Am J Transl Res 2019;11(1):280-289
www.ajtr.org /ISSN:1943-8141/AJTR0081831

Original Article

Efficacy of subcutaneous immunotherapy in a mouse model of Humulus pollen-induced allergic asthma

Rui Kong, Jia Yin

Department of Allergy, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Received June 28, 2018; Accepted December 9, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Objective: The aim of this study was to evaluate the therapeutic effect of subcutaneous injection of high-dose of Humulus pollen allergen on a mouse model of allergic asthma. Method: Humulus pollen-sensitized mice received either regular subcutaneous injections of Humulus pollen allergen extract or control treatment were sacrificed. Airway responsiveness, pulmonary inflammatory cell infiltration, serum cytokine secretion and serum immunoglobulin E (IgE) and G2a (IgG2a) levels were measured. Result: Our results showed that subcutaneous immunotherapy with high-dose of Humulus pollen allergen could obviously relieve the symptoms and improve the pulmonary function in mice with allergic asthma. It also elevated serum IgE and IgG level compared with model group. The Humulus pollen allergens could be identified in serum and the molecular weight of serum proteins that had the maximum binding to allergen were ~25 kDa and ~38 kDa in 7 cases (70%) and 6 cases (60%), respectively. Conclusion: Subcutaneous immunotherapy (high-dose) could effectively alleviate airway hyperresponsiveness and reduce airway inflammatory infiltration in allergic asthmatic mice.

Keywords: Immunotherapy, humulus pollen, allergic asthma, immunoglobulin

Introduction

Asthma is featured by chronic inflammatory response in the airways, with allergic asthma being the most common type. Allergens can induce or exacerbate allergic asthma through an immunoglobulin E (IgE)-dependent immune response. Humulus pollen is an important allergen responsible for the occurrence and development of fall pollen-induced allergic asthma in China [1]. Humulus scandens is mainly distributed in subtropical and temperate zones in Northern Hemisphere. In China, it is mainly distributed in the Northeast, Northern, Central and Southern, Southwest, Shanxi and Gansu regions. Yin reported that Humulus pollen was one of the determinant causes of seasonal allergic rhinitis and asthma [2]. Allergen-specific immunotherapy is currently the only etiological treatment for allergic asthma [3], probably through changing the natural course of the disease, stoping the development of asthma and preventing allergic sensitization to new allergens [4]. At present, Humulus pollen allergen-specific immunotherapy is widely used in clinical practice, but it has certain defects such as low therapeutic concentration, long treatment course and poor patient compliance. In addition, there is no relevant research evaluating the efficacy of specific immunotherapy with high-dose and short-term treatment.

Sun *et al* established a mouse model of Humulus pollen-induced pulmonary inflammation by subcutaneous injection and intranasal instillation of Humulus pollen allergen [5]. This model is useful to investigate the etiopathology of the disease and to find treatments. In this study, a similar animal model of Humulus pollen-induced allergic asthma was established and pulmonary function and allergen specific antibodies in mice were detected. Our study aimed to evaluate the efficacy of high-dose allergen-specific immunotherapy.

Materials and methods

Experimental animals

60 female Balb/c mice (6-8 weeks old, SPF) were provided by the Military Medical Science

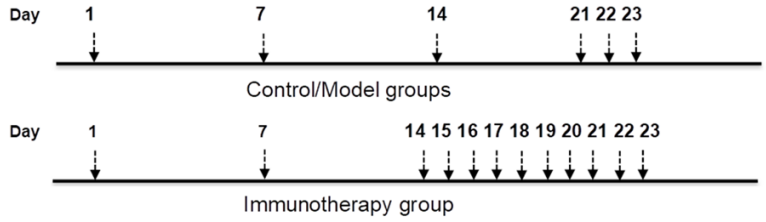

Figure 1. Experimental procedure. Mice in the model and immunotherapy group were intraperitoneally injected with Humulus pollen extract on the 1st, 7th and 14th day, respectively, and were nasally instilled extract on the 21st and 22nd day. Allergen-specific immunotherapy was performed by subcutaneous injections with Humulus pollen extract from the 15th to 23rd day. Mice in the control group were sensitized and stimulated in the same way as model group except for using the same volume of normal saline instead of crude extract of Humulus pollen.

Academy of the PLA, China. Animals were housed in a temperature and humidity-controlled room in the Laboratory Animal Center of Peking Union Medical College Hospital with free access to sterile diet. The temperature was controlled at 22-25°C and the humidity level was maintained at 40-60%. All the animal procedures were approved by the ethics committee of Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College.

Allergen

Humulus pollen was collected from the agricultural areas around Beijing. Liquid nitrogen grinding was used to destroy the cell walls. Pollens were defatted by acetone, extracted with phosphate buffer, filtered through 0.2 μm pore-sized filter membranes, dialyzed with deionized water, then freeze-dried and kept in vials. The concentration of 200 μL redissolution was 7.384 μg/μL (total protein 1.477 mg per bottle).

Experimental procedure

Mice were randomly divided into control, model and immunotherapy groups (20 rats in each group). Mice in the model group were intraperitoneally injected with 300 μL crude extract of Humulus pollen (1 μg/μL) combined with 120 μg aluminum hydroxide adjuvant (V/W: 5/2) on the 1st, 7th and 14th day, respectively. All mice were nasally instilled with 30 μL crude extract of Humulus pollen (10 μg/μL) on the 21st and 22nd day. Mice in the immunotherapy group were desensitized and stimulated in the same way as model group. Allergen-specific immunotherapy was performed by subcutaneous injections with Humulus pollen allergen on the 15th day (100 μL each time at 1 μg/μL, once a day for a total of 8 days, **Figure 1**). Mice in the control group were sensitized and stimulated in the same way as model group except for using the same volume of normal saline instead of crude extract of Humulus pollen. 4% aluminum hydroxide (V/W: 5/2) was added in the normal saline as an adjuvant when sensitization was performed.

10 mice in each group were anesthetized with intraperitoneal injection of 10% chloral hydrate 24 h after last stimulation. Blood samples were drawn from the orbital arteries and serum was collected by centrifuging. Animals were sacrificed by cervical dislocation. The left upper pulmonary lobe was isolated and fixed with formalin, and stained with Hematoxylin-Eosin (HE) and Alcian blue-Period acid Schiff (AB-PAS) staining.

General conditions of the mice were observed including drooping of the head, decreased activity, clustering, piloerection, tachypnea, hunched back, forelimb flexion and allergic conjunctivitis. The frequencies of the above symptoms were compared among groups.

Assessment of airway hyperresponsiveness

10 mice were selected from each group to assess pulmonary function. In order to avoid the influence of acetyl methylcholine chloride (Mch) on other experimental indices, mice that underwent pulmonary function assessment were spared from following sampling.

10 mice were selected from each group to perform aerosol inhalation of methacholine (0.938, 1.875, 3.75, 7.5,15 and 30 mg/mL) for 2 min 24 h after the last allergen stimulation. Pulmonary function index at the corresponding dose was determined by the average enhanced pause (Penh) value within 3 minutes after the inhalation. Penh was calculated following the formula: Penh = [(Te/Tr-1) × (PEF/PIF)]. Te is the expiration time, Tr is the relaxation time, PEF is the peak expiratory flow rate and PIF is the peak inspiratory flow rate multiplied by the constant coefficient 0.67. Relaxation time is

Table 1. Score of pulmonary inflammatory infiltration

Score index	Pathological degree			
	Grade 0	Grade 1	Grade 2	Grade 3
Lymphocytic infiltration of the tracheal wall	None	Follicle not formed	Follicle formed	Obvious follicle
Neutrephil infiltration of the tracheal wall	None	10/HP	10/HP	> 20/HP
Eosinophil infiltration of the tracheal wall	None	10/HP	10/HP	> 20/HP
Airway wall smooth muscle cell hyperplasia	None	< 1/4 circumference	1/4-1/2 circumference	> 1/2 circumference
Tracheal epithelium goblet cell hyperplasia	None	< 1/4 circumference	1/4-1/2 circumference	> 1/2 circumference
Tracheal cilia down and absence	None	< 1/4 circumference	1/4-1/2 circumference	> 1/2 circumference

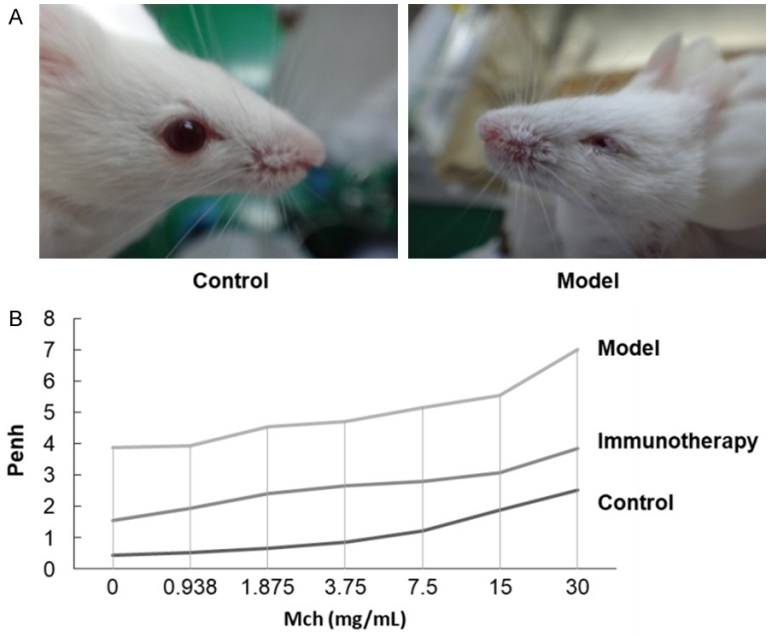

Figure 2. Immunotherapy reduced pulmonary index of Penh. A. Eye symptoms in the model and control groups (erythematous eyelid, tears and runny nose in model group); B. Penh changed with Mch in a dose-dependent manner in three groups. Penh was significantly higher in model group than immunotherapy group in each dose of Mch treatment.

defined when the pressure in the plethysmograph changes from the maximum value to the minimum value (the percentage of the maximum value, usually 40%).

Biochemical measurement

Total IgE detection kit (ab157718, Abcam) was used to detect total IgE and experiments were performed in strict accordance with the manufacturer's instructions.

The experimental procedure for IgE assessment included antigen coating overnight at 4°C, blocked with 1% BSA at 37°C for 2 h, adding diluted test serum at 37°C for 5 h, incubating with enzyme-labeled anti-IgE secondary antibody (ab99574, Abcam) at 37°C for 5 h, coloring, terminating reaction, and reading plate.

The experimental procedure for IgG1 included antigen coating overnight at 4°C, blocked with 1% BSA at 37°C for 2 h, adding diluted test serum at room temperature for 1 h, incubating with enzyme-labeled anti-IgG1 secondary antibody (ab97240, Abcam) at room temperature for 50 min, coloring, terminating reaction, reading plate. Detection of antigen-specific IgG2a (specific IgG2a, sIgG2a) was the same as serum IgG1 with anti-IgG2a secondary antibody (ab97245, Abcam).

Measurements of cytokines

The serum T helper (Th) 1 cytokine interleukin (IL)-12p70, interferon (IFN)-γ, Th2 cytokine IL-5 and IL-13 were detected by commercial kits (ab100699, Abcam; BMS606, eBioscience; ab100711, Abcam and BMS601, eBioscience, respectively).

HE and AB-PAS staining

HE and AB-PAS staining were completed by the Department of Pathology at Institute of Basic Medical Sciences of Peking Union Medical College. The inflammatory pathological changes of mouse airways were evaluated with the Cosio score [6] and the degree of airway mucus secretion was assessed using MOR (ratio of mucus area to bronchial area) [7] (**Table 1**).

Table 2. Comparison of symptom frequencies in the model and immunotherapy groups

Variables	Model			Immunotherapy			Fisher's test	
	+	-	Total	+	-	Total	P	
Head drooping	18	2	20	4	16	20	< 0.0001	
Decreased activity	18	2	20	6	14	20	0.0002	
Clustering	20	0	20	6	14	20	< 0.0001	
Piloerection	20	0	20	4	16	20	< 0.0001	
Nodding breathing	16	4	20	0	20	20	< 0.0001	
Hunched back	14	6	20	0	20	20	< 0.0001	
Forelimb flexion	14	6	20	0	20	20	< 0.0001	
Conjunctivitis	16	4	20	6	14	20	0.0036	
							Pearson test	
							χ^2	P
Total	136	24	160	26	134	160	151.274	0.0000

Table 3. Comparison of symptom frequencies in the immunotherapy and control groups

Variables	Immunotherapy			Control			Fisher's test	
	+	-	Total	+	-	Total	P	
Head drooping	4	16	20	0	20	20	0.1060	
Decreased activity	6	14	20	0	20	20	0.0202	
Clustering	6	14	20	0	20	20	0.0202	
Piloerection	4	16	20	0	20	20	0.1060	
Nodding breathing	0	20	20	0	20	20	1.0000	
Hunched back	0	20	20	0	20	20	1.0000	
Forelimb flexion	0	20	20	0	20	20	1.0000	
Conjunctivitis	6	14	20	0	20	20	0.0202	
							Pearson test	
							χ^2	P
Total	26	134	160	0	160	160	28.299	0.0000

Table 4. Marginal mean[#] of Penh

Groups	Means	Standard error	95% confidence interval	
			Minimum	Maximum
Control	1.152[a]	.135	0.885	1.419
Model	4.957[a]	.136	4.688	5.226
Immunotherapy	2.609[a]	.135	2.342	2.876

[#]Marginal Mean: The Penh's marginal mean in this study refers to the Penh's value after removing the influence of Mch. [a]The covariates appearing in this model are evaluated with the following values: Mch concentration 8.33426 mg/ml.

Immunoblotting

20 μL crude extract of Humulus pollen (1 μg/μL) was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (stacking gel: 80 V for 30 min; separating gel: 150 V for 150 min), Coomassie brilliant blue staining and decolorization. For the immunoblotting, the gel underwent transmembrane at 150 mA for 60 min and incubated with primary antibody (diluted with 5% milk at the ratio of 1:4) at room temperature for 2 h, followed by incubation with anti-IgE secondary antibody (ab995-74, Abcam, diluted with 5% milk at the ratio of 1:1000) at room temperature for 2 h.

Statistical analysis

Frequency of symptoms was tested by Chi-square test. The difference in the pulmonary function indices was evaluated by covariance analysis and the difference in the levels of antibodies and cytokines was analyzed with variance analysis. Pairwise comparison was made using LSD method (with homogeneity of variance) and Benferroni method (with heterogeneity of variance) if the difference was significant. The Mann-Whitney U test was used to compare the pathological scores and the Bonferroni method was used to correct the *P* value. A *P* value < 0.05 was considered significant difference.

Results

General conditions

Allergic symptoms, such as drooping of the head, clustering, decreased activity, piloerection, nodding breathing, hunched back, forelimb flexion and allergic conjunctivitis were observed in all mice, whereas no symptom was observed in the control group (**Figure 2A**). The frequency of each symptom in the immunotherapy group was significantly lower than that in the model group ($P < 0.05$ for 8 indices) (**Table 2**). However, some symptoms of the mice in the immu-

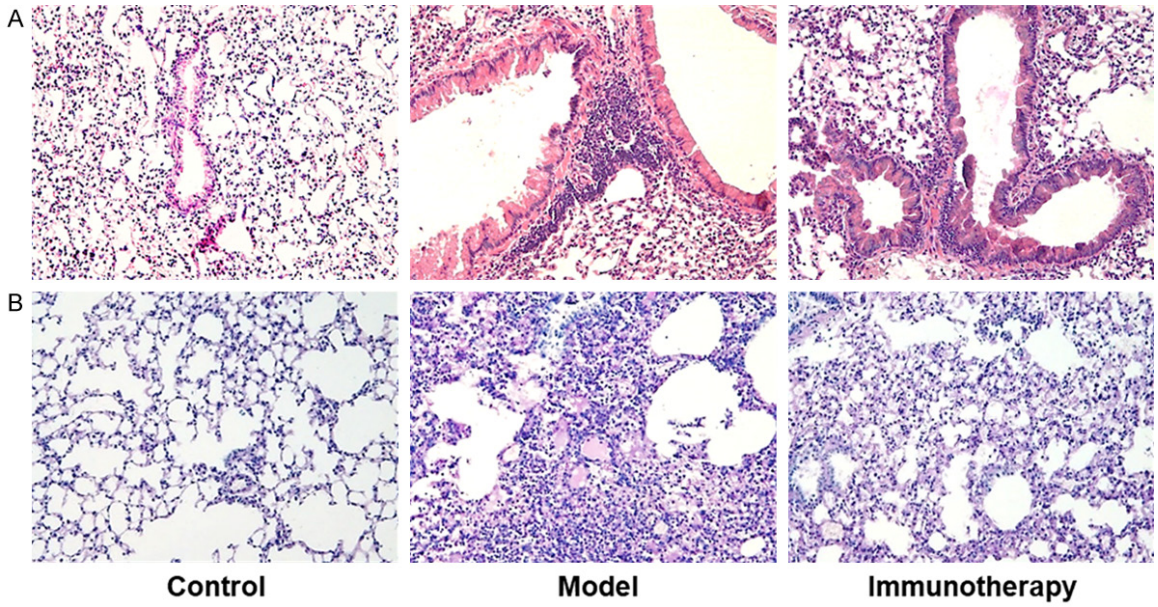

Figure 3. Airway inflammatory cell infiltration and alveolar mucus secretion in three groups (20 ×). A. HE staining; Characteristics like local mucous epithelial shedding, basement membrane thickening, increase in goblet cell numbers, hypertrophy and hyperplasia of vascular smooth muscle cells, infiltrations of eosinophils, lymphocytes and other inflammatory cells in the vascular wall and mucus plugs in the pulmonary alveoli could be observed in the airways in model group; B. AB-PAS staining. Mucus suppository was found in the alveolar cavity of the model group, and the mucus suppository in the immunotherapy group was less than that in the model group.

Table 5. Mann-Whitney U test for the pulmonary pathological scores in the model and immunotherapy groups

Inflammatory indices	Standardized test statistics (Z)	*P* value
Lymphocytic infiltration of the tracheal wall	-0.576	0.631
Neutrephil infiltration of the tracheal wall	0.798	0.425
Eosinophil infiltration of the tracheal wall	-1.990	0.047
Airway wall smooth muscle cell hyperplasia	-0.773	0.439
Tracheal epithelium goblet cell hyperplasia	-3.170	0.002
Tracheal cilia down and absence	-3.502	0.000
Mucous MOR	-2.894	0.004

notherapy group were not completely relieved compared to the control group ($P < 0.05$ for three indices) (**Table 3**).

Immunotherapy reduced pulmonary index of Penh

The Penh value gradually increased with the increase of Mch in a dose-dependent manner in all three groups (**Figure 2B**). Under each Mch concentration, the Penh value of the model group was significantly higher than that of the control group ($P < 0.05$ under each concentration) and it was significantly lower in immunotherapy group than that in model group ($P < 0.05$ under each concentration), indicating that high-dose and short-course immunotherapy could improve the pulmonary function in asthmatic mice. There were significant differences in the marginal value of Penh among three groups when Mch concentration was 8.33 mg/mL (P = 0.000; **Table 4**).

Histopathological changes

Local mucous epithelial shedding, basement membrane thickening, increase in goblet cell numbers, hypertrophy and hyperplasia of vascular smooth muscle cells, infiltrations of eosinophils, lymphocytes and other inflammatory cells in the vascular wall and mucus plugs in the pulmonary alveoli could be observed in the airways in model group (**Figure 3**). Compared with the model group, the goblet cell proliferation, down of cilia and the mucus secretion score were significantly improved in immunotherapy group ($P < 0.01$). There was a tendency that the degree of eosinophil infiltration was less than that in model group, but the score

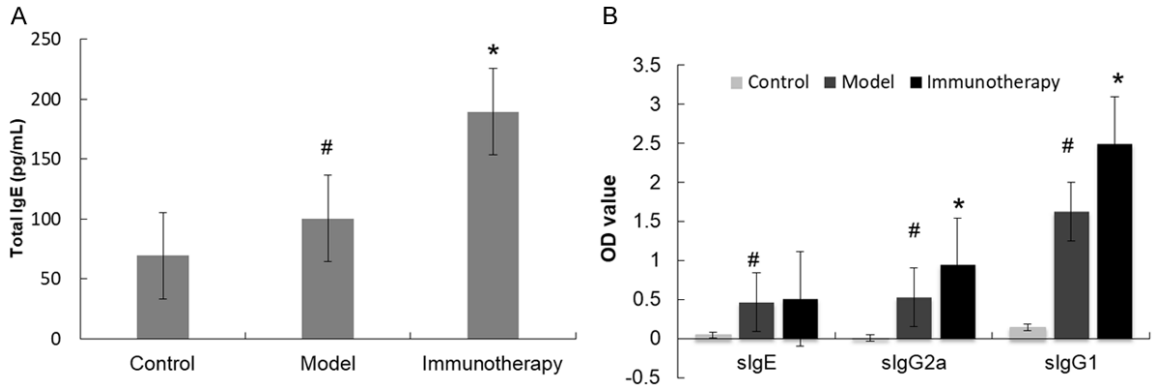

Figure 4. Immunotherapy elevated serum IgE and IgG. A. Serum tIgE levels in the model, immunotherapy and control groups. 10 samples were taken from each group and the values were expressed as mean ± standard error. The measurement was concentration (pg/mL). #P < 0.05 compared with the control group. *P < 0.05 compared with the model group; B. Serum sIgE, sIg2a and sIgG1 levels in the model, immunotherapy and control groups. 10 samples were taken from each group and the values were expressed as mean ± standard error. The tIgE, sIgE, sIgG2a and sIgG1 of the model group were significantly higher than those of the control group. The measurement was OD value. #P < 0.05 compared with the control group. *P < 0.05 compared with the model group.

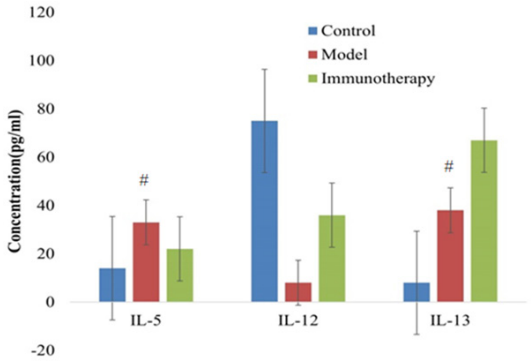

Figure 5. Serum cytokine expression levels in three groups. 10 samples were taken from each group. IL-12 in the model group was significantly lower than that in the control group, but was not significantly different compared to the immunotherapy group. IL-5 and IL-13 in the model group were significantly higher than those in the control group, but were not significant different compared to the immunotherapy group. The values were expressed as mean ± standard error. The measurement was concentration (pg/mL). #P < 0.05 compared with the control group.

showed no significant difference (P = 0.047; **Table 5**).

Immunotherapy elevated serum IgE and IgG

The serum tIgE level was significantly higher in model group than in control group ($P < 0.05$), and it was significantly higher in immunotherapy group than in model group ($P < 0.05$; **Figure 4A**). The serum sIgE level in the model group was significantly higher than in the control group ($P < 0.05$), but was not statistically different when compared with the immunotherapy group ($P > 0.05$; **Figure 4B**). The serum levels of sIgG1 and sIgG2a were significantly higher in the model group than in the control group ($P < 0.05$), and they were significantly higher in the immunotherapy group compared with the model group ($P < 0.05$; **Figure 4B**).

Immunotherapy did not affect serum cytokines

The level of Th1 type cytokine IL-12 in model group was significantly lower than that in control group ($P < 0.05$), but was not significantly different compared to immunotherapy group ($P = 0.10$; **Figure 5**).

The expression level of Th2 type cytokines IL-5 and IL-13 in model group was significantly higher than those in control group ($P < 0.05$), but were not significantly different compared to the immunotherapy group ($P > 0.05$; **Figure 5**).

Protein identification

The molecular weight of major proteins was ~80, ~70, ~38, ~30, ~26, ~15, and ~10 kDa, respectively, in the SDS-PAGE of the crude extract of Humulus pollens (**Figure 6**).

The Humulus pollen allergen could be identified in the serum of 10 mice. The molecular weight of serum proteins that had the maximum binding to allergen was ~25 kDa and

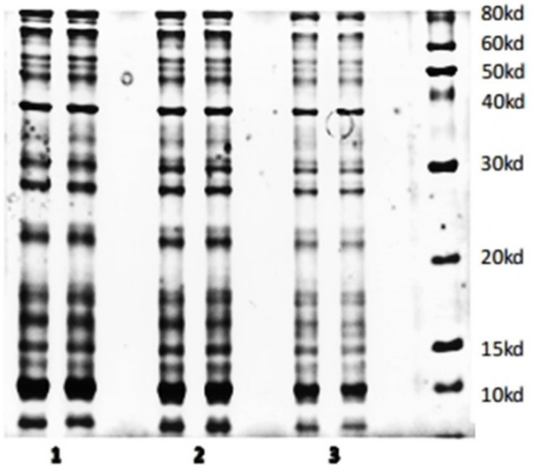

Figure 6. SDS-PAGE of Humulus pollen allergens. The amounts of Humulus protein sample on bands 1-3 were 30 μL (parallel hole), 20 μL (parallel hole) and 10 μL (parallel hole). The concentration was 1.5 μg/μL. The molecular weights of major proteins were ~80, ~70, ~38, ~30, ~26, ~15 and ~10 kDa.

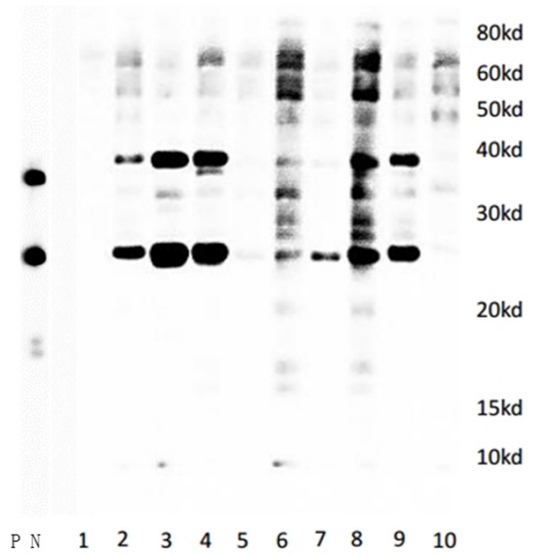

Figure 7. Western blot analysis of Humulus pollen allergen that specifically binds to IgE. 1-10: 10 mice in the model group; P: Positive control; N: Negative control. The molecular weights of serum proteins that had the maximum binding to allergen were ~25 kDa (line 2, 3, 4, 6, 7, 8, 9) and ~38 kDa (line 2, 3, 4, 6, 8, 9). Other low (10-20 kDa), medium (25-35 kDa) and high (50-80 kDa) molecular weight proteins showed poor binding to the allergen.

~38 kDa in 7 cases (70%) and 6 cases (60%), respectively. Other proteins with low (10-20 kDa), medium (25-35 kDa) or high (50-80 kDa) molecular weight showed poor binding to the allergen (**Figure 7**).

Discussion

In this study, we evaluated the efficacy of subcutaneous immunotherapy of high-dose (100 μg) Humulus pollen allergen in a mouse model of allergic asthma induced by Humulus pollen. Penh is a commonly used dimensionless indicator for pulmonary function assessment, which is related to the mouse airway resistance and pressure in the pleura and can be measured by body plethysmography. Our results showed that high-dose and short-course immunotherapy could significantly improve the pulmonary function, alleviate airway inflammatory response and relieve allergic symptoms in asthmatic mice. What we've found is consistent with previous literatures [8, 9].

IgE is involved in the pathophysiological process of type I allergic reaction and plays an important role in the development of airway hyperresponsiveness in patients with asthma [10]. Serum tIgE level is positively correlated with the severity of asthma [11]. The serum tIgE level in the model group was significantly higher than that in the control group, which was consistent with previous studies [12, 13]. It has been reported that the expression of tIgE would either increase [14, 15] or remain steady after immunotherapy [16, 17]. In this study, however, we found that tIgE level in immunotherapy group was significantly elevated compared to that in model group, which was inconsistent with previous studies [16, 17]. IgE is mainly secreted by B cells. Akdis *et al* found that B cells showed no evidence of tolerance in the early stage of treatment [18]. Therefore, the elevated tIgE level after treatment might be related to shorter duration of immunotherapy. In addition, the concentration of allergen used in the present study was obviously higher than previous studies, which in another aspect could partially explain the why the expression of tIgE elevated following treatment. Serum sIgE expression remained steady after immunotherapy, which was also in accordance with previous studies [16, 17]. However, the kinetics of sIgE expression after immunotherapy is still uncertain. Some studies demonstrated that the level of sIgE reduced after immunotherapy [14, 19], whereas Van Ree *et al* suggested that sIgE might increase temporarily in the early stage of treatment and would gradually decline after months or years of treatment [20]. The allergic symptoms of the subjects in these studies

were relieved regardless of the changes in IgE expression level. Yet no clear correlation between serum IgE levels and the remission of allergic clinical symptoms has been established. Changes in the serum IgE level could not explain the reduction of allergen response after immunotherapy.

The level of IgG1 and IgG2a represents the intensity of Th2 and Th1 immune response, respectively [21-26]. The increase of IgG2a level has been regarded as an indicator of effective immunotherapy. It has also been established that allergen could induce immune response to skew Th2-type to Th1-type in asthmatic mice [27-29]. In the present study, IgG2a level significantly increased after immunotherapy, which is consistent with previous study [30]; however, the increase in IgG1 following immunotherapy was inconsistent with previous studies [26, 31]. Bach *et al* [28] suggested that subcutaneous injection could induce Th2-type immune response. Jiménez-Saiz et al suggest that subcutaneous injection of allergens can cause minor lesions, inflammation and the recruitment of activated dendritic cells to draining lymph nodes, a process that induces the production of IgG1+ B cells in lymph node germinal centers and elevate serum allergen sIgG1 [32]. Other study has considered that the presence of non-protein components in crude extracts such as chitin, β-glucose can activate innate immune responses and promote inflammation through immunotherapy injection [33]. In this study, repeated subcutaneous injection of allergens was administrated, and the increase of sIgG1 was likely associated with repeated subcutaneous injection of allergens. Moreover, the immunotherapy process of daily subcutaneous injection might contribute to the induction of Th2-type immune response with elevated IgG1.

IL-5 and IL-13 are two major Th2 cytokines. IL-5 sustains the survival of eosinophil [34] and IL-13 stimulates the antibody class switching to IgE. As shown in the present study, the expression level of IL-5 and IL-13 was significantly higher in the model group than in the control group, suggesting that Th2-type immune response was induced in the model group, which was consistent with previous studies [17, 35]. It was reported that Th2 cytokines were significantly reduced after immunotherapy [27], while other studies showed that Th1 and Th2 cytokines did not change after immunotherapy [17]. In this study, the levels of Th1 cytokine IL-12 and Th2 cytokine IL-5/13 showed increasing tendencies after immunotherapy, although the differences were not statistically significant. It might be reasonable to conclude that both Th1 and Th2 immune responses could be enhanced after immunotherapy. However, this conclusion still merits further researches with lager sample size.

Chinese researchers tested the immunoreactivity of Humulus pollen allergens in the human body and found that Humulus pollen contained more than 20 proteins. The molecular weight of major allergen ranged from 5 to 43 kDa. Proteins with molecular weight of 43-97.4 kDa had stronger immunogenicity but weaker antigenicity [36]. The immunoreactivity of allergens in mice has not yet been reported. Results of this study demonstrated that the molecular weight of main allergens of Humulus pollen-sensitized mice was ~25 kDa and ~38 kDa, which has provided a theoretical basis for the immunotherapy with allergenic components of Humulus allergens.

Despite our discovery, there are still some limitations in this study. The influence of subcutaneous injection on immunotherapy could not be determined because the control of PBS injection was not established in the immunotherapy. In addition, the main allergens of Humulus pollen that sensitized mice were not isolated and the efficacy of immunotherapy was not assessed using these allergens.

In conclusion, high-dose subcutaneous immunotherapy could effectively alleviate airway hyperresponsiveness and reduce airway inflammatory infiltration in allergic asthmatic mice.

Acknowledgements

This work was supported by grants from Beijing Municipal Science and Technology Project (No. Z131100006813047). The sponsor or funding organization had no role in the design or conduct of this research.

Disclosure of conflict of interest

None.

Address correspondence to: Jia Yin, Department of Allergy, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No.1 Shuaifuyuan Wangfu-

jing Dongcheng District, Beijing 100730, China. Tel: 86010-69156346; Fax: 86010-69156346; E-mail: doctoryinjia@126.com

References

[1] Park JW, Ko SH, Kim CW, Jeoung BJ and Hong CS. Identification and characterization of the major allergen of the Humulus japonicus pollen. Clin Exp Allergy 1999; 29: 1080-1086.

[2] Yin J, Yue F, Wang L, He H, Xu T, Li H, Wen L, Sun J, Gu J, Han S, Zhang H and Ye S. Natural course from rhinitis to asthma in the patients with autumnal pollinosis: a clinical study of 1096 patients. Zhonghua Yi Xue Za Zhi 2006; 86: 1628-1632.

[3] Dhami S, Kakourou A, Asamoah F, Agache I, Lau S, Jutel M, Muraro A, Roberts G, Akdis CA, Bonini M, Cavkaytar O, Flood B, Gajdanowicz P, Izuhara K, Kalayci O, Mosges R, Palomares O, Pfaar O, Smolinska S, Sokolowska M, Asaria M, Netuveli G, Zaman H, Akhlaq A and Sheikh A. Allergen immunotherapy for allergic asthma: a systematic review and meta-analysis. Allergy 2017; 72: 1825-1848.

[4] Ross RN, Nelson HS and Finegold I. Effectiveness of specific immunotherapy in the treatment of asthma: a meta-analysis of prospective, randomized, double-blind, placebo-controlled studies. Clin Ther 2000; 22: 329-341.

[5] Li Y, Zhang M, Lu J, Liu Y, Yang X and Sun X. Establishment of a mouse model of humulus pollen allergen-induced allergic asthma. Journal of Xi'an Jiaotong University (Medical Sciences) 2010; 5: 562-565.

[6] Barbera JA, Peinado VI, Santos S, Ramirez J, Roca J and Rodriguez-Roisin R. Reduced expression of endothelial nitric oxide synthase in pulmonary arteries of smokers. Am J Respir Crit Care Med 2001; 164: 709-713.

[7] Aikawa T, Shimura S, Sasaki H, Takishima T, Yaegashi H and Takahashi T. Morphometric analysis of intraluminal mucus in airways in chronic obstructive pulmonary disease. Am Rev Respir Dis 1989; 140: 477-482.

[8] Nieto Garcia A, Nevot Falco S, Carrillo Diaz T, Cumplido Bonny JA, Izquierdo Calderon JP and Hernandez-Pena J. Safety of cluster specific immunotherapy with a modified high-dose house dust mite extract. Eur Ann Allergy Clin Immunol 2013; 45: 78-83.

[9] Irani C, Saleh RA, Jammal M and Haddad F. High-dose sublingual immunotherapy in patients with uncontrolled allergic rhinitis sensitized to pollen: a real-life clinical study. Int Forum Allergy Rhinol 2014; 4: 802-807.

[10] Wu LC and Zarrin AA. The production and regulation of IgE by the immune system. Nat Rev Immunol 2014; 14: 247-259.

[11] Tanaka A, Jinno M, Hirai K, Miyata Y, Mizuma H, Yamaguchi M, Ohta S, Watanabe Y, Yamamoto M, Suzuki S, Yokoe T, Adachi M and Sagara H. Longitudinal increase in total IgE levels in patients with adult asthma: an association with poor asthma control. Respir Res 2014; 15: 144.

[12] Davila I, Valero A, Entrenas LM, Valveny N, Herráez L; SIGE Study Group. Relationship between serum total IgE and disease severity in patients with allergic asthma in Spain. J Investig Allergol Clin Immunol 2015; 25: 120-127.

[13] Schatz M and Rosenwasser L. The allergic asthma phenotype. J Allergy Clin Immunol Pract 2014; 2: 645-648; quiz 649.

[14] Sahin E, Tas E, Dagtekin Ergur EN, Cuhali BD and Gursel AO. [The results of specific immunotherapy for house dust mites in patients with allergic rhinitis]. Kulak Burun Bogaz Ihtis Derg 2008; 18: 79-84.

[15] Siebeneicher S, Reuter S, Wangorsch A, Krause M, Foetisch K, Heinz A, Naito S, Reuter A, Taube C, Vieths S, Scheurer S and Toda M. Epicutaneous immunotherapy with a hypoallergenic Bet v 1 suppresses allergic asthma in a murine model. Allergy 2015; 70: 1559-1568.

[16] Zhang X, Li MR, Wang C, Wang XN, Zhang HL, Lin J, Jin K and Li YC. [Clinical efficacy of a standardized specific immunotherapy against house dust mite in 85 asthmatic children]. Zhonghua Er Ke Za Zhi 2010; 48: 526-530.

[17] Roger A, Depreux N, Jurgens Y, Heath MD, Garcia G and Skinner MA. A novel and well tolerated mite allergoid subcutaneous immunotherapy: evidence of clinical and immunologic efficacy. Immun Inflamm Dis 2014; 2: 92-98.

[18] Akdis CA, Akdis M, Blesken T, Wymann D, Alkan SS, Muller U and Blaser K. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. J Clin Invest 1996; 98: 1676-1683.

[19] Fox EM, Torrero MN, Evans H and Mitre E. Immunologic characterization of 3 murine regimens of allergen-specific immunotherapy. J Allergy Clin Immunol 2015; 135: 1341-1351, e1341-1347.

[20] Van Ree R, Van Leeuwen WA, Dieges PH, Van Wijk RG, De Jong N, Brewczyski PZ, Kroon AM, Schilte PP, Tan KY, Simon-Licht IF, Roberts AM, Stapel SO and Aalberse RC. Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. Clin Exp Allergy 1997; 27: 68-74.

[21] Rabe KF, Calhoun WJ, Smith N and Jimenez P. Can anti-IgE therapy prevent airway remodeling in allergic asthma? Allergy 2011; 66: 1142-1151.

[22] Barry J, Loh Z, Collison A, Mazzone S, Lalwani A, Zhang V, Davidson S, Wybacz E, Garlanda C, Mantovani A, Mattes J, Foster PS and Phipps S. Absence of toll-IL-1 receptor 8/single immunoglobulin IL-1 receptor-related molecule reduces house dust mite-induced allergic airway inflammation in mice. Am J Respir Cell Mol Biol 2013; 49: 481-490.

[23] Ghaemmaghami AM, Gough L, Sewell HF and Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. Clin Exp Allergy 2002; 32: 1468-1475.

[24] Mountford AP, Fisher A and Wilson RA. The profile of IgG1 and IgG2a antibody responses in mice exposed to Schistosoma mansoni. Parasite Immunol 1994; 16: 521-527.

[25] Ormstad H, Groeng EC, Duffort O and Lovik M. The effect of endotoxin on the production of IgE, IgG1 and IgG2a antibodies against the cat allergen Fel d 1 in mice. Toxicology 2003; 188: 309-318.

[26] Zhao J, Li C, Zhao B, Xu P, Xu H and He L. Construction of the recombinant vaccine based on T-cell epitope encoding Der p1 and evaluation on its specific immunotherapy efficacy. Int J Clin Exp Med 2015; 8: 6436-6443.

[27] Shirinbak S, Taher YA, Maazi H, Gras R, van Esch BC, Henricks PA, Samsom JN, Verbeek JS, Lambrecht BN, van Oosterhout AJ and Nawijn MC. Suppression of Th2-driven airway inflammation by allergen immunotherapy is independent of B cell and Ig responses in mice. J Immunol 2010; 185: 3857-3865.

[28] Bach D, Weiss R, Hessenberger M, Kitzmueller S, Weinberger EE, Krautgartner WD, Hauser-Kronberger C, Boehler C, Thalhamer J and Scheiblhofer S. Transcutaneous immunotherapy via laser-generated micropores efficiently alleviates allergic asthma in Phl p 5-sensitized mice. Allergy 2012; 67: 1365-1374.

[29] Chang YS, Kim YK, Kim SH, Park HW, Min KU, Kim YY and Cho SH. Murine subcutaneous immunotherapy models with beneficial immunological and physiological effects. Asia Pac Allergy 2013; 3: 50-58.

[30] Freiberger SN, Zehnder M, Gafvelin G, Gronlund H, Kundig TM and Johansen P. IgG4 but no IgG1 antibody production after intralymphatic immunotherapy with recombinant MAT-Feld1 in human. Allergy 2016; 71: 1366-1370.

[31] Shershakova N, Bashkatova E, Babakhin A, Andreev S, Nikonova A, Shilovsky I, Kamyshnikov O, Buzuk A, Elisyutina O, Fedenko E and Khaitov M. Allergen-specific immunotherapy with monomeric allergoid in a mouse model of atopic dermatitis. PLoS One 2015; 10: e0135070.

[32] Jimenez-Saiz R, Ellenbogen Y, Koenig J, Gordon ME, Walker TD, Rosace D, Spill P, Bruton K, Kong J, Monteiro K, Wen J, Tuomanen EI, Kolbeck R, Chu DK, Waserman S and Jordana M. IgG1(+) B cell immunity predates IgE responses in epicutaneous sensitization to foods. Allergy 2018; [Epub ahead of print].

[33] Hesse L, van Ieperen N, Habraken C, Petersen AH, Korn S, Smilda T, Goedewaagen B, Ruiters MH, van der Graaf AC and Nawijn MC. Subcutaneous immunotherapy with purified Der p1 and 2 suppresses type 2 immunity in a murine asthma model. Allergy 2018; 73: 862-874.

[34] Ishmael FT. The inflammatory response in the pathogenesis of asthma. J Am Osteopath Assoc 2011; 111: S11-17.

[35] Chung MJ, Pandey RP, Choi JW, Sohng JK, Choi DJ and Park YI. Inhibitory effects of kaempferol-3-O-rhamnoside on ovalbumin-induced lung inflammation in a mouse model of allergic asthma. Int Immunopharmacol 2015; 25: 302-310.

[36] Liu Y, Sun XZ, Xu DS, Li W, Feng XL and Xu J. [Purification and analysis of the immunoactivity of humulus pollen allergen]. Sichuan Da Xue Xue Bao Yi Xue Ban 2008; 39: 1027-1031.