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Construction and expression of *Dermatophagoides pteronyssinus* group 1 major allergen T cell fusion epitope peptide vaccine vector based on the MHC II pathway

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Abstract

***Backgound and aims: Dermatophagoides peteronyssinus* is one of the important house dust mites responsible for allergic asthma that can be tentatively managed by specific immunotherapy. The present study was to construct a vector encoding T-cell epitopes of major allergen group 1 of *Dermatophagoides pteronyssinus* as a vaccine delivered by MHC class II pathway.**

***Methods:* the nucleotide sequences of the 3 target genes were synthesized, including TAT, IhC and the recombinant fragment of Der p 1 encoding 3 T-cell epitopes. After amplification of the 3 target fragments by PCR and digestion with corresponding restriction endonucleases, the recombinant gene *TAT-IhC-Der p 1-3T* was ligated using T4 DNA ligase and inserted into the prokaryotic expression vector *pET28a*(+) to construct the recombinant plasmid *pET- 28a(+)-TAT-IhC-Der p 1-3T*, which was confirmed by digestion with restriction endonucleases and sequencing. The recombinant vector was transformed into *E. coli* strain BL21 (DE3) and induced with IPTG, and the induced protein TAT-IhC-Der p1-3T was detected by SDS-PAGE. After purification, the recombinant protein was confirmed by Western blotting and its allergenicity tested using IgE-binding assay.**

***Results:* the recombinant plasmid *pET-28a-TAT-IhC-Der p1-3T* was successfully constructed as confirmed by restriction endonuclease digestion and sequencing, and the expression of the recombinant protein TAT-IhC-Der p1-3T was induced in *E. coli*. Western blotting verified successfull purification of the target protein, which showed a stronger IgE-binding ability than Der p1.**

***Conclusion:* we successfully constructed the recombinant expression vector *pET-28a-TAT-IhC-Der p1-3T* expressing a T-cell epitope vaccine delivered by MHC II pathway with strong IgE-binding ability, which provides**

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CONSTRUCCIÓN Y EXPRESIÓN DE VECTOR DE VACUNA DE PÉPTIDO EPÍTOPO DE FUSIÓN DE CÉLULAS T DE ALÉRGENO PRINCIPAL DEL GRUPO 1 *DERMATOPHAGOIDES PTERONYSSINUS* BASADO EN LA VÍA MHC II

Resumen

***Antecedentes y objetivo:* el *Dermatophagoides peteronyssinus* es uno de los principales ácaros del polvo doméstico responsables del asma alérgica que se pueden administrar provisionalmente para una inmunoterapia específica. El presente estudio busca construir un vector que codifique epítopos de células T del grupo de alérgenos principal, el Grupo 1 de Dermatophagoides pteronyssinus como una vacuna suministrada mediante la vía MHC de clase II.**

***Métodos:* se sintetizaron las secuencias de nucleótidos de los 3 genes objetivo, incluyendo TAT, IhC y el fragmento recombinante de Der p 1 encargado de codificar 3 epítopos de célula T. Después de la amplificación de los 3 fragmentos objetivo por PCR y digestión con endonucleasas de restricción correspondientes, el gen recombinante TAT-IhC-Der p 1-3T se ligó usando T4 DNA ligasa y se insertó en el vector de expresión procariota *pET28a (+)* para construir el plásmido recombinante *pET 28a (+)-TAT-IHC-Der p 1-3T,* que se confirmó por digestión con endonucleasas de restricción y secuenciación. El vector recombinante se transformó en *E. coli* cepa BL21 (DE3) y se indujo con IPTG, y la proteína inducida TAT-IHC-Der p1-3T se detectó mediante SDS-PAGE. Después de la purificación, la proteina recombinante se confirmó por análisis de inmunotransferencia (Western blot) y se probó su alergenicidad usando el ensayo de unión a IgE.**

***Resultados:* el plásmido recombinante *pET-28a-TATIHC-Der p1-3T* se construyó con éxito, se confirmó por digestión con endonucleasas de restricción y la secuenciación y la expresión de la proteína recombinante TAT-IHCDer p1-3T fue inducida en *E. coli.* Purificación con éxito verificada mediante Western blot de la proteína objetivo, que mostró una capacidad de unión a IgE más fuerte que Der p1.**

***Conclusión:* hemos construido con éxito el vector de expresión recombinante pET-28a-TAT-IHC-Der p1-3T que expresa una vacuna de epítopo de células T administrada por vía MHC II con fuerte capacidad de union a IgE. Este trabajo proporciona una base para seguir**

a basis for further study on specific immunotherapy via MHC class II pathway.

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Key words: Dermatophagoides pteronyssinus. *Fused peptide vaccine. Major group 1. Allergen. Prokaryotic expression. T cell epitope. IgE-binding assay.*

estudiando la inmunoterapia específica mediante la vía MHC de clase II.

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Palabras clave: Dermatophagoides pteronyssinus. *Vacuna de pétidos sintéticos. Grupo principal 1. Alérgeno. Expresión procariótica. Epítopos de células T. Ensayo de unión a IgE.*

Introduction

Allergic asthma is a Type I allergy, primarily characterized in clinic by reversible airway obstruction, bronchial inflammation and mucus hypersecretion or the similar symptoms[1,2] and commonly caused by abnormal immunoreactivity against allergens such as one important species — *Dermatophagoides pteronyssinus* (*Der p*). The allergens are found positive in about 80% asthmatics allergic to *Der p* 1[3-6]. Currently, specific immunotherapy (SIT) is considered the only etiological therapy that can ameliorate the allergic symptoms for long period[7-10], yet responsive stimulation of T cells is essential for desensitization and reduction or inhibition of allergen specific IgE[2,11,12]. Previous experiment on the use of human T cells epitope peptide from Fel d 1 allergen for SIT demonstrated a change of the reaction of T cells to these peptides[13]. The potential mechanism may be that the allergen was up-taken through antigen presenting cells (APC), and antigen peptide was generated by hydrolysis of the lysosomal protease. After the MHC II from endoplasmic reticulum was protected by the invariant chain (Ii) and enters the lysosomes, Ii was hydrolyzed, leading to formation of the MHC II molecule with peptide that are finally presented to CD4$^+$T cells[14].Antigen presentation through the MHC II pathway is recognized as a potential technique to the treatment of allergic diseases[15]. Studies have shown that construction and prokaryotic expression of MHC II-peptide fusion protein can promote effective presentation of the peptides[16], because the sequence of TAT, with 11 amino acids (GYGRKKRRQRRR) derived from the human immunodeficiency virus (HIV), can transfer to the proteins from extracellular into intracellular[17], whereas the IhC is short peptide of the first 110 amino acids from human lysosomal Ii, and can target protein at the endosome lysosomes[18,19]. Thus, the vaccine constructed on TAT-IhC-antigen fusion protein basis may effectively alleviate the symptoms of patients with asthma[15].

In present study, we tentatively applied the sequences of TAT, IhC and Der p1, T cell epitopes, to fusion of the gene by molecular biology technique. The fused gene was defined as TAT-IhC-Der p1-3T, which was subjected to prokaryotic expression in vector pET28a (+)-TAT-IhC-Der P1-3T, and further expression and purification by IPTG induction. The purified product was tested for the allergenicity via observation on its binding capacity with IgE, with an attempt to lay a foundation for preparation of such clinical vaccine with high efficacy on MHC II pathways.

Methods

Bacterial strains and plasmids

Escherichia coli DH-5, BL21 (DE3) competent cells and *PET-28a* vector were preservings of our laboratory.

Design and synthesis of the primers

Primers were designed based on the nucleotide sequences of TAT (No: NP_057853.1), IhC (1~110AA) (No: K01144.1) and ProDer p 1 (No: NP_11695.1) released in the GenBank, by using corresponding restriction sites.

The primers for TAT (e-ncoding 11AA) included the upstream primer 5'-GATCTTACGGTCGTAAAAAGCGTCGCCAGCGTCGCCGTGGATCCACTAGT-3' (*BamH* Ⅰ) and the downstream primer 5'-TCGAGACTAGTGGATCCACGGCGACGCTGGCGACGCTTTTTACGACCGTA-3' (*Xho* Ⅰ). And the primers for IhC(1~110AA) were consisted of the upstream prime 5'-GAAGATCTATGGATGACCAGCGCGACC-3' (*Bgl* Ⅱ) and the downstream primer 5'-AAACTAGTGGATCCCCCCTGGGGCAGGGCTCC-3' (*Hind* Ⅲ).

The three T cell epitopes matching with the nucleotide sequence of ProDer p1 (118-146, 175-196 and 206-264) reported previously were synthesized into a complete nucleotide sequence with a length of 324bp, which was defined as Der p1-3T). The primers for PCR amplification comprised 5'-TTAGGATCCCGAACTGTCACTCCCATTCGT-3' (*BamH* Ⅰ) as upstream primer and 5'-AGCCTCGAGTTGGTAACCATTATCGCGTTG-3' (*Xho* Ⅰ) as downstream primer. Gene fusion, together with its synthesis of the fragments, was contracted by Sangon Biotech (Shanghai) Co., Ltd.

Main reagents

DL2000 DNA Marker and T4DNA ligase were purchased from Takara Biotechnology Co., Ltd (Dalian,

China) Instant SK2072 PCR amplification kit (Taq), SanPrep column DNA gel extraction kit, SanPrep column PCR purification kit, 50 BP DNA ladder, protein Marker, restriction endonuclease *BamH* I, *Xho* I enzyme, *Bgl* enzyme, Hind enzyme II iii,DAB color liquid, exposure of ECL kit, cellulose nitrate film, BCA protein assay kit, BSA standard, Coomassie brilliant blue, IPTG, kanamycin, TMB color liquid were products of Sangon Biotech (Shanghai) Co., Ltd. Ni_2^+-NTA affinity chromatography and resin chromatography were purchased from Novagen, Anti-Der p1 antibody was self-preparation, and 16 aliquots of serum samples were previous preservings from patients allergic to huse dust mites in our laboratory. The remaining analytical reagents were domestic products.

Main apparatus

The common apparatus used in this study included ABI2720 PCR (Application Biosystem), High speed refrigerated centrifuge (Beckman, USA), horizontal DNA electrophoresis (Bio-Rad American), vertical BayGene vertical electrophoresis (Baijing, Beijing, China), G:Box gel electrophoresis image analysis system (SYNGENE, UK) and Lex800 ELISA reader (BioTek, USA).

Construction of recombinant vector pET28a (+)-TAT-IhC-Der p 1-3T

TAT specific primers were degenerated at 95°C degeneration and annealed at 55°C to form double chain with cohesive ends, and the vector was prokaryotically expressed in of *pET28a*(+) with *BamH* I and *Xho* I restriction. The total reaction volume was 80 μl consisting of 10×Tango buffer (8 μl), *BamH* I (2 μl), *Xho* I (2 μl), *pET28a*(+) (24 μl) and ddH_2O (44 μl). The digested products were retrieved after enzyme restriction at 37°C for 3 h. T4 DNA ligase was used to connect TAT with pET28a (+) at 16°C for 3 h to develop pET28a (+)-TAT vector. IhC specific primers were used to amplify IhC gene fragment (1~110AA) with polymerase chain reaction(PCR) by reaction conditions at 94°C for 4 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, respectively in total 35 cycles, followed by extension for 10 min at 72°C. Then the PCR products were purfied with commercial kit, and pET28a (+)-TAT and IhC (1~110AA) were subjected to double digestion with *Bgl* II and *Hind* III in terms of the conditions described above. The enzyme products were recovered, and the IhC was connected with the *pET28a* (+)-*TAT* by T4 DNA ligase to generate pET28a (+)-TAT-IhC vector, with the same procedure and condition as noted above. Der p 1-3T fragment was amplified by using specific primers, and *pET28a* (+)-*TAT-IhC* plasmid and Der p 1-3T fragment were double digested by the *BamH* I and *Xho* I with the same reaction protocol as described above. The enzyme products were recovered, and the Der p 1-3T was connected to the *pET28a (+)-TAT-IhC* by T4 DNAligase to create *pET28a (+)-TAT-IhC-Der p 1-3T* vector.

Identification of recombinant vector pET28a (+)-TAT-IhC-Der p 1-3T

Enzyme digestion was performed in a total volume of 80μl,which included 10×Tango buffer(8 μl) *BamH* Ⅰ (2 μl), *Xho* Ⅰ (2 μl), *pET28a (+)-TAT-IhC-Der p 1-3T* (24 μl) and ddH_2O (44 μl) at 37°C for 3h. The digested products were tested by agarose gel electrophoresis, and the positive clones were contracted confirmation and sequencing by Sangon Biotech (Shanghai) Co., Ltd.

Inducing the recombinant protein TAT-IhC-Der P 1-3T expression

The *pET-28a*(+)-*TAT-IhC-Der P1-3T* recombinant plasmids were transferred to *E.coli* BL21 (DE3), the competent cells, and then applied to culture plate containing LB solid culture medium diluted in 100 mg/L $Kana^+$ by single colony, which was cultured overnight at 37°C by 200 r/min. The ratio of cells to culture medium was adjusted to 1: 50, and set to 0.6 by OD_{600} measurement. IPTG (final concentration: 1 mmol/L) was added to further culture the cells for 4-5 h at 200r/min. Then, 1.5 ml bacteria liquid was taken, and centrifuged at 4°C by 10000 r/min for 5min. The supernatant was removed, and 100 μl (2×) protein sample buffer was added and mixed thoroughly. The solution was boiled for 10 min, and 20 μl of it was taken and treated in 12.5% SDS-PAGE, followed by Kaumas brilliant blue staining before photographing.

Purification of recombinant protein TAT-IhC-Der P1-3T

E. coli BL21 (DE3) strains containing *pET-28a* (+)-*TAT-IhC-Der p 1-3T* recombinant plasmid were cultured on a large scale; and the cells were collected after precipitation. Ni^{2+}-NTA resin was used to isolate and purify the recombinant TAT-IhC-Der p 1-3T as the user instructions. , and the purity was determined by SDS-PAGE.

Identification of recombinant protein TAT-IhC-Der p 1-3T

BCA protein assay kit and BSA standard were used to determine the protein concentration according the user protocol. After quantifying the protein, 10 μg total protein was taken for separation by 12.5% SDS-PA-

GE, and then transferred to nitrocellulose membrane containing 1% BSA TBST (50 mmol/L Tris; pH 7.5; 150mmol/L NaCl; 0.1%Tween-20), which was blocked for 2 h at room temperature. Der p 1 antibody diluted in 1: 500 was applied to the membrane that was cultured overnight at 4°C, followed by rinsing three times with TBST(10 min/each rinse). Second HRP-Goat anti-rabbit IgG (1: 5000 dilution) was added to the membrane, which was incubated for 40 min at 37°C, subjected to three rinses (10 min/each rinsing) and exposure by ECL technique.

Determination of TAT-IhC-Der P 1-3T

The binding capacity of *TAT-IhC-Der P* 1-3T with IgE was tested using ELISA as procedures described in previous literature. Der p 1 was initially applied to a 96-well plate (500 ng/each well) that was coated overnight at 4°C, and TBST was used to rinse the plate 5 times (100 μl/each well). Then the plate was blocked with TBST containing 1% BSA (150 μl/each well) for 1 h at 37°C. Sera from the asthmatics were added by dilution ratio of 1:8, and incubated for 1 h at 37°C. The plate was rinsed 5 times with TBST, and added with TMB substrate and reacted for 20 min at 37°C. The reaction was terminated by stop buffer (50 μl/each well), and measured at $A_{450\ nm}$.

Results

Identification of recombinant vector pET28a (+)-TAT-IhC-Der p 1-3T

The recombinant plasmid *pET28a (+)-TAT-IhC-Der p 1-3T* was doubly digested with *BamH* I and *Xho* I, and electrophoresis revealed a gene strip with approximate 800 bp (Fig. 1), which indicated that the recombinant plasmids were successfully constructed. By sequencing and NCBI-BLAST results, the size of the recombinants was 687 bp, which was consistent with that of TAT-IhC-Der p 1-3T gene.

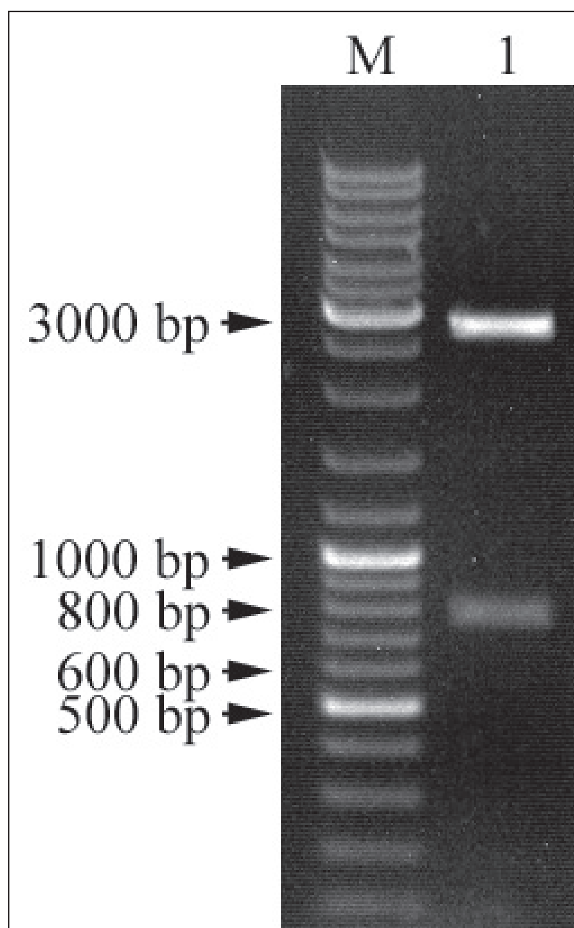

Fig. 1.—Endonuclease digestion with BamH I and Xho I for recombinant plasmid pET28a (+)-TAT-IhC-Der p1-3T.
M: DNA Marker;
1: Products of pET28a (+)-TAT-IhC-Der p1-3T digested by BamH I and Xho I.

Expression of the recombinant protein TAT-IhC-Der P 1-3T

In order to observe whether TAT-IhC-Der P 1-3T could induce expression, we transfected the recombinant vector *pET28a*(+)-TAT-IhC-Der p 1-3T with *E. coli* BL21 (DE3). After induction by IPTG at its optimal final concentration of 1 mmol/L and confirmation by SDS-PAGE, the strip indicating TAT-IhC-Der p 1-3T protein was exposed (Fig. 2), suggesting that this protein was successfully induced. The relative molecular mass was about 25000, which is consistent with the theoretical value. Single band was evident after treatment by Ni_2^+-NTA resin (Fig. 2).

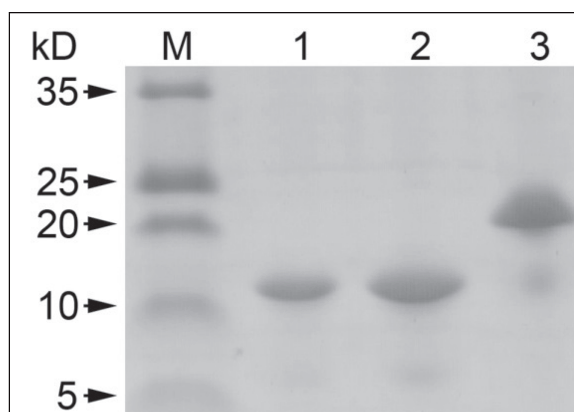

Fig. 2.—Detection of recombinant proteins Der P1-3T, TAT-Der P1-3T, and TAT-IhC-Der P1-3T purified from E. coli BL21(-DE3) with Ni+-NTA chromatography.
M: Protein marker;
1. Purified recombinant protein Der P1-3T;
2. Purified recombinant protein TAT- Der p1-3T;
3. Purified recombinant protein TAT-IhC-Der P 1-3T.

Western blot detection of recombinant protein TAT-IhC-Der p 1-3T

To verify the purified recombinant protein band for TAT-IhC-Der p 1-3T, Western blot was performed by using Der p 1 polyclonal antibody. The results demonstrated an evident strip for TAT-IhC-Der p1-3T instead of the control *E. coli* BL21 (DE3) (Fig. 3). The size of the recombinant was comparable with that on SDS-PAGE, which indicated that TAT-IhC-Der p 1-3T was successfully purified, and can be used for further test.

TAT-IhC-Der P 1-3T ELISA IgE binding ability test

Recombinant protein TAT-IhC-Der p 1-3T was prokaryotically expressed and tested for its binding capacity with IgE in 16 aliquots of sera from patients sensitized by house dust mites. ELISA assay demonstrated a higher binding power of TAT-IhC-Der p 1-3T to bind to IgE over single Der p 1[(1.35±0.33) *vs*. (0.80±0.19)] ($P<0.001$). The findings showed that TAT-IhC-Der p 1-3T had excellent allergenicity.

Discussion

SIT is considered the only etiological therapy to modify the allergic disease process[2,20], and can effectively induce the production of allergen specific IgG_1 and IgG_4, thus leading the reduced antibody levels of allergen specific IgE, and blocking the occurrence of type I hypersensitivity[21]. SIT not only reduces the allergic sensitization, but also the incidence of asthma after treatment[22]. An early study on SIT by using recombinant pollen allergen Bet V 1 with T cell epitopes being reserved while B cell epitopes being reduced had reduced the allergen specific IgE production, and prevented the allergens from binding to IgE and/or stimulated inability of body to induce antigen-specific T cells[23]. These findings suggest that optimal SIT should be involved in vaccines with hypoallergenic (reduced B cell epitope), enhanced immunogenic (increased T cell epitope vaccine)[2]. Prickett *et al* once proved the efficacy of CD_4^+T cell epitope peptide derived from Ara h 1 allergen by stimulating the mononuclear cells from peripheral blood of patients allergic to peanuts[24], and Mackenzie *et al*. (2013) used the dominant T cell epitope peptide extracted from the ovalbumin of chicken egg for SIT, and found that this peptide had effectively inhibited the production of allergen specific IgE and eosinophilia[25].

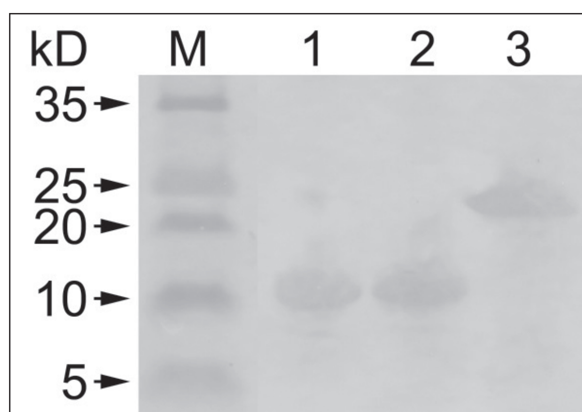

Fig. 3.—Western blot detection of purified recombinant proteins Der p1-3T, TAT- Der p1-3T, and TAT-IhC- Der p1-3T from E. coli strain BL21 (DE3) with Ni+-NTA chromatographic column. M: protein marker;
1. Purified recombinant protein Der p1-3T;
2. Purified recombinant protein TAT-Der p1-3T;
3. Purified recombinant protein TAT-IhC-Der p1-3T.

Exogenous antigen uptaken by APCs can be degraded and bound with MHC II, and activated after presenting to CD_4^+T cells, which leads to antigen specific immune responses. The effect of SIT by MHC II pathway has been confirmed[15]. However, the efficacy may be boosted if MHC II pathway can be presented through multiple T cell epitope peptide fusion protein.

In view of the TAT and IhC functions as well as the role of T cell epitopes in SIT, we tentatively constructed recombinant TAT-IhC-Der p 1-3T and prokaryoticaly expressed it in vector *pET-28a-TAT-IhC-Der p 1-3T*. After induction by IPTG and SDS-PAGE analysis, we found that this fusion allergen can be effectively expressed. Further Western blot assay proved that the allergens were successfully purified. Following experiments showed that the capacity of TAT-IhC-Der p 1-3T in binding with IgE is superior to Der p 1, and our findings are inconsistent with previous reports. Karamloo[26] constructed a chimeric protein from the melittin, which contains Api M 1, Api M 2 and Api m3 T cell epitopes, and successfully removed the B cell epitopes from the antigen. And his following test proved that the chimeric protein had lower binding potential with IgE than the natural allergen. Contrarily, our TAT-IhC-Der P 1-3T protein had enhanced power of allergen, this is probably associated with the overlapping of T cell and B cell epitopes in Der p 1[27], and emergence of new epitopes of B cell configuration or linear epitope. Yet the exact mechanism remains unclear.

In summary, we successfully obtained the fusion protein that can be intracelluarly transferred and anchored in the lysosomes, and enhance T cell epitope peptide. This work may lay a foundation for vaccine development in therapy of asthmatic patients caused by house dust mites, particularly the vaccine on SIT basis.

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Disclosure of conflict of interest

None.

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