able to suppress the production of both T_H1 and T_H2 cytokines.⁹ Further studies have demonstrated impaired suppressive capacity of Treg cells in atopic patients, especially during the pollen season.¹⁰ Children who have outgrown cow's milk allergies have increased numbers of circulating Treg cells compared with children with persistent active allergic disease.¹¹

Allergen immunotherapy with standardized extracts administered in a specialist setting is a safe form of treatment, although the administration of a native allergen might occasionally give rise to IgE-mediated systemic reactions and severe anaphylaxis. To avoid IgE-mediated allergic reaction, use of nonallergenic peptides has been recommended.

Alternative immunotherapy strategies include the use of novel adjuvants to potentiate the ability of allergen vaccines to induce T_H2-to-T_H1 immune deviation. Immunostimulatory sequences appear to be effective as an adjuvant for murine and human T_H1 responses when directly conjugated to the allergens.^{12,13} An immunostimulatory sequence with ragweed allergen (Amb a 1) conjugation suppressed murine airway eosinophilia and hyperresponsiveness.¹⁴ A short course of 6 escalating doses of the conjugate in ragweed-sensitive adults reduced nasal mucosal eosinophilia and interleukin (IL) 4 expression and increased interferon γ (IFN- γ) expression on nasal rechallenge with ragweed allergen.¹⁵

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IL-10 has numerous potential antiallergic properties, including suppression of mast cells, eosinophils, and T-cell responses, as well as acting on B cells to induce heavy chain class switching to IgG4.¹⁶ Recent studies have indicated that successful immunotherapy is associated with augmentation of Treg cells, especially IL-10⁺ Treg cells.¹⁷ The fungal immunomodulatory peptide fve (FIP-fve) is an immunomodulatory protein isolated from *Flammulina velutipes*. Various therapeutic effects of FIP-fve have been noted, including immune regulation, antitumor effects, hepatoprotective effects, antiviral effects, antioxidant effects, cholesterol lowering, hypoglycemia induction, and appetite suppression. Furthermore, FIP-fve suppresses anaphylaxis, enhances transcription of IL-2 and IFN- γ , and inhibits the development of food allergy reactions in mice.^{18–20}

In our previous studies, the combination of FIP-fve and *Dermatophagoides pteronyssinus* group 2 allergen (Der p 2) peptide inhibited Der p 2–induced airway inflammation in a mouse model.^{21,22} Nasal immunotherapy with Der p 2 peptides and FIP-fve caused immune deviation of the T_H2-to-T_H1 response. The production of salivary immunoglobulin A and suppression of Der p–induced airway inflammation also occurred after nasal immunotherapy with Der p 2 and FIP-fve. Accordingly, we conducted an investigation of the modulatory effects of FIP-fve and Der f 2 N-peptides on Treg cells in humans.

MATERIALS AND METHODS

Patient Selection

A total of 11 patients with allergic rhinitis who received treatment at the Allergy Clinic at Taichung Veterans General Hospital, Taichung, Taiwan, were recruited for this study. Allergic rhinitis was defined as a history of sneezing, rhinorrhea, and nasal stuffiness associated with exacerbation, resulting from allergen exposure and concomitant skin reactivity to exacerbating allergens. All patients were sensitive to *D pteronyssinus* as determined by positive skin test results and mite specific IgE measurement using the Pharmacia CAP System (Uppsala, Sweden). A titer of antigen specific IgE higher than 0.35 kU/L was considered to be a positive skin test result. Only those patients with positive response to *D farinae* for both skin test and specific IgE measurement were included in this study, and those individuals with negative responses to both tests were selected as controls. Only 1 individual had a history of bronchial asthma in addition to allergic rhinitis. Seven nonallergic individuals from the hospital staff were recruited as controls. The study was approved by the institutional research board of Taichung Veterans General Hospital, and informed written consent was obtained from each participant.

Skin Prick Test and Measurement of Specific IgE

Study participants underwent skin prick testing with *D farinae* (Greer Laboratories, Lenoir, North Carolina) and recombinant Der f 2 at a concentration of 100 μ g/mL in phosphate-

buffered saline that contained 50% glycerol after the end point titration as previously described.²³ Briefly, the test was performed with a sterile disposable SharpTest applicator (Greer Laboratories). The concentration of Der f 2 in the commercially available *D farinae* extract was 0.8 μ g per 10 μ g of crude extract. Phosphate-buffered saline that contained 50% glycerol and 1.0 mg/mL of histamine were used as negative and positive controls, respectively. Reactions of 3 $\times$ 3 mm or greater were considered positive.

Measurements of IgE specific to *D farinae* were performed using the Pharmacia CAP System. The assay procedure was automatically performed, and the results were calibrated against the World Health Organization standard for IgE. Concentrations greater than 0.35 kU/L were considered positive.

Preparation of Der f 2, FIP-fve, and Der f 2 N-Peptides Recombinant Proteins

Recombinant Der f 2 and FIP-fve were expressed as previously described, except that the expression system was shifted to the yeast *Pichia pastoris*. The Der f 2– and FIP-fve–encoding DNA were generated by polymerase chain reaction (PCR). The PCR-amplified DNA fragments were cut with *EcoRI/NotI* and ligated in-frame to the respective sites of the pPIC9K vector (Invitrogen, San Diego, California). The constructs were transformed into *Escherichia coli* TOP10F and sequenced again to confirm the identity of the inserts. The pPIC9K-Der f 2 and FIP-fve constructs were then linearized with *SalI* and transformed into the *P pastoris* strain GS115 according to the instruction manual. Recombinants were grown at 28°C in yeast extract peptone dextrose medium for 48 hours using an orbital shaker at 200 rpm. Cells were then centrifuged and transferred to buffered methanol-complex medium, pH 6.0. Culture supernatants were harvested after 96 hours and partially purified by sequentially precipitation with 50% ammonium sulfate. The precipitate was dissolved in 0.02M PBS, pH 7.2, and desalted using Amicon Ultra PL-10 (Millipore, Bedford, Massachusetts). Der f 2 N-terminal peptide fragments (Der f 2 N-peptides) containing the first 63 amino acids of Der f 2 (1–63) were constructed. The PCR product was ligated into the pGEM-T vector (Promega, Madison, Wisconsin), double digested with *BamI/SalI*, and subcloned into pET-30a. The construct was confirmed by DNA sequencing. *E coli* BL21 (DE3) cells were transformed with the plasmid pET-30a-Der f 2 N-peptides. The Der f 2 N-peptides were expressed as 6 $\times$ His-tagged proteins and purified according to the manufacturer's instructions (Novagen, Valencia, California).

Purification and Culturing of Treg Cells

For cell separation and culture, we obtained 50-mL blood samples with heparinized syringes and separated the peripheral blood mononuclear cells (PBMCs) by density centrifugation using Ficoll-Paque Plus density gradient (Pharmacia Biotech, Freiburg, Germany). We isolated CD4⁺ T cells by negative immunomagnetic selection, followed by positive

selection for CD25⁺ T cells by magnetic cell sorting (Miltenyi, Gladbach, Germany). We enriched CD4⁺ cells to a median of 96% pure and CD25⁺ to a median of 70% pure. We then cultured the cells in 5-mL culture tubes at a density of 1×10^6 cells/mL in RPMI 1640 medium with penicillin, streptomycin, and L-glutamine (Gibco, Grand Island, New York) for 6 days.

The CD4⁺CD25⁺ T cells were cultured with medium, Der f 2 alone, FIP-fve alone, FIP-fve plus Der f 2, or FIP-fve plus Der f 2 N-peptides. The PBMCs were incubated with mitomycin C (50 μ g/mL) as antigen-presenting cells to the CD4⁺CD25⁺ cells. At day 6, phytohemagglutinin A (PHA; 1 μ g/mL) was added and the cells were cultured for another 2 days.

To identify the percentage of CD4⁺CD25⁺Foxp3⁺ among the CD4⁺CD25⁺ T cells, 3-color flow cytometry (FACSCalibur; Becton-Dickinson, Heidelberg, Germany) was used to analyze the Treg cells using the following monoclonal antibodies: CD4-phycoerythrin-Cy5 mouse IgG1, CD25-allophycocyanine mouse IgG1 (BD Pharmingen, San Diego, California), and Foxp3–fluorescein isothiocyanate (eBioscience, San Diego, California) (Fig 1). Monoclonal antibody IL-10–peridinin chlorophyll protein and monoclonal antibody TGF- β –peridinin chlorophyll protein were used as the fourth color to identify the subpopulations of IL-10⁺ Treg cells and TGF- β ⁺ Treg cells, respectively.

PBMC Proliferation

The PHA-stimulated PBMCs were cultured with Treg cells for 7 days, then fixed and permeabilized with reagents from the bromodeoxyuridine (BrdU) flow kit (Becton-Dickinson and Pharmingen) according to the manufacturer's instructions. To expose incorporated BrdU, the DNA was digested with 30 μ g of DNase (Sigma Chemical Company, St Louis, Missouri) per well for 1 hour at 37°C. After staining with fluorescein isothiocyanate–conjugated anti-BrdU antibody, 20,000 events were acquired using flow cytometry (FACS-Calibur; Becton-Dickinson). Data were analyzed with CellQuest software (Becton-Dickinson).

Cytokine Measurements

Culture supernatant was analyzed simultaneously for 4 different cytokines, IL-2, IL-5, IL-10, and IFN- γ , using a bead-based Flowcytomix (Bender MedSystems, Burlingame, California). Briefly, 25 μ L of each analyte and various concentrations of each cytokine standard were added to 25 μ L of antibody-conjugated beads in 96-well plates. After incubation for 1 hour, the plates were washed and 50 μ L of a biotin-conjugated secondary antibody solution was added to each well, followed by incubation for another hour. The plates were then washed and 50 μ L of streptavidin-conjugated phycoerythrin was added to each well and incubated for 1 hour. After the final wash, the contents of each well were

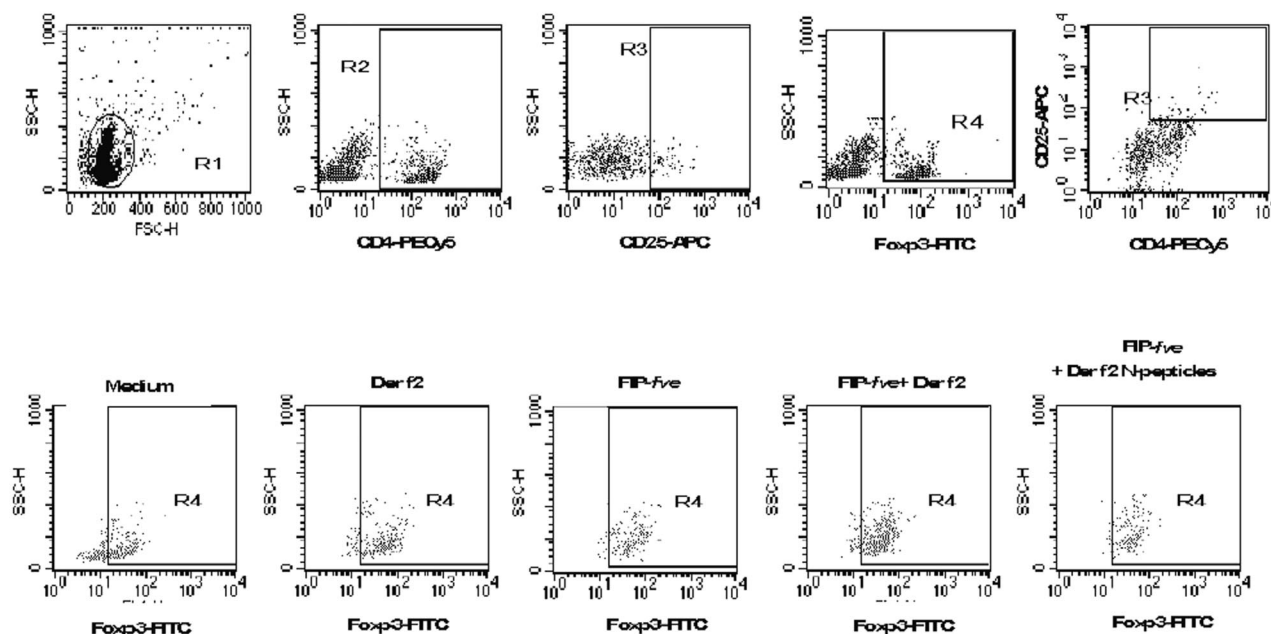


Figure 1. CD4⁺CD25⁺ Foxp3⁺ T cells were stained and analyzed by using 3-color flow cytometry. CD4⁺CD25⁺ T cells and mitomycin C–treated antigen-presenting cells were cultured with medium, Der f 2, fungal immunomodulatory peptide fve (FIP-fve), FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides. The cells were stained with (1) anti-CD4-phycoerythrin-Cy5 and (2) anti-CD25-allophycocyanine for cell surface markers and then (3) anti-Foxp3–fluorescein isothiocyanate for transcription factor. Foxp3⁺ T cells among CD4⁺CD25⁺ T cells were stained and analyzed using 3-color flow cytometry.

resuspended in 200 μ L of assay buffer and placed in 12 $\times$ 75-mm polystyrene tubes and then read in a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, California). The flow cytometer was equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions at 575 and 685 nm. Cytokine concentrations were calculated by comparison with standard curves for each cytokine derived from the cytokine standards assayed in the same manner.

Statistical Analysis

Results are expressed as an arithmetic mean (SEM). Differences between values before and after treatment were analyzed with the paired *t* test. Differences between the groups were analyzed with the unpaired *t* test. Differences were considered statistically significant if *P* < .05.

RESULTS

Effect of Der f 2 N-peptides in Conjunction With FIP-fve on the Subpopulation of the Foxp3⁺CD4⁺CD25⁺ Treg cells

The percentage of Foxp3⁺CD4⁺CD25⁺ Treg cells among CD4⁺CD25⁺ T cells was analyzed. CD4⁺CD25⁺ T cells were separated and cultured with medium, Der f 2, FIP-fve, FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides in the presence of antigen-presenting cells. The results showed that the percentage of Foxp3⁺ Treg cells increased after culture. Compared with the medium control, the percentage of Foxp3⁺CD4⁺CD25⁺ Treg cells was significantly increased in the presence of FIP-fve plus Der f 2 and FIP-fve plus Der f 2 N-peptides from allergic patients (*P* = .01). No significant increase was found after stimulation from nonallergic individuals (Fig 2). In the presence of Der f 2 or FIP-fve alone, no significant increase was found after Der f 2 and FIP-fve stimulation in allergic vs nonallergic groups (Fig 2).

Effect of Der f 2 N-peptides in Conjunction With FIP-fve on the Subpopulations of IL-10⁺ Treg cells and TGF- β ⁺ Treg cells

The percentages of IL-10⁺ Treg cells and TGF- β ⁺ Treg cells among Foxp3⁺CD4⁺CD25⁺ T cells were analyzed. Results showed that the percentages of IL-10⁺ Treg cells and TGF- β ⁺ Treg cells were 61.3% (7.8%) and 64.6% (7.9%) in the medium control from allergic individuals and 64.4% (9.8%) and 65.4% (8.9%) in the medium control from nonallergic individuals, respectively. No significant differences were found in the percentages between the groups. Compared with the medium control, the percentages of IL-10⁺ Treg cells and TGF- β ⁺ Treg cells both significantly increased after stimulation with FIP-fve plus Der f 2 and FIP-fve plus Der f 2 N-peptides in allergic individuals (*P* < .05). However, no significant increase was found in either IL-10⁺ Treg cells or TGF- β ⁺ Treg cells from nonallergic individuals. Only IL-10⁺ Treg cells were significantly increased by FIP-fve stimulation alone in allergic individuals (Fig 3A and B).

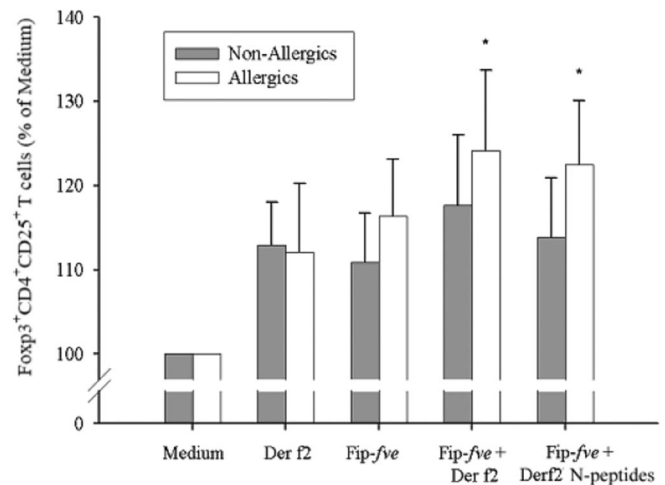


Figure 2. Effect of Der f 2 N-peptides in conjunction with fungal immunomodulatory peptide fve (FIP-fve) on the percentage increase of Foxp3⁺ Tregulatory (Treg) cells derived from allergic and nonallergic individuals. CD4⁺CD25⁺ T cells and mitomycin-treated antigen-presenting cells were cultured with or without Der f 2, FIP-fve, FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides. The percentage increase of Foxp3⁺ Treg cells among CD4⁺CD25⁺ T cells was analyzed. Data represent mean (SEM) of 11 allergic and 7 nonallergic individuals. **P* = .01 compared with medium control.

Effect of Treg Cells Induced by Der f 2 N-peptides in Conjunction With FIP-fve on PBMC Proliferation

Der f 2- and FIP-fve-induced Treg cells were evaluated for their immunomodulatory functions. PBMCs were cultured with Treg cells in the presence of PHA. Cell proliferation was determined by the uptake of BrdU. The data were presented as percentages of BrdU⁺ cells in PBMCs. The results showed that all the Treg cells obtained from either Der f 2- or FIP-fve-stimulated PBMCs inhibited PHA-stimulated PBMC proliferation. Compared with PHA-stimulated PBMCs, the percentage of BrdU⁺ cells in PBMCs was down-regulated by Treg cells derived from both allergic and nonallergic individuals. However, statistically significant inhibition was only obtained in Treg cells derived from allergic individuals (*P* < .05). No differences were found in the inhibitory functions among those Treg cells (Fig 4).

Effect of Treg Cells Induced by Der f 2 N-peptides and FIP-fve on the Cytokine Secretion of PBMCs

Der f 2- and FIP-fve-induced Treg cells were evaluated for immunomodulation of cytokine secretion from PBMCs. PBMCs were cultured with Treg cells in the presence of PHA. Supernatants were collected for cytokine measurements. The results showed that the secretion of IL-10, IL-5, IFN- γ , and IL-2 did not significantly change after coculture with Treg cells induced by Der f 2 and FIP-fve. No statistically significant differences were found between the allergic and nonallergic individuals (Fig 5).

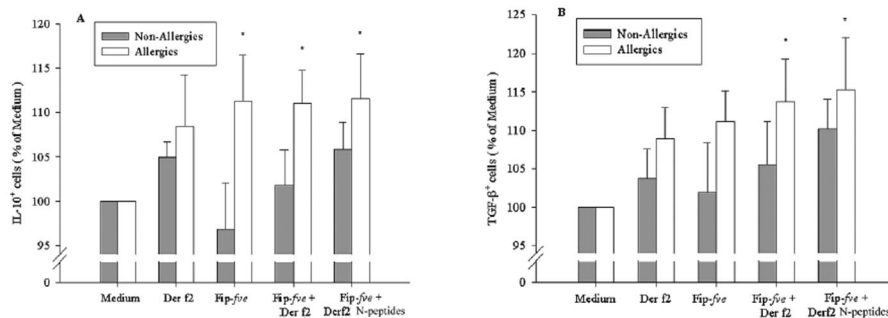


Figure 3. Effect of Der f 2 N-peptides in conjunction with fungal immunomodulatory peptide fve (FIP-fve) on the percentage increase of interleukin 10 (IL-10⁺) and transforming growth factor β (TGF- β ⁺) cells in the Foxp3⁺ T regulatory (Treg) cells. CD4⁺CD25⁺ T cells and mitomycin-treated antigen-presenting cells were cultured with or without Der f 2, fungal immunomodulatory peptide fve (FIP-fve), FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides. The percentage increase of IL-10⁺ (A) and TGF- β ⁺ (B) cells in the Foxp3⁺ Treg cells were analyzed. **P* < .05 compared with medium.

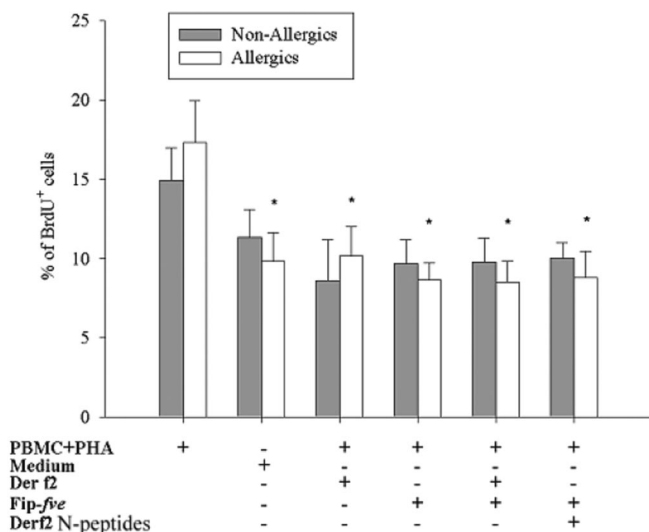


Figure 4. Effect of Der f 2 N-peptides in conjunction with fungal immunomodulatory peptide fve (FIP-fve) on the T regulatory (Treg) cell function as determined by peripheral blood mononuclear cell (PBMC) proliferation. Treg cells obtained from allergic and nonallergic individuals were separated after being cultured with or without Der f 2, FIP-fve, FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides. Cells were tested for phytohemagglutinin A (PHA)-stimulated PBMC proliferation as determined by bromodeoxyuridine (BrdU) incorporation. Data are presented as mean (SEM) of the percentage of BrdU⁺ cells. **P* < .05 compared with PBMCs plus PHA.

DISCUSSION

Our studies demonstrated that in the peripheral blood of allergic patients not only can IL-10- and TGF- β -producing Foxp3⁺CD4⁺CD25⁺ Treg cells be up-regulated, but also the inhibitory functions of these cells can be demonstrated in the presence of Der f 2 N-peptides in conjunction with FIP-fve. It was reported that pulmonary CD4⁺CD25⁺ Treg cells were impaired in pediatric asthma patients and failed to suppress proliferation and production of T_H2-associated cytokines and chemokines.²⁴ In our study, the percentage of Foxp3⁺ Treg

cells among CD4⁺CD25⁺ T cells was significantly increased among allergic patients by Der f 2 N-peptides in conjunction with FIP-fve, indicating that Der f 2 N-peptides up-regulate the subpopulation of Treg cells.

Adaptive Treg cells can be induced in the peripheral blood, and their suppressor effects can be mediated through TGF- β and IL-10.²⁵ The high levels of IL-10 and TGF- β produced by Treg cells mediate the inhibition of primary naive T cells in vitro.²⁶ In our study, both IL-10- and TGF- β -secreting Treg cells were identified and up-regulated. However, only those Treg cells derived from allergic individuals were augmented by Der f 2 in conjunction with FIP-fve, indicating that Treg cell regulation might be allergen specific.

Several studies implied that the suppressive function of Treg cells is compromised in a variety of atopic diseases in a manner dependent on natural exposure to allergens or products produced by skin-colonizing bacteria.^{27,28} We found similar results in our study. PHA-stimulated PBMC proliferation was suppressed by Treg cells derived from allergic patients but not from nonallergic individuals, despite the finding that all the cytokines secreted by PBMCs were not significantly inhibited by Treg cells and remained similar in both allergic and nonallergic individuals. This discrepancy might be due to the overactivation of PBMCs by PHA. The proliferation of PHA-stimulated PBMCs could be inhibited by Treg cells, but cytokines in PBMC supernatants showed no significant differences in both groups. These results indicated that PHA-stimulated cell proliferation might not be correlated with the cytokine production. Further studies, using non-PHA-stimulated PBMCs are necessary to clarify the effect of FIP-fve and Der f 2 N-peptides on the Treg cell function.

It has been reported that the suppression of T_H2 activity can be modulated by allergen-specific immunotherapy.¹³⁻¹⁵ We previously showed that nasal immunotherapy with Der f 2 peptides and FIP-fve alleviated symptoms in dust mite allergic mice.²¹ In the present study, Der f 2 in conjunction with FIP-fve could up-regulate the Treg cells derived from allergic individuals. Therefore, we hypothesized that the antiallergic immune response of FIP-fve and Der f 2 might function

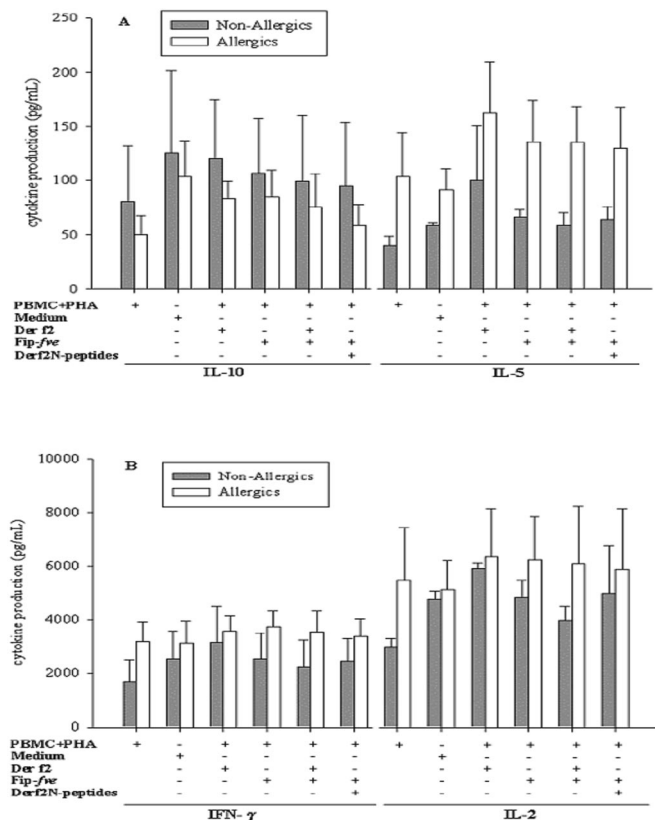


Figure 5. Effect of Der f 2 N-peptides in conjunction with fungal immunomodulatory peptide fve (FIP-fve) on the T regulatory (Treg) cell function as determined by the cytokine secretion by peripheral blood mononuclear cells (PBMCs). Treg cells obtained from allergic and nonallergic individuals were separated after being cultured with or without Der f 2, FIP-fve, FIP-fve plus Der f 2, and FIP-fve plus Der f 2 N-peptides. Cells were tested on the cytokine secretion from phytohemagglutinin (PHA)-stimulated PBMCs. The concentrations of interleukin (IL) 10, IL-5, IL-2, and interferon γ (IFN- γ) in the culture supernatant were compared between the treatment groups.

through the enhancement of Treg cell proliferation and activation.

Neither Der f 2 nor FIP-fve up-regulated Treg cells, but there were modulatory effects by Der f 2 in conjunction with adjuvant. Whether Der f 2 and FIP-fve affect Treg cells directly or secondarily through other immunoregulatory cells remains to be determined. Although both Der f 2 and Der f 2 N-peptides had a modulatory effect with FIP-fve on the increase of Foxp3⁺CD4⁺CD25⁺ Treg cells, the high allergenic property of Der f 2 might limit its clinical application. The fragment of Der f 2 N-peptides that do not bind specific IgE may be more suitable for clinical use.

In conclusion, not only the subpopulation of Foxp3⁺CD4⁺CD25⁺ cells but also IL-10- and TGF- β -producing Treg cells can be up-regulated in vitro after culture with Der f 2 N-peptides in conjunction with FIP-fve. Although Treg cells are insufficient in allergic individuals, they can be up-regulated by Der f 2 N-peptides with FIP-fve, indicating

that these agents can be used for therapeutic applications. The nonallergenic Der f 2 N-peptides with FIP-fve might be more suitable for immunotherapy to avoid IgE-mediated reactions.

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