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Original Article

Construction of the recombinant vaccine based on T-cell epitope encoding Der p1 and evaluation on its specific immunotherapy efficacy

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Abstract: Specific immunotherapy (SIT) is currently recognized as the only etiological therapy to ameliorate asthmatic symptom. The current study was aimed at evaluating the immune effect of vaccine MAT3T designed on MHCII pathway, which includes T cell fusion peptide encoding *Dermatophagoides pteronyssinus* class 1 allergen (Der p1). We initially cloned the nucleotide sequences of TAT, IhC and 3 segments of T cell epitope coding for Der p1, and reassembled these sequences in linear manner to form fusion gene named MAT3T, which was applied to immunize the asthmatic models of mice induced by Der p1 allergen for tentative SIT. ELISA results showed that MAT3T was able to increase the level of IFN-γ in BALF and allergen specific antibody IgG_{2a} in serum, while decrease the level of IL-13 in BALF and allergen specific antibody IgE and IgG_1. Pathological confirmation further revealed that the inflammatory reactions and inflammatory cell infiltration were totally reduced in lung tissue of mice after MAT3T treatment. Our results show that the recombinant allergen MAT3T can effectively correct the imbalance of Th1/Th2, and MAT3T may be used as candidate vaccine against asthma on SIT basis.

Keywords: *Dermatophagoides pteronyssinus*, class 1 allergen, allergic asthma, chimeric allergens

Introduction

Allergic asthma is a chronic airway inflammation attributed to a variety of cells, including mast cells, eosinophils and helper T lymphocytes. This inflammation is characterized by variable and recurring symptoms of reversible airflow obstruction, airway hyperpesponsiveness, limited airflow, chest distress and cough. Allergic asthma tends to be more prevalent worldwide and has become one of the important global health concerns [1], and is easily affected by genetic susceptibility, pathways to expose to allergens, contacted dose of allergen, even allergen configuration and other factors [2, 3]. However, allergic asthma and other allergic disorder are strongly predisposed to allergens of *Dermatophagoides farinae* (Der f) and *Dermatophagoides pteronyssinus* (Der p) [4, 5], from which the major group 1 and group 2 allergen (Der pl/Der f1, Der p2/Der f2), important air transmissive allergen, can bind as high as 70% of the IgE in patient serum [6].

It is well acknowledged that the pathogenesis of asthma attack is associated with Th1/Th2 cells imbalance that is marked by predominance of type 2 cytokines secreted by Th2 cells. Th2 cells are important roles to change the course of allergic asthma through inducing B cells to produce allergen specific IgE and secret cytokines of IL-4, IL-5 and IL-13, eventually leading to recruitment and infiltration of eosinophils, basophiles and mast cells [7, 8]. Additionally, asthma attack is also associated with presence of Th17 cells and regulatory T (Treg) cell balance [9-12].

Specific immunotherapy (SIT) represents the exclusive allergen specific and disease modifying approaches to allergic patients [13, 14]. This is achieved through inducing the allergen specific immune tolerance to finally improve the disease course and alleviate allergy symptoms [15, 16]. However, some adverse events, ranging from local skin wheal, areola, sclerosis and necrosis to general symptoms, such as life-

threatening anaphylactic shock, laryngeal edema, bronchospasm, systemic erythema, urticaria and angioedema, may occur in SIT. This makes it hard for wider clinical application [17, 18]. Nevertheless, these side effects can be counteracted by designing a peptide vaccine with dominant $CD4^+$T cell epitope specific to the allergen [19-21].

Theoretically, antigen peptides are generated by hydrolysis of the proteolytic enzyme in the lysosomes bound with the antigen presenting cell (APC) to which after the antigen being presented. As the major histocompatibility complex (MHC) class II molecules from endoplasmic reticulum and being protected by the invariant chain (Ii) bind with the lysosomes, Ii is hydrolyzed, and the complexes produced after binding of MHC II with the segments of antigen peptides are presented to $CD4^+$T cells [22]. Endogenous allergen expression occurs after fusion of the exogenous allergen with Ii chain, and is efficiently delivered by MHC II presentation pathway, consequently enhancing the SIT efficacy [23]. Crameri *et al* [24] had successfully designed a modular-antigen translocating (MAT) vector containing the first 110 amino acids of the invariant human chain (IhC) that was introduced into TAT and Ii, in which TAT (GYGRKKRRQRRR) represents a protein transduction peptides of human immunodeficiency virus (HIV) and is biological role capable of transferring extracellular proteins into the cells [25, 26]. IhC is the first 110 amino-acids of short peptide sequences of Ii for targeting the fusion proteins to endosomal/lysosomal compartments [23, 27]. Once the allergen gene is inserted into MAT vector, the chimeric gene containing allergen TAT-IhC occurs, and the allergen TAT-IhC as a vaccine for SIT is achieved after prokaryotic expression. The experiment showed that the chimeric allergen TAT-IhC can effectively correct the Th1/Th2 imbalance in patients with asthma via promoting secretion of Th1 type cytokines and IL-10, yet decreasing secretion of Th2 type cytokines and IL-2 [24]. These finding suggests that vaccines based on MHC II presentation pathway can be effective strategy by SIT for allergic asthma.

In current study, we attempted to bind the nucleotide sequences of 3 period of T cell epitope coding for Der p1 in linear manner to form fusion gene, and insert it into MAT vector to design chimeric gene MAT-Der p1-3T, i.e., MAT3T. After prokaryotic expression of the above gene, we tentatively used the MAT3T as a vaccine for SIT in mouse models of allergic asthma with an attempt to provide a basis for the vaccine development via evaluation of the efficacy in mice.

Materials and methods

Animals

Forty female BALB/c mice, aged from 6 to 8 weeks and weighed between 18 and 22 g, were purchased from the Center of Comparative Medicine, Yangzhou University (Licence: SCXK SU 2007-0001). All of the mice were provided with food and water under specific-pathogens free conditions. Animal studies were approved by the Research Ethics Board of Wannan Medial College.

Construction of recombinant plasmid

The three known T cell epitopes (119-147, 176-197 and 207-265, GenBank: AAB60215.1) encoding ProDer p1 were linearly combined into Der p1-3T nucleotide sequence (354 bp). Amplification of the Der p1-3T nucleotide sequence was performed by forward primer and reverse primer of 5'-GAGCGGCCGCAACTGTCACTCCCATTCGTATG-3' (*Not* I) and 5'-GACTCGAGTTGGTAACCATTATCGCGTTGA-3' (*Xho* I), respectively. The sequence of primers, TAT and $IhC_{1\text{-}110}$ were all cloned by Sangon Biotech, Shanghai, China. pET28a-MAT vector was designed as previous description [24]. The PCR product of Der p1-3T was purified and inserted into the pET28a-MAT vectors to create recombinant plasmid pET28a-MAT3T, and nucleotide sequences were identified by sequencing.

Expression and purification of the recombinant antigens

Recombinant plasmid pET28a-MAT3T was transfected *into E. coli* BL21 competent cell (TIANGEN, No: CB105-02). Antigen expression was induced by 1mmol/L Isopropyl-β-thiogalactopyranoside (IPTG). The total protein was purified under denaturing conditions with Ni^+-NTA affinity column chromatography kit (Invitrogen, Carlsbad, CA, USA). The purified proteins MAT3T were assessed by SDS-PAGE using 12.5% polyacrylamide gel and Coomassie Blue staining. Protein concentration was measured by the Brandford kit according to the manufac-

turer instructions (Sangon Biotech, Shanghai, China).

Western blotting

A total of 2 μg purified MAT3T proteins were analyzed on 12.5% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA), which was incubated in blocking buffer (0.05% dried mile; 50 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 0.1% Tween-20) overnight at 4°C. Then, the membranes were incubated in monoclonal rabbit antibody as primary antibodies by ratio of 1:500 for 2 h (Sangon Biotech, Shanghai, China) at 37°C, and rinsed 3 times in the blocking buffer (10min each). HRP-goat anti rabbit IgG (Sigma-Aldrich Co. LLC.) was used as second antibody by ratio of 1:5000, followed by 3 washes in blocking buffer (10 min each). Transferred proteins were visualized using DAB Horseradish Peroxidase Color Development Kit (Sangon Biotech, Shanghai, China) in PBS (pH 7.2) according to the manufacturer instructions.

Determination of the binding MAT3T with IgE

The serum samples in our study were obtained in the affiliated Yijishan Hospital of Wnnan Medical College from patients allergic to mites, and all patients signed informed consent. The clinical trial was approved by Institutional Review Board of Wannan Medial College. Determination of the biding capacity of MAT3T with IgE was performed as previous description [28].

Development of the asthmatic models in mice and SIT

Forty BALB/c mice were randomly assigned to four groups (n=10 for each), i.e. PBS group (negative controls), asthma group, Der p1 group and MAT3T group. Mice were sensitized with intraperitoneal injection of 10 μg relevant allergen in 100 μL PBS (pH7.2) containing 2% (w/v) $Al(OH)_3$ at day 0, 7 and 14, respectively. The PBS group was exclusively treated with PBS. At day 21, the animals were challenged by nebulized inhalation of Der p1 (0.5 μg/ml) for 30 min for successive 7 days. The PBS group was challenged by PBS. SIT was daily performed for 30 min prior to aerosol inhalation from day 25 to 27. Der p1 group and MAT3T group given intraperitoneal injection of Der p1 and MAT3T containing 10 μg relevant allergen for specific immune therapy. PBS group and asthma group was challenged by PBS. After the final challenge, blood was collected via inner canthus in 24 h in all mice, and stored at -80°C for following use.

Collection of the bronchoalveolar lavage fluid (BALF)

All mice were anaesthetized with 10% chloral hydrate (0.2-0.3 ml) through intraperitoneally injection. Collection of the bronchoalveolar lavage was done via tracheal cannula under aseptic conditions. Then pre-cooled PBS by dose of 0.4 ml, 0.3 ml and 0.3 mL (total 1 mL), respectively was applied to irrigate the trachea till recovery rate greater than 85%. The liquid collected was centrifuged at 5000 × g for 5 min, and the supernatant was obtained and stored at -80°C for following use.

Detection of cytokines in BALF and antibodies in serum

ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to detect the levels of cytokines IFN-γ and IL-13 in BALF, and antibodies of specific allergens IgE, IgG_1 and IgG_{2a} in serum according to the manufacturer instructions.

Histopathological examination of the lung tissue

Lung tissues were fixed with 10% formaldehyde solution and embedded in paraffin, and stained with hematoxylin and eosin (HE) after conventional sectioning. The sections were observed under microscope (BX51, Olympus, Japan) for the histopathological changes.

Statistical analysis

The data in each group were expressed as mean ± SD, and analyzed using SPSS 16.0. Differences between groups were analyzed using the one-factor analysis of variance. F inspection was applied for comparison among several groups while t or t' inspection were applied for comparison between two groups. A P-value of less than 0.05 was accepted as significant.

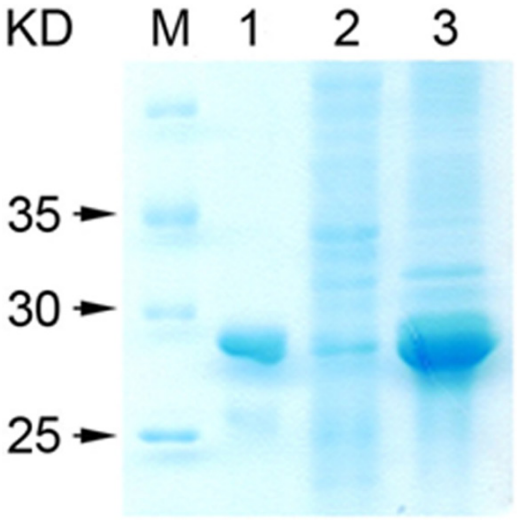

Figure 1. Detection of recombinant protein MAT3T expressed in *E. coli* strain BL21 (DE3). M: protein marker; Lane 1: Purified recombinant protein MAT3T; Lane 2: Total protein of *E. coli* strain BL21 (DE3) non-induced by IPTG; Lane 3: Total protein of *E. coli* strain BL21 (DE3) induced by 1 mmol/L IPTG.

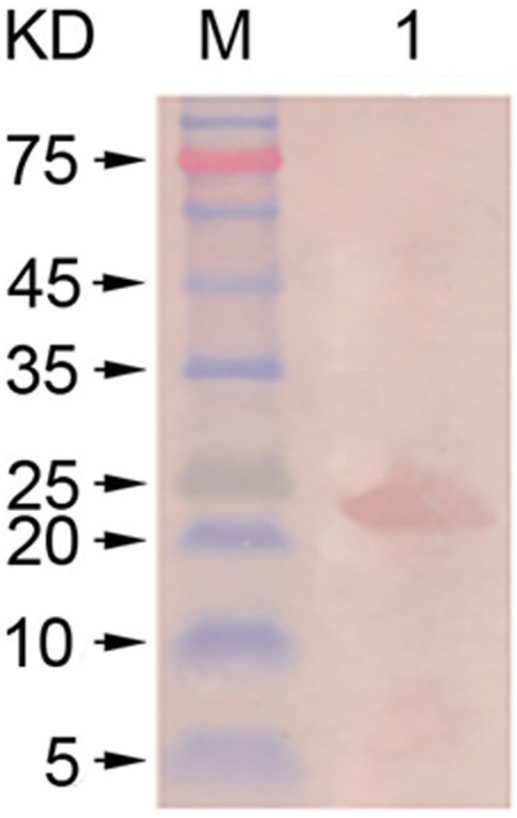

Figure 2. Western blot analysis of the purified MAT3T. M: protein marker; Line 1: Purified recombinant protein MAT3T.

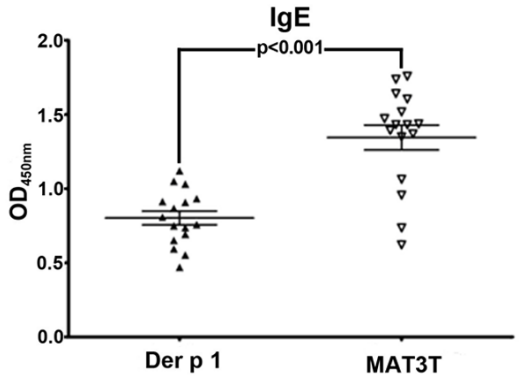

Figure 3. Binding capacity of MAT3T with IgE.

Result

Identification of the recombinant plasmid pET28a (+)-MAT3T

The recombinant plasmid pET28a (+)-MAT3T was identified by restriction enzyme digestion with BamH I and Xho I. Successful recombinant plasmid was verified by 687 bp after nucleotide sequencing of MAT3T.

Detection of recombinant protein MAT3T

In order to examine the expression of fusion protein, recombinant plasmid pET28a (+)-MAT3T was transfected into *E. coli* BL21 (DE3) competent cell, and then induced by 1mmol/L IPTG and purified by Ni^{2+}-NTA. Single MAT3T protein band was exposed at 28 kD by SDS-PAGE examination (**Figure 1**).

Western blot analysis of recombinant protein MAT3T

Western blot analysis of the recombinant MAT3T was performed by using anti-rabbit polyclonal antibodies as primary antibodies. The results showed that the band was absent in the control group without IPTG induction, yet present in MAT3T purification group (**Figure 2**). The size appeared consistent with SDS-PAGE text, which indicated successful recombinant protein MAT3T.

Testing of the binding capacity of MAT3T with IgE

96-well plate was coated with Der p1 and MAT3T protein, and ELISA was used to verify

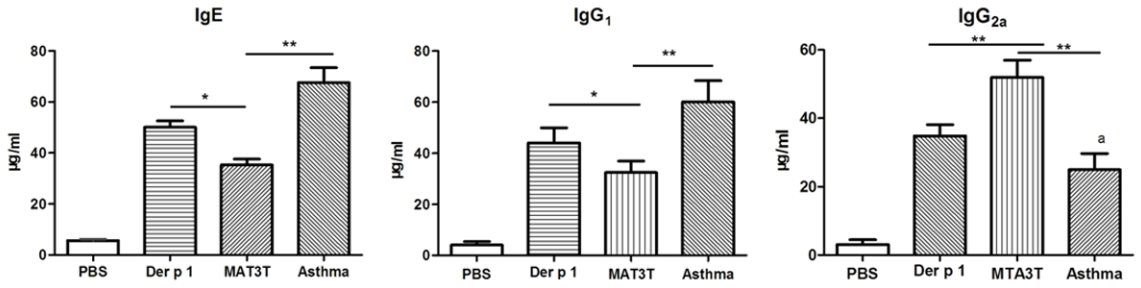

Figure 4. Changes of allergen-specific antibodies IgE, IgG_1 and IgG_{2a}. Note: *: $P < 0.05$; **: $P < 0.01$.

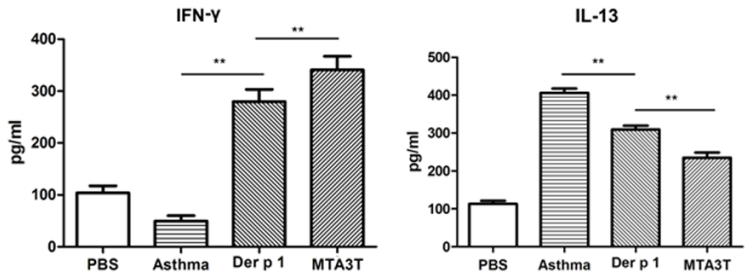

Figure 5. Level of IFN-γ and IL-13 in bronchoalveolar lavage fluid. Note: *: $P < 0.05$; **: $P < 0.01$.

the binding capacity of MAT3T with IgE. The results showed that the binding with IgE (0.80±0.19) was significantly higher than Der p1 (1.35 ± 0.33) ($P < 0.001$) (**Figure 3**), suggesting that MAT3T may have a better allergenicity.

Level changes in f allergen-specific antibodies

In order to verify the effect of MAT3T on asthma mice, we determined the level changes with ELISA concerning antigen-specificity IgE, IgG_1 and IgG_{2a} in serum from each mouse. The results showed that serum IgE level was lower in MAT3T group (35.26±5.02 μg/mL) than that of asthma group (67.56±13.01 μg/mL, $P < 0.01$) and Der p1 group (50.02±5.75 μg/mL, $P < 0.05$). The levels of IgG_1 in MAT3T group, asthma group and Der p1 group were 32.38±4.53 μg/mL, 60.07±8.06 μg/mL and 44.03±5.91 μg/mL, respectively, in which the level was significantly lower in MAT3T group ($P < 0.01$) and in Der p1 group ($P < 0.05$). However, the level of antibody IgG_{2a} in MAT3T group (51.9±5.06 μg/mL) was obviously higher than that of asthma group (24.95±4.71 μg/mL, $P < 0.01$) and Der p1 group (34.89±3.22 μg/mL, $P < 0.01$) (**Figure 4**). These findings revealed that MAT3T and Der p1 can effectively down-regulate antigen-specificity IgE, IgG_1 antibodies, yet boost IgG_{2a} antibody for asthma mice.

Level changes in IFN-γ and IL-13 in BALF

The level of IFN-γ in BALF from mice in specific immunotherapy MAT3T group (340.7±26.63 pg/ml) and Der p1 group (279.9±23.3 pg/ml) was obviously higher than that of asthma group (49.55±10.27 pg/mL) ($P < 0.01$), and the difference was significant between MAT3T group and Der p1 group ($P < 0.01$) (**Figure 5**). The level of IL-13 in BALF was (234.8±30.93 pg/mL) in MAT3T group, which was distinctly lower than asthma group, Der p1 group and PBS group ($P < 0.01$) (**Figure 5**).

Histopathologic changes of the lung tissue

Examination of the lung tissue sections for each group of mice demonstrated that inflammatory cell infiltration, vasculitis, mildly thickened bronchial wall, partially fractured or shed epithelial cells, or occasional fibrosis hyperplasia occurred in the bronchial, sub-mucosal vessels and surrounding lung tissues. Contrarily, pathological changes were not significant for the mice in PBS group whose bronchia, alveolar structure and mucosal membrane at the bronchial wall remained relatively intact without evident inflammatory cell infiltration. The inflammatory response and cell infiltration in the lung tissues of mice were greatly alleviated after Der p1 and MAT3T specific immunotherapy as compared to the asthma group, and the reduced inflammation was more evident in MAT3T group than Der p1 group (**Figure 6**).

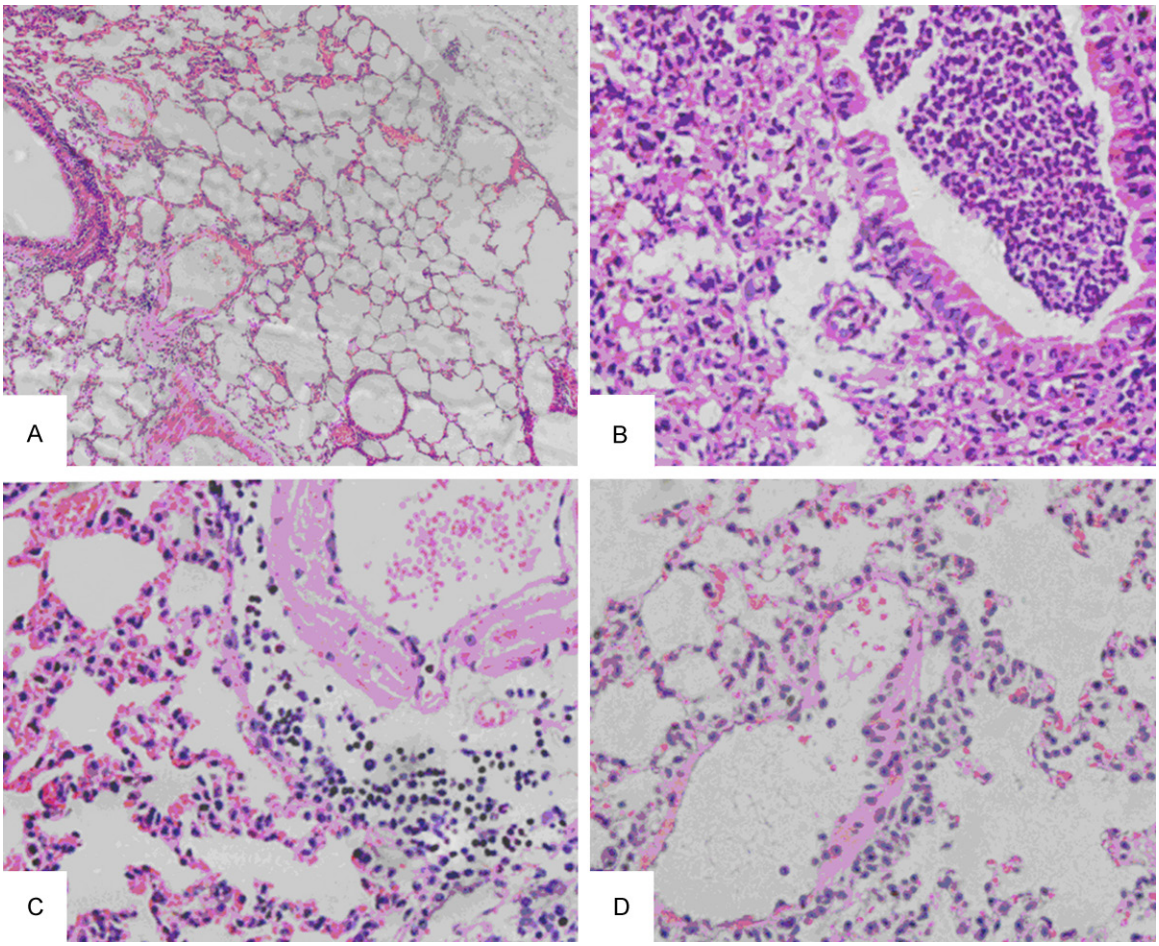

Figure 6. Observation of lung tissue sections (HE staining, ×400). A: PBS group; B: Asthma group; C: Der p1 group; D: MTA3T group.

Discussion

SIT is recognized as the sole allergen specific and causative treatment for allergic diseases [29], because it can countercheck type I hypersensitivity through induction of secretion of allergen specific antibody IgG and effective reduction of allergen specific antibody IgE level in patients with such disorder [30]. Since T cell multiplication can be stimulated by T cell epitopes, a linear amino acid sequence, recombined pollen allergen Bet v1 with T cell epitopes being preserved and B cell epitopes being modified can reduce the level of allergen specific antibody IgE, and prevent the allergens from binding with IgE, thus inducing the disability of antigen specific T cell by SIT [31]. This suggests that the best strategy for SIT shall depend on designing a vaccine with properties of hypoallergenicity (e.g. reducing B cell epitopes) and high immunogenicity (e.g. T cell epitomes being preserved or increased) [32]. Prickett *et al* [33] had sccessfully stimulate the proliferation of mononuclear cells in the peripheral blood in patient with $CD4^+$ T cell epitope coding for allergen Ara h1, an allergy extracted from peanut, and Mackenize *et al* [34] also confirmed that T cell epitope encoding ovalbumin(OVA) of chicken can inhiit the secretion of antigen specific anbibody IgE and profiferation of eosnophils in SIT.

Previous reports proved that TAT can transfer extracellular proteins into cell [25], and IhC can target protein to the endosome/lysosome [24]. The Chimeric allergens MAT3T (including TAT, Ihc and 3 period of T cell epitope coding Der p1) are designed successful in our study, which was identified by SDS-PAGE and Western blot. In order to detect this allergenicity of recombination allergens MAT3T, we used ELISA methods to detection the combining ability between

allergen and IgE in serum from patients who suffer from allergic asthma with mites. It turned out that allergen MAT3T have higher allergenicity compared with allergen Der p1, this maybe because of the hidden B cell epitope in MAT3. Wu *et al.* [35] also confirmed that T cell epitope encoding Der p2 could induce large amount of IgE when SIT, this T cell epitope has hidden B cell epitope.

In this study, with the purpose of analysis the vaccine effectiveness of T cell epitope fused gene based on MHC II past way to SIT, MAT3T was taken as the vaccine to do SIT in allergic asthma mouse, the level of IFN-γ and IL-13 in BALF and antigen specific antibody IgE, IgG_1 and IgG_{2a} in serum were analysis. It turned out that MAT3T can raised effectively the level of IFN-γ secreted by Th1, while reduce the level of IL-13 secreted by Th2. Furthermore, the levels of IgE and IgG_1 in mouse cured by MAT3T for SIT were lower than other groups significantly, as well as lower than Der p1 group; while the level of IgG_{2a} was higher than other groups. The result illustrated MAT3T could adjust Th1/Th2 imbalance to cure allergic asthma. This result was consistent with the report of Crameri *et al.* [24].

In total, this fusion protein MAT3T obtained in our research can transfer extra-cellular proteins into cell, fixed protein onto lysosome and enhance T cell epitope. This study laid the foundation for developing of vaccine and clinical diagnosis in SIT.

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Disclosure of conflict of interest

None.

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