Int J Clin Exp Pathol 2015;8(1):137-145
www.ijcep.com /ISSN:1936-2625/IJCEP0002842

Original Article

Analysis of T-cell epitopes of Der f3 in *Dermatophagoides farina*

Na Li[1*], Haifeng Xu[2*], Hongyu Song[1], Kexia Wang[2], Chaopin Li[1]

[1]Department of Medical Parasitology, Wannan Medical University, Wuhu 241002, Anhui, China; [2]Department of Pathogenic Biology, Anhui University of Science and Technology, Huainan 232000, Anhui, China. [*]Equal contributors and co-first authors.

Received September 28, 2014; Accepted November 26, 2014; Epub January 1, 2015; Published January 15, 2015

Abstract: House dust mites (HDM) are most important indoor allergens for humans. Der f3, one of the potent allergens with allergenicity, is derived from *Dermatophagoides farina (D. farinae)*, and exhibits strong allergenicity that was confirmed in our previous work. The current study was undertaken to determine the localization of T-cell epitope of Der f3. We initially developed the T-cell fraction from BALB/c mice sensitized with recombinant Der f3 to determine the T-cell epitopes in the murine models, and performed T cell proliferation assay with 25 synthetic overlapping peptides of Der f3. The results indicated that T-cell reactive region of murine were assigned on amino acid range 41-60, 101-120, 161-180 and 201-220, respectively. In addition, we did T-cell proliferation experiment, respectively using the 4 murine T-cell epitope peptide and the human T-cell lines from three patients allergic to mite allergens in order to verify homogenous T-cell epitopes in humans. The results indicated that the amino acid sequences of 41-60, 101-120 and 161-180 had induced T cell proliferation in humans, yet 201-220 failed to. These findings suggest that T-cell epitope in Der f3 is located in the amino acid sequences of 41-60, 101-120 and 161-180, respectively. T-cell epitope localization detected in our study may provide a basis for development of animal therapeutic model and peptide vaccine for asthma.

Keywords: Allergen, asthma, house dust mite, Der f3, T-cell epitope

Introduction

Type I allergic diseases, including allergic asthma, allergic rhinitis and atopic dermatitis are increasingly prevalent, and becoming one of global health problems, which affected as high as 10% to 30% of the world population [1]. House dust mites (HDM) are major source of inhalant allergens responsible for allergic asthma. Extensive studies have been done to understand the biological, chemical and structural properties of such mite allergens, and most characterized allergens are from Dermatophagoides farinae (D. farinae) and Dermatophagoides pteronyssinu (D. pteronyssinu) that are coexisting in most geographical regions [2-4]. Secretions, feces and body degradation products of HDM can cause human allergic diseases [5, 6]. There are about 30 reported allergen components produced by dust mite group 1 and 2 families being recognized as primarily important allergens reported inland and overseas [7-9]. T cell epitopes on Der 1 and Der 2 groups have been intensively analyzed. Der f3, the group 3 allergen, isolated from D. farinae is one of these allergenic proteins confirmed in our previous work to have strong allergenicity. The physiological role of Der f3 being a serine protease with trypsin-like activity remains unknown [10-12], though it can activate the kallikrein-kinin system involved in various allergic reactions in human plasma [13]. It was reported that Der f3 could combine with the specificity antibody IgE in serum of patients with allergic asthma at a rate of 16%-100% [10-12], which suggests that Der f3 is a valuable antigen to design peptide vaccine for treatment of asthma.

Asthma is a chronic airway inflammatory disease, characterized by lung leukocyte and eosinophilic infiltration accompanied by mucus

Figure 1. 25 synthetic overlapping peptides of Der f3.

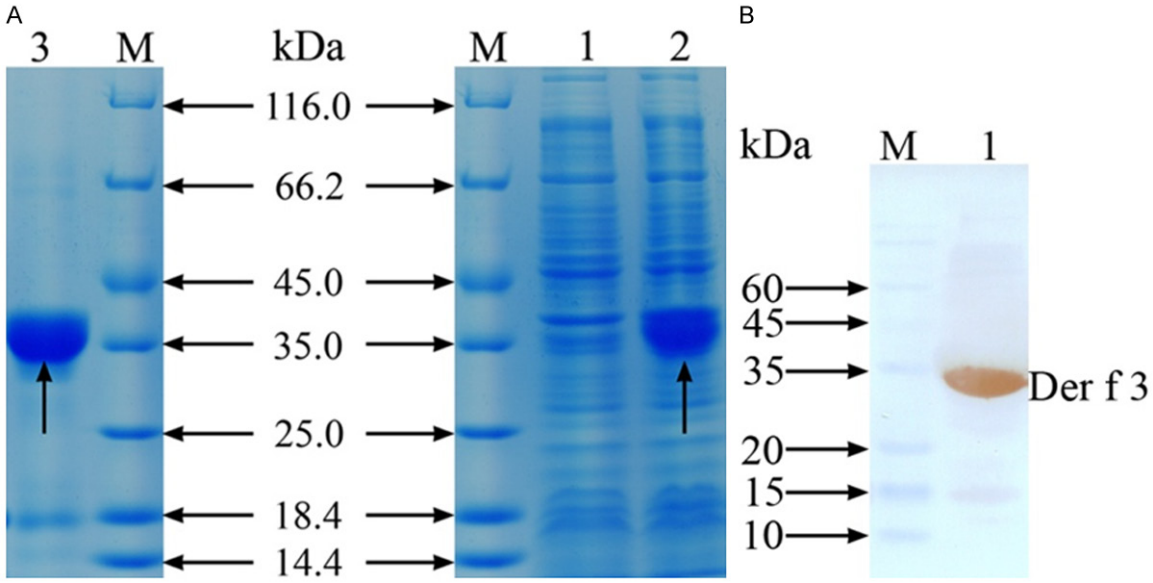

Figure 2. SDS-PAGE and Western blotting analysis of Der f3. (A) Purified recombinant proteins (0.5 mg each) were run on a 12.5% polyacrylamide gel, and then visualized by CBB staining. M: Molecular weight marker; 1: BL21 with Der f3 but without IPTG; 2: Small dose of Der f3 expression; 3: Purified Der f3. (B) Immunoblot analysis of purified recombinant allergens Der f3. After completion of SDS-PAGE (as shown in A), fractionated protein on the gel was transferred onto a PVDF filter, and then stained with human anti-Der f3 serum and rabbit anti-human IgG antibody respectely. M: Rainbow marker simultaneously transferred onto the filter. 1: Purified Der f3.

hypersecretion and airway hyperresponsiveness [14, 15], and closely associated with activated T cells in the lung. Previous studies proved that differentiation of Th0 cells towards Th2 is capable of leading to imbalanced Th1/Th2, a key factor attributable to asthma attack [16] as a result of changed cytokine levels secreted by Th1 and Th2 in the blood of such victims. Th1 cells are generally recognized to generate IFN-γ, IL-2 and other cytokines. IFN-γ functions a lot in B lymphocytes of mouse in synthesizing IgG2a, and can effectively suppress the production of IgE induced by IL-4. Contrarily, Th2 cells are responsible for secretion of IL-4 and IL-5 cytokines [17-20].

Allergen specific immunotherapy (SIT) represents exclusive current etiology therapy that can modify the allergic disease process. It is based on the principles by desensitizing therapy for allergic disease through administration of appropriate concentrations of allergen extracts that has been shown to be reproducibly effective in carefully selected patients. However, traditional vaccines, including crude extracts, mixed allergens, complete antigen protein, or subunit vaccine, are associated with a series of defects, such as serious adverse effects, lower safety in use, high cost and complex development processes, for which a variety of strategies have been tried to eliminate such negative effects. At present, much interests in SIT have focused on the epitope vaccine basis [21-23]. Since an epitope vaccine is decided by the dominant number of T-cell epitope, thus, analysis and identification of T cell epitopes [24-26] are the key to successful research and development of the new epitope

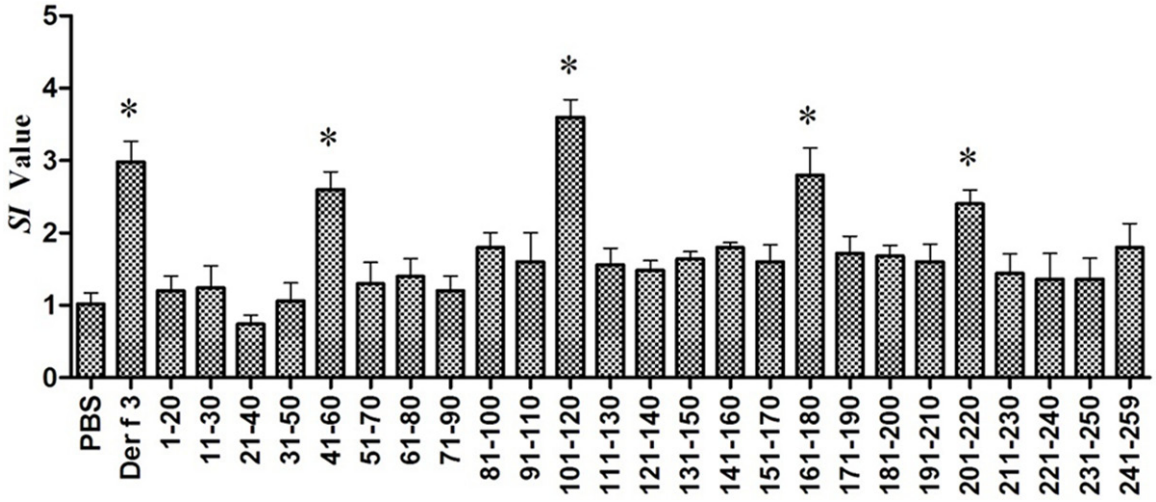

Figure 3. Stimulation index of 25 overlapping synthetic peptides to T-cell lines of asthma mice. *, SI>2.

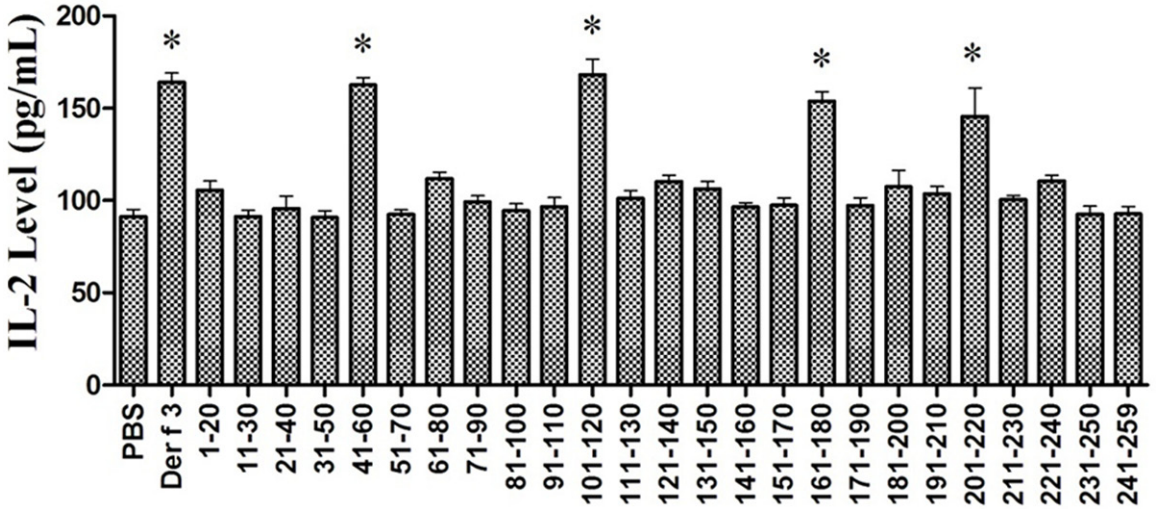

Figure 4. Changes of IL-2 levels in the cultured supernatants from the T-cell lines of asthma mice. *, vs PBS group, P<0.05.

vaccine. Nevertheless, individual epitope peptide varies a lot in diverse region, and epitope vaccines prepared from different categories of dust mite allergens involves highly complex processes and technology. In order to successfully develop an epitope vaccine library that is based on SIT strategy and wider recommendation for majority of the sufferers, T cell epitopes shall be characterized. However, current interests are still focused on the T cell epitope of Der f1, Der p1, Der f2, Der p2, Mag 1 and Mag 3 [7, 27-31]. Der f3, one of the dust mite allergens, has strong allergenicity and trypsin activity, and is capable of inducing serious asthma attack. Yet few reports are available on characterization of T cell epitope of Der f3. In the present study, we firstly identified the T cell epitopes of Der f3 by using amino acid overlapping technique for synthesis of Der f3 peptide, and performed T cell proliferation assay in vitro, screening of active peptides, with an attempt to lay a foundation for preparation of broad-spectrum and epitope vaccine with high specificity.

Materials and methods

Expression and purification of Der f3

The pET28a(+)-Der f3-carrying E. coli BL21 (Preserved in Department of Medical Para-

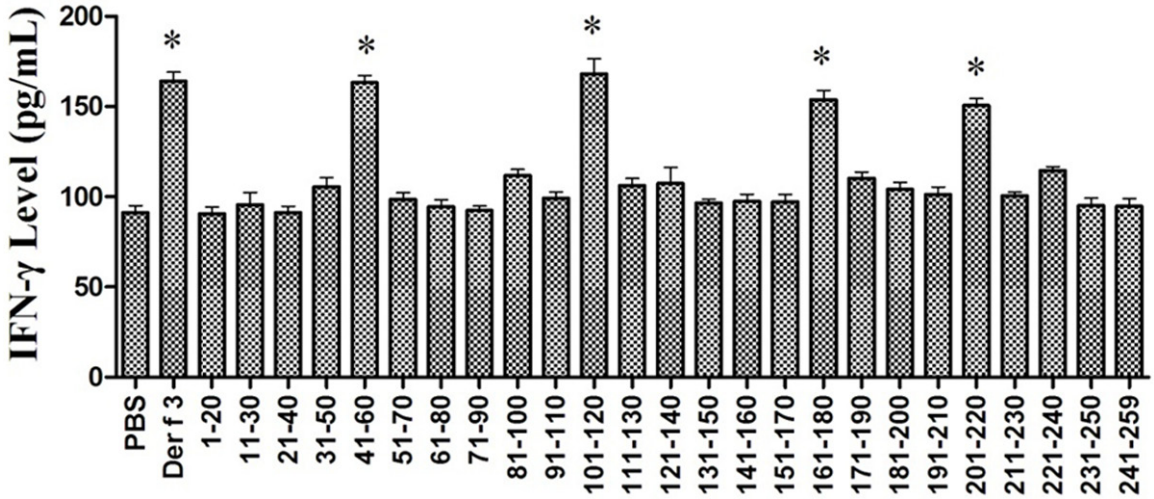

Figure 5. Changes of IFN-γ levels in the cultured supernatants from the T-cell lines of asthma mice. *, vs PBS group, P<0.05.

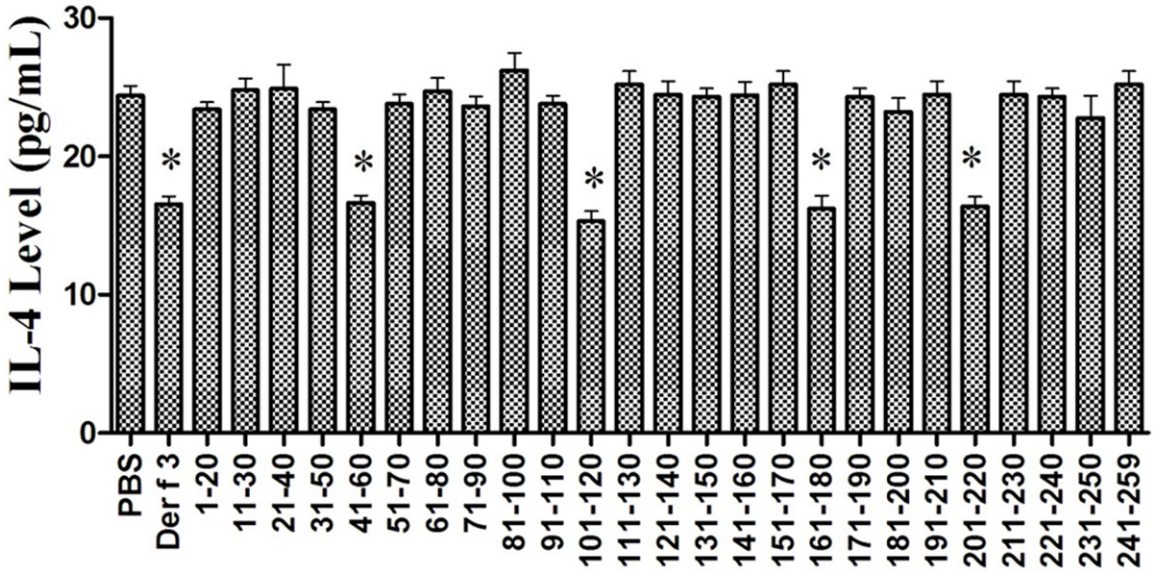

Figure 6. Changes of IL-4 levels in the cultured supernatants from T-cell lines of asthma mice. *, vs PBS group, P<0.05.

sitology, Wannan Medical College) were inoculated into 2 ml LB plates containing 50 μg/ml of kanamycin at 37°C overnight. 1 ml of the cultured solution was taken and added into a new glass tube containing 500 ml LB fluid containing kanamycin, further cultured for 4 h at 37°C. One hundred mmol/L of isopropyl-β-D-thiogalactopyranoside (IPTG, final 1 mmol/L) was added to express the protein of Der f3. After additional 5 h incubation at 37°C, the E. coli cells were harvested by centrifugation. The collected cells were rinsed for 20 min with inclusion body liquid I (10 mol/L Triton X-100, 50 mmol/L Tris-HCl, 150 mmol/L NaCl and 10 mmol/L EDTA) 150 ml at 4°C and centrifuged at 10000 g for 10 min, and then the supernatant was discarded. The cells were was rinsed again with inclusion washing liquid II (2 mol/L Urea, 50 mmol/L Tris-HCl, 150 mmol/L NaCl), then discarding the supernatant. The precipitation was dissolved with 50 mL inclusions dissolving liquid (8 mol/L Urea, 50 mmol/L Tris-HCl, 150 mmol/L NaCl), the supernatant was saved after being centrifuged at 10000 g for 15 min 4°C.

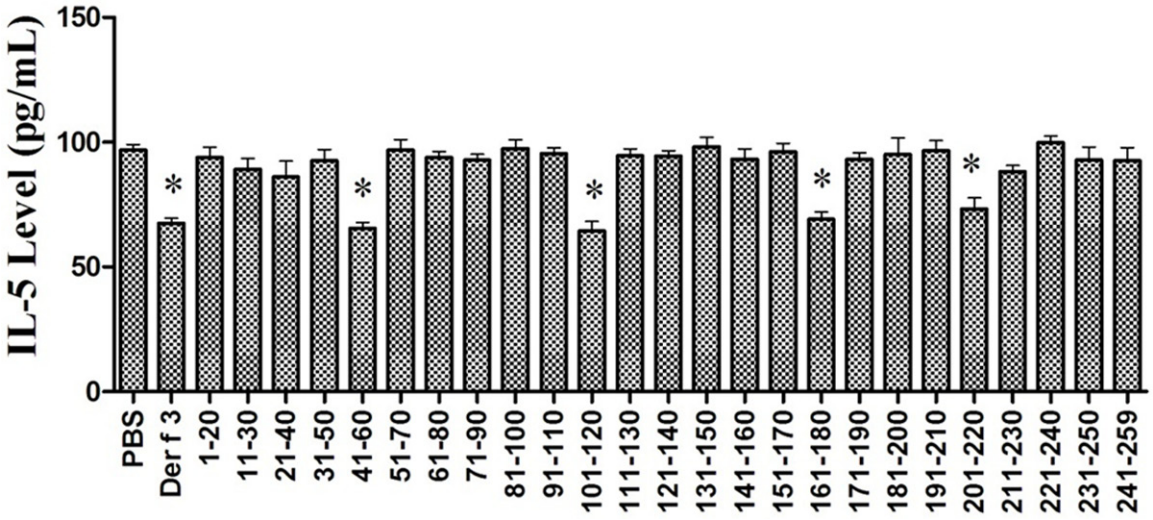

Figure 7. Changes of IL-5 levels in the cultured supernatants from the T-cell lines of asthma mice. *, vs PBS group, $P<0.05$.

The supernatant was passed through the Ni-NTA column which was washed by buffer solution (6 mol/L Urea, 50 mmol/L Tris-HCl, 200 mmol/L NaCl and 30 mmol/L midazole) to obtain the purified Der f3 protein.

SDS-PAGE and Western blotting analysis

Blood samples were obtained under the consent from 3 Der f3-allergic inpatients (Department of Respiratory Medicine of Yijishan Hospital, Wannan Medical College), who had positive skin reaction. Another 3 negative blood samples were obtained from one of the author and two students from our college, who demonstrated negative skin reaction by prick test. The Medical Ethics Committee of Wannan Medical College approved collection of the samples and the informed consent was oral obtained from the study participants.

The unpurified and purified Der f3 protein samples were loaded (20 μl per well) onto a 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) respectively. Fractionated proteins in the gel were electronically transferred onto a PVDF membrane (Sangon Biotech, Shanghai, China) for 2 h at 100 V, and then incubated with primary human anti-Der f3 serum previously prepared overnight at 4°C, followed by incubation with the horseradish peroxidase (HRP)-rabbit anti-human IgG antibody (Sangon Biotech).

Experimental animals and immunization

A total of 40 BALB/c female mice weighing 18-22 g at 6 to 8 weeks old were purchased from Comparative Medicine Center of Yangzhou University (Jiangsu, China). All animal experiments were performed in accordance with the Chinese regulations for animal protection and in adherence with the experimental guidelines and procedures approved by the institution or the National Research Council guide for the care and use of laboratory animals. Thirty of 40 mice were injected intraperitoneally with Der f3 protein on 0, 7 and 14 days, and then challenged with atomization inhalation from day 21. One week after the final intervention, mice were sacrificed and T-cell fraction was obtained from the spleen cells by purifying with nylon wool column as previously described [7]. Another 10 ones were sacrificed and used for antigen presenting cells (APC).

Peptide synthesis

Peptide synthesis was performed using 20-mer synthetic peptides (Sangon Biotech), 10-mer overlapping at 10 amino acid intervals, as the user instructions. Total 25 peptides from p1-20 to p240-259 were identified (**Figure 1**).

T-cell stimulation assay

Polyclonal Der f3-specific T-cell lines were prepared prior to T-cell stimulation assay. To estab-

Table 1. Cytokine level changes in the T-cell line (Case 1)

Groups	IL-2 (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)	IL-5 (pg/mL)
PBS	98.38±11.76	90.49±10.19	23.12±4.89	92.47±10.56
Der f3	174.78±20.89	165.18±18.59	16.11±5.65	68.13±12.74
41-60	170.04±16.32*	177.79±18.69*	15.33±3.89*	70.67±10.88*
101-120	179.77±17.89*	188.05±16.99*	17.40±7.89*	65.77±11.76*
161-180	168.22±25.63*	156.44±26.43*	16.32±5.89*	67.67±20.89*
201-220	125.11±20.72	93.48±15.41	21.11±5.89	89.38±14.43

Data are presented as the mean ± SD. *P<0.05 vs. PBS group.

Table 2. Cytokine level changes in the T-cell lines (Case 2)

Groups	IL-2 (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)	IL-5 (pg/mL)
PBS	88.33±10.82	78.32±10.43	22.55±2.66	96.65±17.89
Der f3	154.68±22.77	143.43±17.87	14.32±1.89	58.43±20.33
41-60	160.45±20.43*	140.56±16.87*	13.76±3.76*	60.11±14.65*
101-120	190.54±18.67*	159.65±23.65*	14.65±2.65*	55.65±5.89*
161-180	188.28±17.89*	158.37±25.37*	16.32±0.89*	69.44±7.75*
201-220	110.27±16.95	82.35±12.69	25.45±6.66	87.45±12.21

Data are presented as the mean ± SD. *P<0.05 vs. PBS group.

lish murine T-cell line, 2×10^5 spleen cells T-cells from Der f3-immunized mice were mixed with Der f3 (5 mg : ml) and 2×10^5 mitomycin C (MMC)-treated splenocytes from ten non-immunized mice as antigen presenting cells (APC). Then the cells were cultured for 5 days in AIM-V serum-free medium (GIBCO: BRL, Grand Island, NY, USA) in incubator (37°C, 5% CO_2, 95% O_2). After the stimulation, expanded antigen-specific T-cells were maintained by standard procedure as previous descriptions. To prepare human T-cell lines, 2×10^5 peripheral blood mononuclear cells (PBMC) from newly diagnosed mite-allergic asthmatic donors (judged by skin test, diameter of erythema >20 mm) were stimulated with Der f3 (5 mg : ml) for 7 days in AIM-V medium. Then the proliferated cells (2×10^5) were co-cultured for additional 7 days with antigens, r-human IL-2 (10 U : ml, Sangon Biotech), and freshly prepared 2×10^5 MMC-treated PBMC as APC. To analyze murine or human T-cell response, 2×10^5 T-cell line was cultured with 2×10^5 fresh APC and antigens (5 mg : ml recombinant antigens, or 1 mg : ml synthetic peptides) for 5 days. The levels of IL-2, IFN-γ, IL-4 and IL-5 in the supernatant of cultures were detected by ELISA 5 days later. Then the sample was measured using microplate reader at 450 nm wavelength, with calculating the stimulation index (SI) after adding CCK-8 (10 μl/well, SI values >2.0 being positive).

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used for statistical analysis to determine differences between groups. *P* value is <0.05 was considered to indicate a statistically significant result.

Results

Expression and purification of Der f3

Der f3 was successfully expressed. SDS-PAGE (**Figure 2A**) and Western Blotting (**Figure 2B**) confirmation demonstrated that these proteins are Der f3.

Experimental mice and immunization

The 30 mice developed typical symptoms of asthma attack, including intense restlessness, deep and fast breath, nodding like breathing, convulsions, scratching in 20 min on the first day of atomization inhalation of the Der f3, and the symptom started earlier with the increased dosage of inhalation excitation.

Localization determination of murine T-cell epitopes using synthetic overlapping peptides

To determine the murine T-cell determinant region, we performed stimulation assay using 25 synthetic overlapping peptides (1-20 to 240-259; 20-mer, 10-mer overlapping at ten amino acids intervals), which covered entire amino acid sequence of the Der f3 molecule. As shown in **Figures 3-7**, significant T-cell response was observed in culture wells stimulated with peptides 41-60, 101-120, 161-180 and 201-220. In contrast, no significant proliferative response was observed in wells stimulated with other peptide samples. These results suggested that murine T-cell determinant was assigned within

Table 3. Cytokine level changes in the T-cell lines (Case 3)

Groups	IL-2 (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)	IL-5 (pg/mL)
PBS	94.45±16.33	93.55±12.82	24.33±3.89	96.33±12.83
Der f3	145.78±17.66	125.55±19.89	13.22±7.54	68.44±14.44
41-60	150.45±24.87*	160.56±18.84*	15.32±2.89*	70.65±15.85*
101-120	180.56±23.65*	148.65±17.98*	17.43±5.62*	65.63±10.55*
161-180	160.43±17.66*	136.33±20.89*	16.54±2.76*	67.64±12.64*
201-220	93.43±10.89	96.54±17.43	23.43±693	97.74±5.89

Data are presented as the mean ± SD. *P<0.05 vs. PBS group.

amino acid range 41-220 of the Der f3 molecule.

T-cell reaction region for mite-allergic patients partially overlapped with those identified in Der f3 primed BALB/c mice

We next determined the human T-cell determinants using the three mite-sensitized asthmatic donors responsive to Der f3 with four mice T cell epitopes. Proliferation assay revealed that the three T-cell determinant regions (41-60, 101-120 and 161-180) of mice were also able to trigger significant T-cell proliferation of human's. This proliferative response was evident in the sera of three patients, and the results suggest that three Der f3 T-cell determinant region in Der f3-immunized BALB/c mice was commensurate with that of mite-allergic individuals; except for the significant T-cell proliferation being not observed when stimulated with peptides 201-220 in the three subjects (**Tables 1-3**).

Discussion

Asthma is a chronic airway inflammatory disease characterized by lung leukocyte and eosinophilic infiltration accompanied by mucus hypersecretion and airway hyperresponsiveness [14]. It was estimated that about 80% of asthmatic patients were allergic to dust mites, one of the most important factors. Although the allergenic properties of Der f3 were characterized several years ago, yet its T-cell epitopes fail to be determined to date, since allergen specific immunotherapy (SIT) is generally recognized as the only gene therapy for this entity via desensitization for the subjects who are administered with increasing doses of allergen extracts. However, traditional vaccines have a series of defects. At present, many researches on SIT mainly concentrated in the epitope vaccine, for these vaccines are high immunogenic and hypoallergenic. Importantly, this kind of vaccine may not only promote the Th1 cell proliferation in the cellular immune by correcting Th1/Th2 imbalance in the process of asthma, but reduce the potential harm to body due to the humoral immunity aiming at allergen itself.

An epitope, also known as antigenic determinant, is recognized by corresponding antibody or specific antigen receptor, and the main chemicals recognized by immune system. T cell epitope is the clips that can be identified and combined specificity by T-cell surface receptors, or antibody [32]. T cell epitope can stimulate the Th1 cells secreted cytokines IL-2, IFN-γ after combining with the activated antigen presenting cells (APC), and these cytokines could further promote the differentiation of Th1 cells to produce more Th1 cells and inhibition of the proliferation of Th2 cells, which could promote Th0 cells to differentiate into Th1 cells to modify imbalanced the Th1/Th2 in the course of asthma attack to achieve asthma symptom relief [16, 25]. Conventional determination of the T-cell epitope peptide relies on overlapping technique that may lead to reduced miss-detection of the epitopes by analysis of the whole cell epitope peptides, and improved detection accuracy. Furthermore, elucidation of T-cell activation mechanism against important mite allergen is pivotal for the development of specific immunotherapy, and T-cell epitope analysis is a primary issue of this study.

To solve the previous problems, we conducted an analysis on the murine and human T-cell reactive region on the Der f3 polypeptide. Initial proliferation assay with 25 synthetic overlapping peptides of Der f3 indicated that T-cell reactive region was assigned within amino acid 41-60, 101-120, 161-180 and 201-220. ELISA measurement of the cytokine levels in culture supernatant showed higher IL-2 and IFN-γ, lower IL-4 and IL-5, which indicated that T-cell reactive region was assigned within amino acid 41-60, 101-120, 161-180 and 201-220. We

also determined human T-cell determinant using specific T-cells from mite-allergic patients with four mice T-cell epitopes, and somehow found that amino acid range 201-220 identified by the murine T-cell epitope in Der f3 failed to be recognized by T-cells from three patients. This may be explained by that T cell epitopes of Der f3 allergen were distributing within amino acid 41-60, 101-120, 161-180. The cytokine levels in culture supernatant measured by ELISA showed high levels of IL-2 and IFN-γ, yet low levels of IL-4 and IL-5. This indicates that T cell epitope can adjust Th1/Th2 imbalance in the process of asthma and improve the treatment effect. However, further investigation on unique T-cell epitope region determined in our work remains necessary prior to development of the vaccine on Der f3 basis in large scale for clinical use.

Acknowledgements

This research was supported by National Natural Science Foundation of China (Nos. 8127-0091, 30872367).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chaopin Li, Department of Medical Parasitology, Wannan Medical University, Wuhu 241002, Anhui, China. Tel: +86 553-393-2587; E-mail: cpli001@126.com

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