

CRM197-Coupled Der p 2 Peptides Suppress Allergic Airway Inflammation in a Der p 2-Induced Asthma Mouse Model

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Keywords

Cross-reacting material 197 · House dust mite · Allergen-specific immunotherapy · Der p 2 · Mouse model of asthma

Abstract

Allergic diseases affect more than 25% of the global population. Der p 2 is the major allergen of the house dust mite (HDM) *Dermatophagoides pteronyssinus*. Allergen-specific immunotherapy is the only treatment to change the course of allergic diseases. In this study, two synthesized Der p 2 peptides coupled to cross-reacting material 197 (CRM197) showed reduced IgE reactivity and allergenic activity. CRM197-coupled Der p 2 peptides induced rDer p 2-specific IgG1 antibodies in mice, which could inhibit HDM-allergic patients' IgE binding to rDer p 2. The immunity effects of CRM197-coupled Der p 2 peptides were studied in an rDer p 2-induced asthma mouse model. CRM197-coupled Der p 2

peptides can suppress asthmatic airway inflammation in this model. Analysis of IL-4, IL-5, and IFN- γ levels in bronchoalveolar lavage fluid revealed that the suppression was associated with a shift from a Th2 to a Th1 response. Thus, CRM197-bound Der p 2 peptides exhibited less allergenic activity than the rDer p 2 allergen, which preserved immunogenicity and may be candidates for mite allergy vaccines.

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Introduction

Allergic diseases are common systemic diseases caused by inhalation, ingestion, or exposure to allergens [1]. House dust mite (HDM) allergy, one of the most common allergies worldwide, affects more than 50% of all allergic patients [2]. The major allergen of the HDM *Dermatophagoides pteronyssinus*, Der p 2, is recognized by

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approximately 90% of mite-allergic patients. Der p 2 mainly exists in the excreta of HDMs, which represents a protein of 129 amino acids with a molecular weight of 14 kD and without N-glycosylation sites [3]. T cell epitopes are distributed in the whole sequence of Der p 2, while B cell epitopes mainly exist in Der p 2 proteins as spatial epitopes [4, 5]. Der p 2 represents an important component for a vaccine for HDM allergy.

Allergen-specific immunotherapy modifies the allergen-specific immune response and the course of disease and has been shown to have long-lasting effects [6, 7]. Current therapeutic allergy vaccines are still prepared from relatively poorly defined allergen extracts. However, the ingredients of crude allergen extracts are inconsistently produced by different manufacturers and different batches. Many unrelated ingredients mixed in the effective ingredients of drugs cause side effects in the treatment [8]. Many strategies based on gene recombination technology are developed to optimize the active ingredients of crude extracts of allergens and minimize those side effects [9, 10]. However, retained IgE epitopes of recombinant allergen still caused side effects in the treatment of sensitized patients [11]. Recombinant mutants and deletion variants of the conformational IgE epitopes of group 2 allergens have been considered as Der p 2 hypoallergenic vaccine candidates for immunotherapy [12, 13]. Furthermore, hypoallergenic fragments of Der p 2 have been engineered, which is also important to the vaccine development of Der p 2 peptides [14, 15]. Two peptides (aa 21–51 and aa 62–103) showed no relevant IgE reactivity, allergenic activity, and induced lower Der p 2-specific T-cell activation than Der p 2 [15]. It was reported that allergens with a length of about 30 amino acid residues are coupled to the carrier protein to form a complete antigen, which can induce the production of specific IgG antibody against the complete allergen [16, 17]. Carrier-bound allergen peptide vaccines are composed of an allergen-unrelated carrier protein which provides T-cell help for the production of antibodies against the peptides covalently linked with the carrier [18]. Recently, keyhole limpet hemocyanin (KLH) was chosen as the carrier protein conjugated with Der p 2 polypeptides [15]. Although the immunogenicity of KLH is high, it is rarely used in the clinic.

The carrier protein cross-reacting material 197 (CRM197) is widely used in many vaccines against infectious diseases [19]. The CRM197 protein is a variant of diphtheria toxin with a glycine to glutamic acid substitution at position 52 (G52E) [20]. CRM197 rapidly activates CD4⁺ T cells with a heterogeneous Th1 and Th2 cytokine

profile for activating B cells and regulating the quantity of the induced antibodies [21]. Generally, CRM197 was shown to have reduced toxicity while retaining the same inflammatory and immune-stimulant properties as diphtheria toxin, allowing it to be used as a suitable carrier protein for the development of conjugate vaccines. In this study, synthesized Der p 2 polypeptides were conjugated with CRM197, which retain the similar immunogenicity as Der p 2 protein and reduce its allergenicity. The IgE reactivity of the CRM197-coupled Der p 2 peptides were studied with serum from HDM-allergic patients, and their allergenic activity was studied by basophil degranulation experiments *in vitro*. Mice were immunized to study whether CRM197-coupled Der p 2 peptides can induce IgG1 antibodies and inhibit IgE reactivity of allergy patients. Moreover, CRM197-coupled Der p 2 peptides could suppress allergic airway inflammation in an rDer p 2-induced asthma mouse model.

Materials and Methods

rDer p 2, CRM197-Conjugated Der p 2 Peptides, and Sera from Allergic Patients

rDer p 2 was expressed in *Escherichia coli* strain BL21(DE3) and purified as described [22], and CRM197 was kindly provided by Keynova Inc. (Shandong, China). Two peptides of the Der p 2 sequence (P1 fragments: aa 21–51; P2 fragments: aa 62–103) were synthesized (Chinapeptides Inc., Shanghai, China). Each of the Der p 2 peptides was coupled to CRM197 using succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB, Pierce, ThermoFisher Scientific). Sera from HDM-allergic patients (*n* = 10) were selected according to case history, skin prick testing, and serological analysis as described [23]. Sera from non-allergic (NA) individuals were included for control purposes. Peripheral blood samples were obtained from Shenzhen Hospital, Peking University. The study was approved by the ethics committee of the Shenzhen Hospital, Peking University, and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

IgE Reactivity of Dot-Blotted rDer p 2 and Der p 2 Peptides

The concentration of Der p 2, CRM197, and the CRM197-conjugated Der p 2 peptides was measured using the Micro BCA Assay Kit (Pierce, Rockford, IL, USA). Aliquots containing 0.4 µg of purified rDer p 2, CRM197-conjugated Der p 2 peptides, or CRM197 were dotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany), and IgE reactivity was tested with sera from 10 HDM-allergic patients as described [14].

Immunization of Mice

Six-week-old female BALB/c mice were purchased from Guangdong animal experimental center (Guangzhou, China). The mice were supplied with a standard laboratory diet. All experiments were carried out according to animal use protocols approved by the Committee for the Ethics of Animal Experiments, Shenzhen Peking University-The Hong Kong University of Sci-

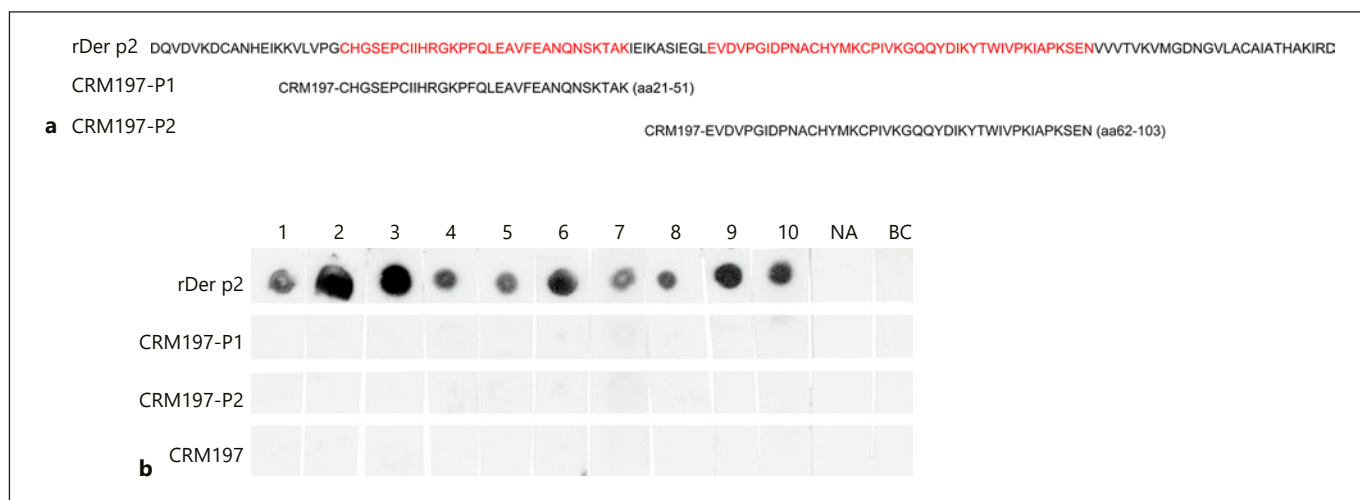


Fig. 1. a Amino acid sequence of Der p 2. Synthetic Der p 2-derived P1 and P2 are indicated. **b** IgE reactivity of rDer p 2 and CRM197-conjugated Der p 2 peptides. Dot-blotted rDer p 2, CRM197-conjugated Der p 2 peptides 1/2, and CRM197 were studied for IgE reactivity with sera from 10 HDM-allergic patients (1–10), serum from an NA individual and BC. Bound IgE antibodies were detected with a secondary HRP-labelled anti-human-IgE antibody via chemiluminescence.

ence and Technology Medical Center (SPHMC; protocol No. 2011-004). Six groups consisting of 5 mice each were monthly immunized subcutaneously in the neck with PBS, 30 µg rDer p 2, 30 µg CRM197, 30 µg CRM197-P1, or 30 µg CRM197-P2, absorbed to 200 µL of AluGel-S (SERVA Electrophoresis, Heidelberg, Germany). Namely, these were the PBS groups (negative control), rDer p 2 group (positive control), and CRM197 and CRM197-P1/2 group (immunotherapy with CRM197 or CRM197-conjugated Der p 2 peptides). Blood samples were collected the day before each immunization and stored at -80°C .

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) plates (Nunc, Roskilde, Denmark) were coated with rDer p 2 (100 µg/well in PBS) overnight at 4°C . The plates were washed twice with PBST and blocked in 200 µL of 5% BSA blocking buffer for 1 h at room temperature. Then, 100 µL of mouse anti-rDer p 2 antiserum, each of the mouse anti-Der p 2 peptide antisera (anti-peptide 1 and anti-peptide 2), or the corresponding pre-immune sera (1:100 dilution in PBST, 0.5% BSA) were added to the plates and incubated for 1 h at 37°C . To test IgG1 antibody responses in mice, bound human IgG1 antibodies were detected with horseradish peroxidase (HRP)-coupled goat anti-mouse IgG1 antibodies (Abcam, USA) diluted 1:2,500 in PBST, 0.5% BSA. To test the inhibition of allergic patients' IgE binding to rDer p 2 with peptide-specific antibodies, after mice antisera were added and the plates were washed, they were incubated with 100 µL of HDM-allergic patients' sera (1:10 dilution in PBST, 0.5% BSA) for 1 h at 37°C . Bound human IgE antibodies were detected with HRP-coupled mouse anti-human IgE antibodies (Abcam) diluted 1:2,500 in PBST, 0.5% BSA. The percentage inhibition of IgE binding was calculated as follows: $100 - (\text{ODs}/\text{ODp}) \times 100$, where ODs and ODp represent the extinctions after pre-incubation with the mouse immune serum and pre-immune serum, respectively.

Establishment of Asthmatic Mouse Models

Thirty BALB/c mice were randomized into six immunized groups equally, including the rDer p 2 group, CRM197 and CRM197-P1 groups, CRM197-P2 group, and two PBS groups. Mice in one PBS group were intraperitoneally injected with 100 µL PBS suspension (containing 2% $\text{Al}(\text{OH})_3$, pH 7.4) on days 0, 7, and 14, respectively. The remaining five groups were individually sensitized with rDer p 2 dissolved in 100 µL containing 2% $\text{Al}(\text{OH})_3$ PBS suspension liquid (pH 7.4) via intraperitoneal injection at a dose of 20 µg on days 0 and 7, respectively. From day 14, the PBS group were challenged only with PBS suspension, finally named the PBS/PBS group. The five treatment groups were sensitized with 0.5 mg/mL rDer p 2 dissolved in PBS suspension for 30 min every 2 days for 14 days consecutively, finally named the PBS/rDer p 2 group, rDer p 2/rDer p 2 group, CRM197/rDer p 2 group, CRM197-P1/rDer p 2 group, and CRM197-P2/rDer p 2 group.

Collection and Analysis of the Bronchoalveolar Lavage Fluid

The mice were killed 24 h after the final challenge with an intraperitoneal injection of 10% chloral hydrate. Then the airways were exposed for easy endotracheal cannula. Bronchoalveolar lavage fluid (BALF) was collected through application of the pre-chilled sterile PBS, a total of 1 mL of which was applied in a fractionated dose of 0.3, 0.3, and 0.4 mL to irrigate the trachea. BALF from each animal was pooled in a plastic tube, cooled on ice, and centrifuged at 150 g at 4°C for 5 min. Cell pellets were resuspended in the same buffer and stored at -80°C for determination of cytokine production. The concentrations of IL-4, IL-5, and IFN- γ were assayed by commercial ELISA kits according to procedure protocols (eBioscience, USA). The absorbance was measured at 450 nm in a microplate reader. With Wright Staining, 200 inflammatory cells were observed and eosinophils were counted randomly from different visual fields in the bright field of the microscope.

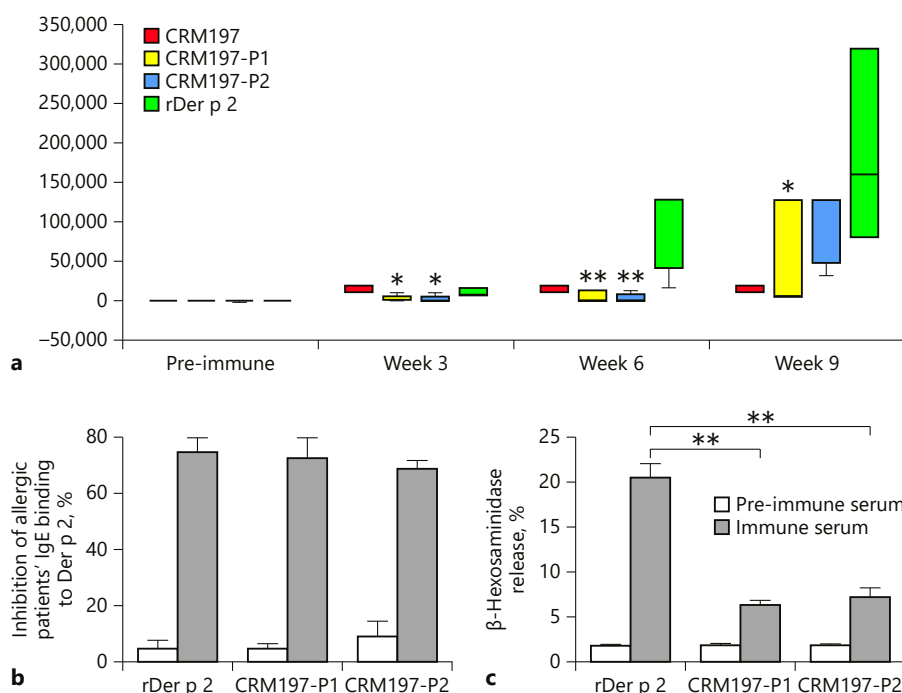


Fig. 2. **a** Der p 2-specific IgG1 antibody level responses of mice immunized with CRM197-coupled Der p 2 peptides were lower than those of mice immunized with rDer p 2. Groups of 5 mice each were immunized with rDer p 2 or CRM197-P1/P2 peptides at 3-week intervals. The IgG1 antibody levels are displayed for groups of 5 mice as a box plot where 50% of the values are within the boxes and non-outliers are between the bars. Lines within the boxes indicate the median values. * $p < 0.05$, ** $p < 0.01$, versus the rDer p 2 group. **b** Inhibition of allergic patients' IgE binding to rDer p 2 with mouse anti-CRM197-coupled Der p 2 peptides antisera was slightly lower than that with mouse anti-rDer p 2 antisera. To test the inhibition of allergic patients' IgE binding to rDer p 2 with peptide-specific antibodies, after 100 μ L of mouse antisera were added and the plates were washed before being incubated with 100

μ L of HDM-allergic patients' sera (1:10 dilution in PBST, 0.5% BSA) for 1 h at 37°C. Bound human IgE antibodies were detected with HRP-coupled mouse anti-human IgE antibodies (diluted 1:2,500 in PBST, 0.5% BSA). The results are shown as the percentage inhibition of IgE binding. **c** Der p 2-specific basophil degranulation of RBL-2H3 cells loaded with sera from mice immunized with CRM197-coupled Der p 2 peptides was lower than that with mouse anti-rDer p 2 antisera. RBL-2H3 cells were loaded with serum IgE from mice obtained before (pre-immune serum) or after immunization (immune serum) with rDer p 2 or CRM197-coupled Der p 2 peptides. The rDer p 2-specific release of β -hexosaminidase is displayed as a percentage of the total release on the y axis. Results are displayed as mean values $\pm$ SD from each mouse group. * $p < 0.05$, ** $p < 0.01$.

Staining of the Lung Tissue Sections

Lung tissues were isolated from each mouse in all groups, and fixed in 10% paraformaldehyde overnight at 4°C. The tissues were then embedded in paraffin and sectioned using a conventional technique. After HE staining, the sections were examined under the light microscope for the infiltration of the lymphocytes and eosinophils, as well as the incidence of edema and bronchial epithelial damage.

Rat Basophil Leukemia Cell Degranulation Assay

The sera from mice were exposed to rat basophil leukemia cells RBL-2H3 seeded in 96-well plates for 2 h at 37°C. The cells were

washed twice and then stimulated with rDer p 2. Total hexosaminidase release was induced by the addition of 10% Triton X-100 (Merck, Darmstadt, Germany). The releases of β -hexosaminidase in the cultures were measured using a microplate reader.

Statistical Analyses

Results are represented as the mean $\pm$ SEM. Statistical significance between two groups was estimated by using the two-tailed Student *t* test. *p* values < 0.05 were considered significant.

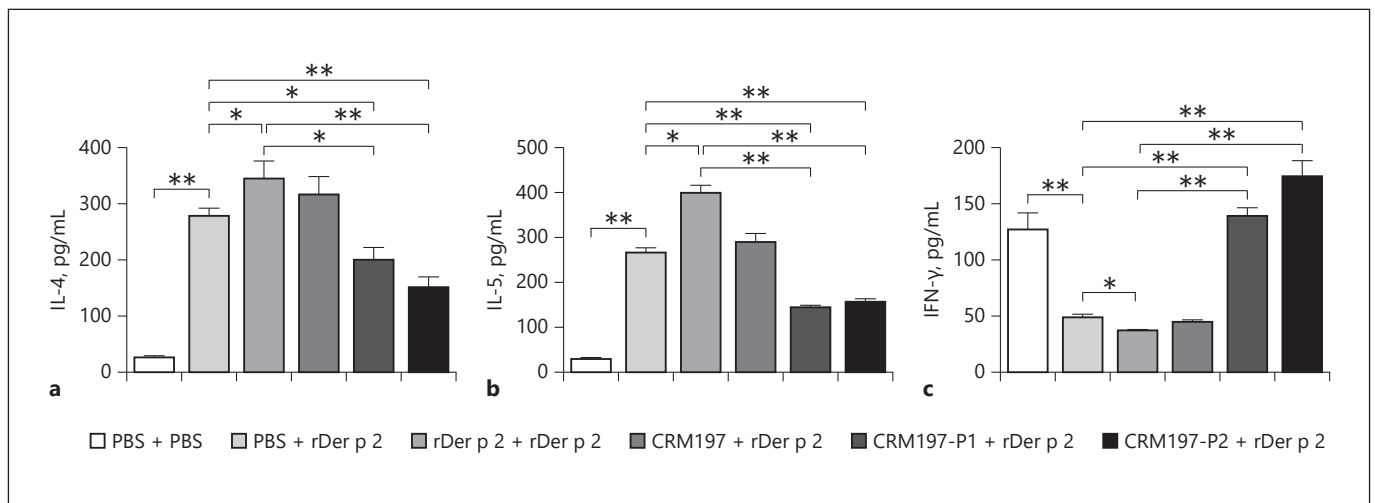


Fig. 3. CRM197-conjugated Der p 2 peptides enhanced IFN- γ production and inhibited IL-4 and IL-5. **a** The IL-4 level was lower in BALF of mice immunized with CRM197-coupled Der p 2 peptides, compared with that of mice immunized with rDer p 2. **b** The IL-5 level was lower in BALF of mice immunized with CRM197-cou-

pled Der p 2 peptides, compared with that of mice immunized with rDer p 2. **c** The IFN- γ level was higher in BALF of mice immunized with CRM197-coupled Der p 2 peptides, compared with that of mice immunized with rDer p 2. Values represent the mean or mean $\pm$ SEM of 5 mice in each group. * $p < 0.05$, ** $p < 0.01$.

Results

CRM197-Conjugated Der p 2 Peptides Showed No Relevant IgE Reactivity and Allergenic Activity

Two peptides of Der p 2, namely P1 and P2, were synthesized and coupled with CRM197 (Fig. 1a). The IgE-binding capacity of the complete rDer p 2 allergens was compared with that of CRM197-conjugated P1 and P2 peptides in non-denaturing IgE-binding dot-blot assays with sera from 10 HDM-allergic individuals. All patients showed a different intensity of anti-rDer p 2 IgE reactivity, while patients showed no IgE reactivity to the CRM197-conjugated Der p 2 peptides or to CRM197 (Fig. 1b). When serum from an NA person or buffer without serum (BC) was used, no IgE reactivity to rDer p 2 or to CRM197-conjugated Der p 2 peptides was found (Fig. 1, lanes NA, BC).

Immunization with CRM197-Coupled Der p 2 Peptides Induced Der p 2-Specific IgG1 Antibodies, Which Also Inhibited Mite-Allergic Patients' IgE Binding to the rDer p 2 Allergen

The IgG1 reactivities of mice immunized with CRM197, rDer p 2, and the CRM197-coupled peptides were studied. Der p 2-specific IgG1 responses induced with the individual CRM197-coupled peptides were lower and were detected later than those induced with rDer p 2 (Fig. 2a). Moreover, it was found that antibodies raised

by immunization of mice with Der p 2 peptides could inhibit HDM-allergic patients' IgE binding to rDer p 2 in ELISA competition experiments (Fig. 2b). Mouse anti-rDer p 2 antibodies inhibited patients' IgE binding to rDer p 2. Interestingly, the inhibitions of mice antibodies induced by immunization with CRM197-P1 and CRM197-P2 allergen were similar with the inhibitions of mouse anti-rDer p 2 antibodies.

To compare the in vivo allergenicity CRM197-coupled Der p 2 peptides with rDer p 2, the induction of Der p 2-specific reagenic IgE antibodies was studied by loading RBL-2H3 cells with the sera from immunized mice (Fig. 2c). Immunization with rDer p 2 raised Der p 2-specific degranulation by approximately 20%. By contrast, a very low level of β -hexosaminidase release was observed when RBL-2H3 cells loaded with mouse anti-peptide IgE were exposed to CRM197-coupled Der p 2 peptides (Fig. 2c).

CRM197-Coupled Peptides Could Skew the Allergic Th2 Response to Th1 Response and Attenuate Allergic Inflammation Induced by HDM

Mice were immunized with CRM197-conjugated Der p 2 peptides, rDer p 2, and PBS, respectively. Then allergic asthma symptoms were induced by rDer p 2 sensitization and nasal drip challenge. To characterize the polarization of the immune response after immunization with the CRM197-coupled peptides, IL-4, IL-5, and IFN- γ were measured as indications of Th2 and Th1 responses, re-

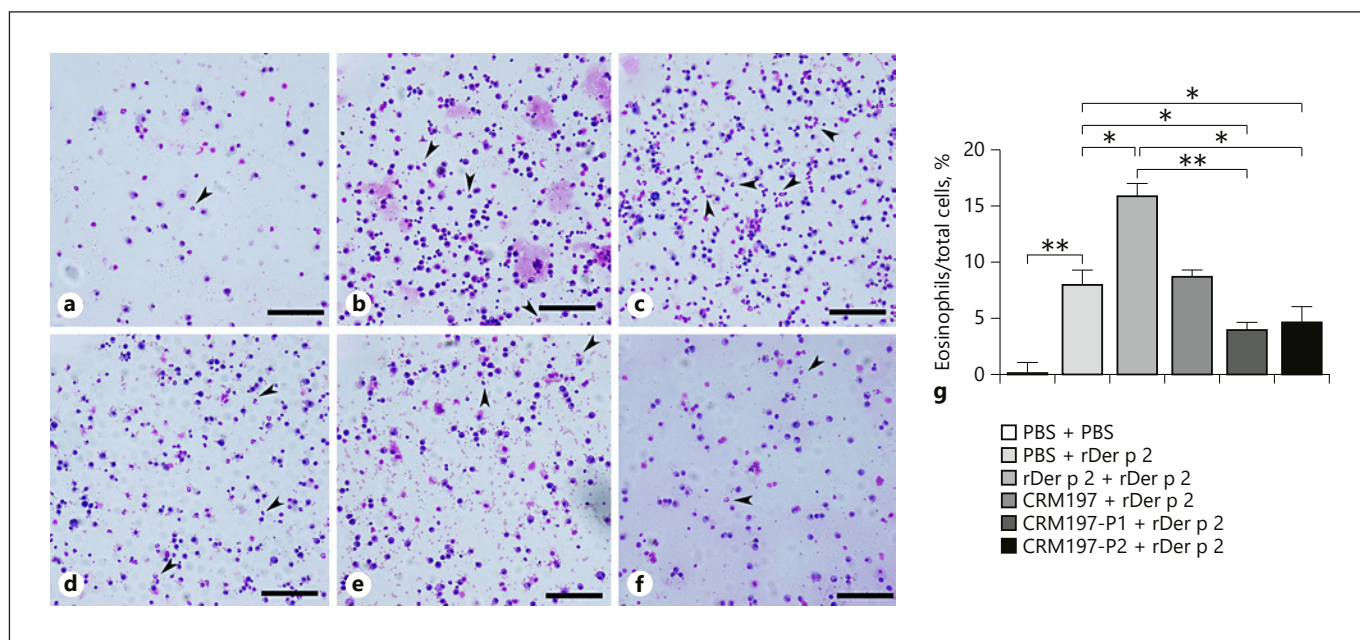


Fig. 4. Total cell and eosinophil counts in BALF decreased significantly after being immunized with CRM197-conjugated Der p 2 peptides. With Wright staining, inflammatory cells observed randomly by light microscopy were shown in the PBS/PBS group (a), PBS/rDer p 2 group (b), rDer p 2/rDer p 2 group (c), CRM197/rDer p 2 group (d), CRM197-P1/rDer p 2 group (e), and CRM197-P2/rDer p 2 group (f). **g** Statistical analysis of three independent experiments. * $p < 0.05$, ** $p < 0.01$. Scale bars, 100 μm . Arrowheads indicate eosinophils.

spectively. As shown in Figure 3, a higher IL-4 level was seen in the rDer p 2 group than the PBS group, while a lower IL-4 level was observed in the CRM197-P1/P2 group in BALF. The level change of IL-5 remained similar to IL-4 in each group, and the difference was significant. As for Th1 cytokine, however, the CRM197-P1/P2 group had a significantly higher level of IFN- γ than the PBS group as well as the rDer p 2/rDer p 2 group. Therefore, mice immunized with the CRM197-coupled peptides induced the release of IFN- γ and reduced the release of IL-4 and IL-5. This indicated that the treatment to the established allergic mice model could skew the allergic Th2 response to Th1 response.

Since the inflammatory cells, especially eosinophils, play an important role in allergic diseases, the total cells and eosinophils of BALF were obtained and counted after the anesthesia of each mouse. As shown in Figure 4, the ratio of eosinophils/total cells was significantly higher in the rDer p 2/rDer p 2 mice than in PBS/rDer p 2 and CRM197/rDer p 2 mice. Moreover, after being immunized with the CRM197-coupled peptides, the ratio of eosinophils/total cells was decreased markedly. This suggested that the CRM197-coupled peptides could attenuate allergic inflammation induced by HDM.

CRM197-Coupled Peptides Generally Improved the Pathological Condition of the Lung Tissues

Light microscopic examination of sections after HE staining demonstrated that the lung tissues in the rDer p 2/rDer p 2 group (Fig. 5c) showed notably broadened interalveolar septum, thickened bronchial walls, partially fractured bronchial epithelial cells, proliferated goblet cells, lymphocytes surrounding the entire bronchia, inflammatory cell infiltration of primary eosinophils, dilated small vessels, edema at the vascular walls, and exuded erythrocytes within the lung tissues. The pathological condition was generally improved in the CRM197-coupled peptides group, since inflammatory cell infiltration into the lung tissues was significantly alleviated in the CRM197-coupled peptides group with a relatively well-restored airway epithelial structure as compared with the rDer p 2/rDer p 2 group (Fig. 5e, f). Moreover, immunization with the CRM197-coupled peptides had effectively inhibited the inflammatory cell infiltration into the airways (Fig. 5e, f), which led to excellent recovery of the airway epithelial structure that appeared similar to that of the negative controls (Fig. 5a).

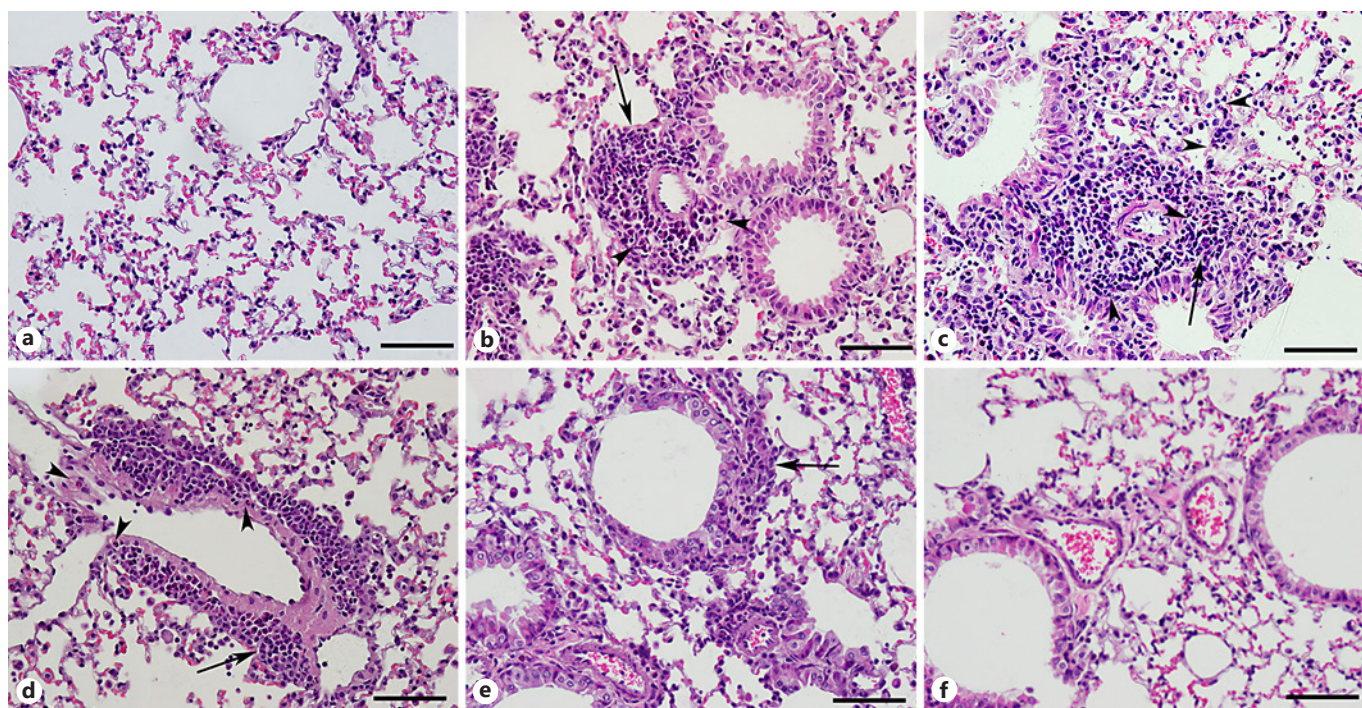


Fig. 5. Histological analysis of lung sections stained with HE 24 h after the final antigen challenge (×400) in BALB/c mice. Lung tissue sections by light microscopy are shown in the PBS/PBS group (a), PBS/rDer p 2 group (b), rDer p 2/rDer p 2 group (c), CRM197/rDer p 2 group (d), CRM197-P1/rDer p 2 group (e), and CRM197-P2/rDer p 2 group (f). Scale bars, 100 μm. Arrowheads and arrows indicate eosinophils and inflammatory cell infiltration, respectively.

Discussion

A large number of protective IgG antibodies have been produced in patients with allergic diseases after desensitization treatment, which prevent IgE antibodies from binding allergens in the environment and prevent the body from producing allergic reactions. It was generally considered as one of the intrinsic mechanisms of allergen immunotherapy. At present, the crude extract of the original allergen is used in allergen immunotherapy, which has a risk of systemic allergic symptoms after the treatment. Because the components in the crude extract bind to IgE of the allergy patients, degranulation of mast cells and basophils cause a systemic allergic reaction. CRM197 is widely used as a vaccine carrier protein in the clinic [19]. In this study, Der p 2 peptides were conjugated with CRM197 to form a complete antigen, which affected the immune response of the body to Der p 2 peptides. All mice immunized with CRM197-P1 or CRM197-P2 produced high titers of protective IgG1 antibody. As the competitive ELISA experiments showed, these protective antibodies could effectively inhibit the

binding of IgE and rDer p 2 in the serum of dust mite allergic patients. Moreover, CRM197-coupled peptides reduced the IgE binding ability and allergenicity. Therefore, a complete antigen with CRM197 greatly improved the safety of the anti-allergic vaccine.

The Th2-type immune response is one of the main characteristics of allergic asthma [24]. The Th1 cytokine level in BLAF of mice immunized with CRM197-P1 and CRM197-P2 increased significantly, while the Th2 cytokine level in BLAF decreased significantly. This suggested that the Th1-type immune response played a dominant role. It is generally believed that the Th1-type immune response promotes the production of protective antibody IgG and inhibits the production of IgE [25]. Th2 cytokines such as IL-5 were associated with eosinophil recruitment and invasion [26]. Toll-like receptors play an important role in the primary immunity reaction. Some biological adjuvants bind to Toll-like receptors and induce a mainly secondary Th1-type immune response, which was consistent with our finding of CRM197-coupled Der p 2 peptides [27]. At the same time, it was proven that CRM197 coupled with Der p 2

peptides induces less specific IgE antibody against rDer p 2. Reduced production of allergen-specific IgE antibodies is of great importance in allergen immunotherapy. On the one hand, IgE on the surface of mast cells binds to allergens and induces degranulation. On the other hand, the delayed phase of allergic diseases is directly related to the antigen presentation of B cells to T cells mediated by IgE antibody [28].

In conclusion, the allergenicity of CRM197-coupled Der p 2 peptides was decreased significantly. CRM197-Der p 2 peptides can induce the symptoms of asthma and a Th1-type immune response in mice, which also increases the specific IgG and decreases IgE. This suggested that the CRM197-coupled Der p 2 peptide has the potential to prevent and treat allergic diseases.

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Statement of Ethics

The study was approved by the ethics committee of the Shenzhen Hospital, Peking University, and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants. All mouse experiments were carried out according to animal use protocols approved by the Committee for the Ethics of Animal Experiments, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center (SPHMC; protocol No. 2011-004).

Disclosure Statement

The authors declare that there are no conflicts of interest.

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