

Original Article

Repetitive application of recombinant Pla a 1 reduces the airway inflammation and hyperresponsiveness in *P. acerifolia* sensitized mice

Shengyu Wang^{1,3}, Guizuo Wang¹, Xinming Xie¹, Yuanyuan Wu¹, Xiuzhen Sun², Manxiang Li¹

¹Department of Respiratory Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, PR China;

²Department of Respiratory Medicine, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, PR China;

³Department of Respiratory Medicine, The First Affiliated Hospital of Xi'an Medical University, Xi'an, PR China

Received October 5, 2016; Accepted November 17, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: *Purpose:* To evaluate whether repetitive injection of rPla a 1 can decrease the airway inflammation and hyperresponsiveness in *P. acerifolia* sensitized mice. *Materials and Methods:* After sensitization, mice were injected intraperitoneally with rPla a 1 for five times. Lung tissues from the mice were collected for histopathology analysis, period acid-schiff (PAS) and in situ hybridization (ISH) to detect the expression of IL-10 and FoxP3. Airway responsiveness was assessed in the whole body plethysmograph. Bronchoalveolar lavage fluids (BALF) were harvested from the mice to analyze the composition of inflammatory cells. Blood samples were collected to measure levels of *P. acerifolia* specific IgE (sIgE) and IgG (sIgG). Additionally, cytokine productions (IL-10, IL-13 and IL-17) from the spleen tissue homogenate were measured. *Results:* Intraperitoneal administration of rPla a 1 attenuated the airway inflammation and mucus production caused by *P. acerifolia* sensitization, reduced the airway responsiveness, and promoted the expression of IL-10 and FoxP3 in the lungs. In addition, intraperitoneal administration of rPla a 1 notably induced the production of sIgG in the serum and decreased IL-13 and IL-17 in the spleen tissue homogenate. *Conclusion:* Repetitive application of rPla a 1 reduces the airway inflammation and hyperresponsiveness in *P. acerifolia* sensitized mice, which may serve as an impetus for further research on the use of recombinant protein in the management of human allergic asthma.

Keywords: Allergic asthma, mice model, airway responsiveness, immunological tolerance

Introduction

The prevalence of Type I hypersensitivity has increased rapidly in recent years. Nearly one-fifth of the populations suffer from allergic rhinitis, conjunctivitis and asthma. Airborne inhalant allergens are partially responsible for this trend [1]. *Platanus acerifolia* (*P. acerifolia*) is a deciduous tree, often found in urban areas world-wide because of its resistance to air pollution and disease. Therefore, *P. acerifolia* pollen allergy has become a crucial health problem due to large amounts of airborne tree pollen detected in many cities around United States, Europe and China [2-4]. Accurate diagnosis and effective immunotherapy are important for these patients sensitive to *P. acerifolia*. However, current treatments and approaches strongly rely on the utilization of standardized

allergenic extracts containing complicated mixtures of allergens. It is essential to identify, isolate, and characterize *P. acerifolia* proteins on IgE-mediated allergic response.

P. acerifolia pollen is constituted by three identified pollen allergens [5]: two major allergens are Pla a 1 and Pla a 2, and the third minor allergen is Pla a 3. Pla a 1 represents approximately 60% of the total IgE binding of *P. acerifolia* pollen extract, and has been accounted for 84% of *P. acerifolia* related allergic reaction in patients [6]. Pla a 2, on the other hand, is a glycoprotein, accounting for 52% of the total IgE-binding capacity of *P. acerifolia* [7]. Lastly, Pla a 3 is an aeroallergen with compounding IgE effects in patients with food allergies [8]. Therefore, Pla a 1 should be mainly responsible for *P. acerifolia* related allergic reaction.

rPla a 1, airway inflammation and hyperresponsiveness

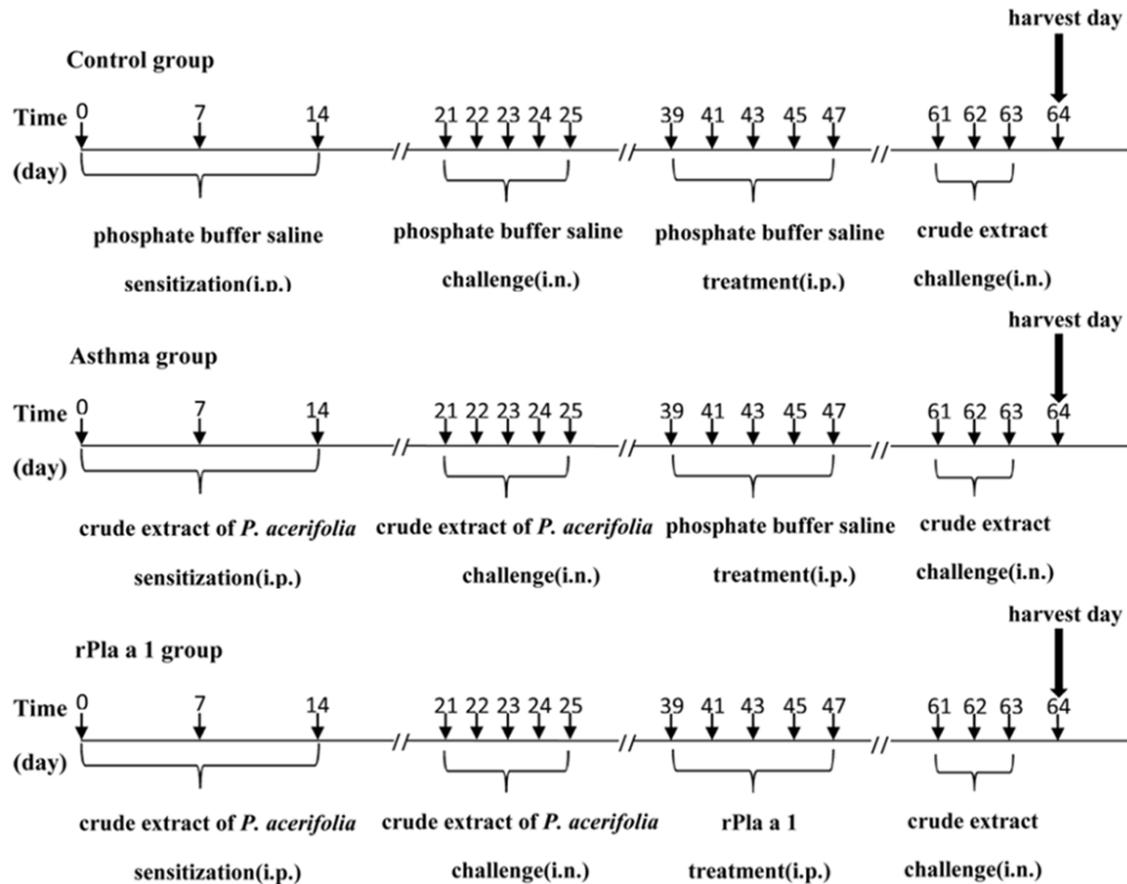


Figure 1. Schematic diagram illustrated the protocol for sensitization, challenge, and drug administration.

Immunological tolerance is the failure to mount an immune response to an antigen and can be generated through nasal and airway administration of antigen [9, 10]. Thus, we hypothesized that the airway inflammation and hyperresponsiveness in *P. acerifolia* sensitized mice can be effectively attenuated by repetitively exposing to recombinant Pla a 1 (rPla a 1), which had been previously produced by using *E. coli* Rosetta transfected with pET44a-Pla [4]. Meanwhile, we further explored the possible mechanism so as to provide an effective and novel method for treating the chronic inflammatory airway diseases.

Materials and methods

Expression and purification of rPla a 1

E. coli Rosetta (Novagen, Madison, WI, USA) was transformed with pET44a-Pla and grown at 37°C in LB medium containing 100 mg/ml Ampicillin. Expression of recombinant protein was induced by the treatment with 0.1 mM

Isopropyl-thio-D-galactopyranoside (IPTG) or 2 g/L Lactose for 4 h at 37°C. The refolded protein was then collected and stored in storage buffer 1 (20 mM Tris-HCl, pH 6.6, 300 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol) or storage buffer 2 (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The details followed the previous protocol [4].

Pollen crude extract preparation

Mature *P. acerifolia* flowers were collected from Xi'an Jiaotong University campus. Flowers were sun dried for two days and made into the crude extract following previously published protocol [11]. Extract was stored at -80°C and protein concentration was determined by the Bradford's method [12].

Experimental mice model

Female specific-pathogens free BALB/c mice, 3-4 weeks age and weight of 16±2 g, were purchased from Animal Center Laboratory of Xi'an

rPl_a 1, airway inflammation and hyperresponsiveness

Jiaotong University, Xi'an, China. Animal experiments were performed according to the European Community Guidelines for Care and Use of animals and approved by the Ethic Committee for Animal Use of Xi'an Jiaotong University Health Center (No.01459).

Following a three day acclimation period, mice were assigned to three groups: control, allergic asthma and rPl_a 1 treatment group (12 mice/group). Mice in asthma and rPl_a 1 group were sensitized intraperitoneally (i.p.) at Day 0, Day 7 and Day 14 with 300 µg of crude extract of *P. acerifolia* constituted in 2 mg Al(OH)₃. Seven days after the last sensitization, an intranasal instillation challenge was done with 300 µg/50 µl of crude extract of *P. acerifolia* for five consecutive days (Days 21, 22, 23, 24 and 25) when mice were anesthetized with intraperitoneal injection of 0.8% pentobarbital sodium (60 mg/kg). The allergen extract was instilled into nares with a micropipette. Mice in control group were injected intraperitoneally and challenged intranasally with equal volume of phosphate buffer saline (PBS) in the corresponding course. At two weeks after the last intranasal instillation challenge, 300 µg of rPl_a 1 was injected intraperitoneally every other day for 5 days (Days 39, 41, 43, 45 and 47). The mice in control and asthma group were treated with equal volume of PBS. Two weeks after the final treatment, mice in three groups were intranasally challenged again with 300 µg/50 µl of crude extract of *P. acerifolia* (Days 61, 62 and 63). On Day 64, all mice were anesthetized with the previous method and sacrificed by cervical dislocation. The experimental protocol outlining the treatments was shown in **Figure 1**.

Pulmonary assessment of enhanced pause (Penh)

Airway responsiveness of mice was assessed by barometric whole body plethysmography in response to methacholine (Mch) challenge according to the previously described protocol [13]. Briefly, non-anesthetized mice with spontaneous breath were placed in the plethysmograph (EMKA Technologies, France) and PBS aerosol was administered to form baseline readings over 3 min. Mch (acetyl-methylcholine chloride; Sigma-Aldrich) was then nebulized with increasing, serial 2-fold concentrations from 3.125-50 mg/ml for 3 min each dose to induce bronchoconstriction. In this model, the extent of bronchial response of individual

mouse was quantified as Penh. Airway responsiveness of mice was expressed as the fold increase for each concentration of Mch compared to Penh values after PBS challenge.

Determination of antigen-specific IgE and IgG in serum

96-well plates (Boster, Wuhan, China) were coated with *P. acerifolia* crude extract (in 10 mg/ml carbonate buffer pH 9.6) over night at 4°C. After washing with TBS-Tween (TBST), the plates were blocked with 1% BSA for 2 h at room temperature. Serum samples (diluted 1:10 for IgE and 1:1000 for IgG) were applied to each well and the plates were incubated overnight at 4°C. After 5 washes, the plates were incubated with anti-mouse IgE and IgG antibodies (1 µg/ml; Boster, Wuhan, China) for 4 h at room temperature followed by an additional 1 h incubation with peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Boster, Wuhan, China). Following incubation, ABTS substrate (1 mg/ml azinoethylbezthiozoline sulfonic acid substrate; Boster, Wuhan, China) was added to each well and absorbance was measured at 450 nm. The amount of *P. acerifolia* specific IgE (sIgE) and IgG (sIgG) were calculated using a standard curve of a reference protein (Boster, Wuhan, China).

Histopathology, periodic acid-schiff (PAS) and in situ hybridization of Lungs

Left whole lung of each mouse was fixed with 10% neutral-buffered formalin and embedded in paraffin. 5 µm thick sections then were cut and stained with hematoxylin and eosin (H&E) and periodic acid-schiff staining (PAS) for the assessment of goblet cells and mucus production. The intensity of inflammatory infiltration was graded on a semi-quantitative scale from 0 to 3.

Right whole lung was used for mRNA analysis of IL-10 and FoxP3 using *in situ* hybridization kit (Boster, Wuhan, China). All sections were given an immunohistochemical score (IHS) as previously described [14]. The grading of the slides was performed by 2 individuals blinded to the experimental protocol.

Levels of IL-10, IL-13 and IL-17 in spleen tissue homogenate

Spleens from mice were harvested aseptically, minced and filtered through sterile filters.

rPl_a 1, airway inflammation and hyperresponsiveness

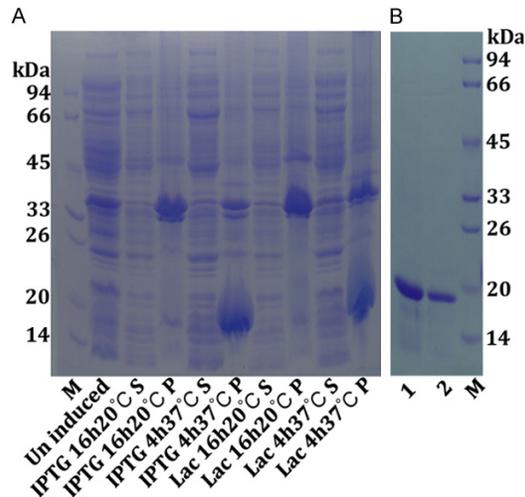


Figure 2. Expression, purification and identification of recombinant Pl_a 1. A. Supernatant and pellet were induced separately by IPTG and Lactose at 20 °C for 16 h or at 37 °C for 4 h. The production of rPl_a 1 (18 kDa) was observed in the pellet of *E.coli* in both buffers at 37 °C for 4 h. S: Supernatant; P: Pellet; M: Protein marker. B. rPl_a 1 was purified in two different buffers. Buffer 1: 20 mM Tris-HCl, pH 6.6, 300 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol; Buffer 2: 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA. M: Protein standard.

Smashed tissue were resuspended in sucrose buffer (Boster, Wuhan, China) and centrifuged for 5 min. The supernatant was reserved and the sediment was minced again. This process was repeated 3 times. Finally, tissue homogenates were sonicated and centrifuged, and the supernatant was used to measure the levels of IL-10, IL-13 and IL-17 using mouse ELISA kits (Boster, Wuhan, China).

Bronchoalveolar lavage fluids (BALF)

BALF were collected as previously described [15]. Lungs were washed twice with 0.8 mL cold PBS and nearly 1.4 mL of erythrocyte-free BALF were recovered from each lung. Samples were spun onto microscope slides, air dried and stained with Wright's staining solution [16]. At least 200 cells were counted and differentiated by light microscopy according to the standard morphologic criteria. The total number of counted cells was set to 100%, and the percentages of eosinophils, macrophages, lymphocytes and neutrophils were expressed as relative value.

Statistical analysis

The statistical significance of differences between control and experimental groups was

calculated using either a Student's t test for unpaired data or an ANOVA test followed by Fisher's protected least significant difference tests for paired datasets. The results were reported as means of experimental replicates \pm SE, unless indicated otherwise. A value of $P < 0.05$ was considered statistically significant.

Results

rPl_a 1 was expressed and purified

To investigate the production of rPl_a 1 by transforming *E.coli* Rosseta system with pET44a-Pl_a, we electrophoretically separated the components of the product supernatant and pellet samples. Only pellets from *E.coli* treated with IPTG or Lac showed 18 kDa electrophoretic band, which was predicted to be rPl_a 1 (Figure 2A). 18 kDa refolded rPl_a 1 protein was extracted from gel and further analyzed using SDS-PAGE analysis (Figure 2B). A single electrophoretic band with a targeted molecular mass of 18 kDa was visible, which suggests a monomeric protein.

Repetitive injection of rPl_a 1 attenuates the airway inflammation

Lung tissues from the earlier described three groups were obtained to analyze the airway inflammation in Figure 3A. The level of inflammation was graded based on the location of the inflammatory cells. Lung tissue from asthma group (Figure 3Ab and 3Ac) showed massive infiltration of the inflammatory cells around the bronchi and blood vessels (dotted arrow). Meanwhile, the goblet cells (solid arrow) and mucus production (red color) in asthma group were more notable than control group (Figure 3Aa). Inflammatory reactions were scored as grade 2, which was significantly higher than control group ($P < 0.05$). The inflammatory cells filtration, the goblet cells and mucus production in rPl_a 1 group (Figure 3Ad) were significantly reduced in comparison to asthma group as scored as having grade 1 inflammatory reactions ($P < 0.05$).

Repetitive injection of rPl_a 1 increases the expression of IL-10 and FoxP3

To investigate the molecular aspects of inflammation, we next measured mRNA levels of IL-10 and FoxP3 which are important modulators of inflammation in the lung in Figure 3B. The mRNA levels of IL-10 (Figure 3Bc) and FoxP3

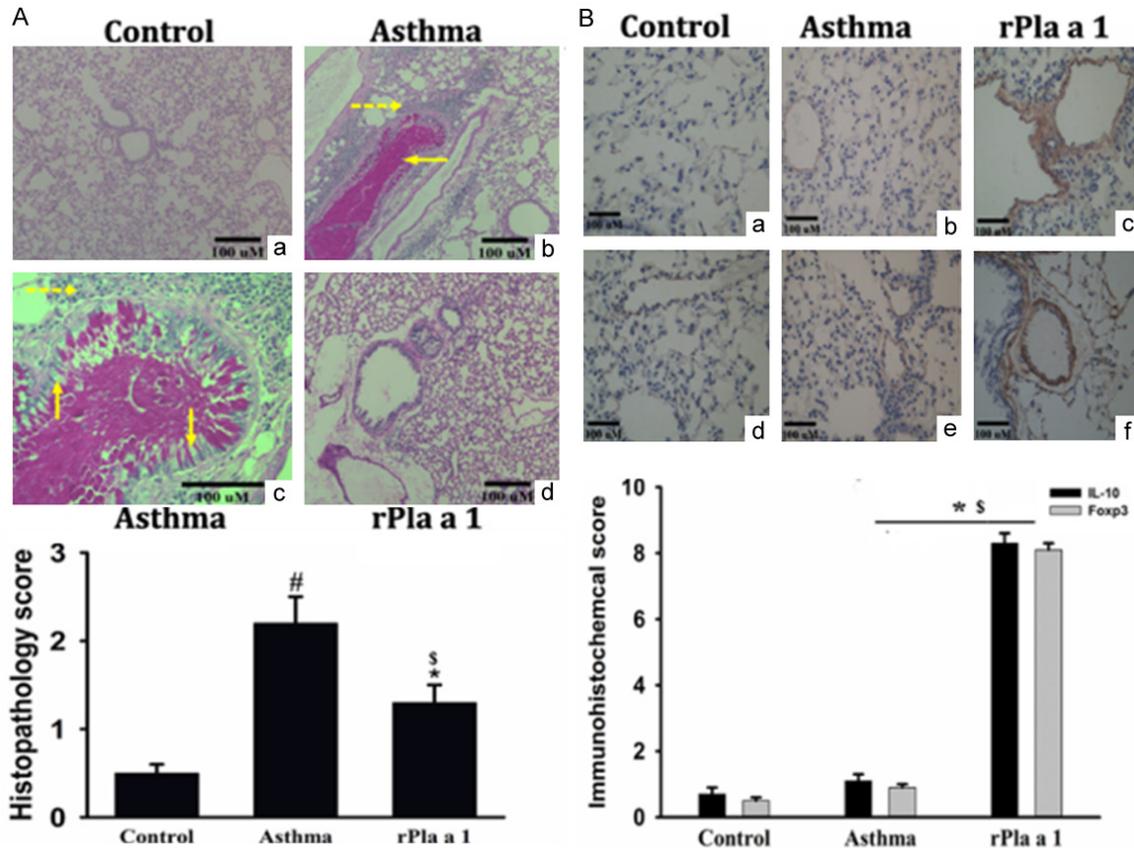


Figure 3. Representative lung sections from the mice. (A) H&E, PAS staining and histopathology score of control, asthma and rPl a 1 group. (a) No infiltration of inflammatory cells in control group were scored as grade 0. (b and c) Massive infiltration of inflammatory cells around the airway (dotted arrow) in asthma group were scored as grade 2. The goblet cells (solid arrow) and mucus production (red color) were more notable than control group. (d) Decline of inflammatory cells around the airway in rPl a 1 group were scored as grade 1. (B) Expression of IL-10 and FoxP3 mRNA in the lung via in situ hybridization. More mRNA expression of IL-10 (c) and FoxP3 (f) were shown in rPl a 1 group than asthma group (b and e). No significance between asthma and control group (a and d) was found. Error bars represent standard error of mean (n=6 samples). Symbol (#) indicates significance between asthma and control group (P<0.05); Symbol (*) indicates significant differences between rPl a 1 and asthma group (P<0.05); Symbol (\$) indicates significant differences between rPl a 1 and control group (P<0.05).

(Figure 3Bf) in rPl a 1 group were significantly elevated compared to IL-10 (Figure 3Bb) and FoxP3 (Figure 3Be) in asthma group (P<0.05). However, this trend between asthma and control group (Figure 3Ba and 3Bd) was not significantly. Repetitive administration of rPl a 1 benefits the production of IL-10 and FoxP3 in the mice lung.

Repetitive injection of rPl a 1 reduces the airway responsiveness

To understand whether rPl a 1 treatment reduced the airway responsiveness, the effect of rPl a 1 on *P. acerifolia* induced airway activity was assessed using whole body plethys-

mography. Mice that were sensitized with *P. acerifolia* developed airway hyperresponsiveness (AHR). Compared to mice in control group, sensitized mice demonstrated a dose-dependent elevation in Penh in response to Mch (Figure 4A, P<0.05). After the treatment of rPl a 1, the bronchial responsiveness was significantly lowered compared to asthma group (Figure 4A, P<0.05).

Repetitive injection of rPl a 1 decreases the inflammatory cells in BALF

To further investigate the anti-inflammatory role of rPl a 1 in *P. acerifolia* sensitized mice model. We looked at the effect of the protein in

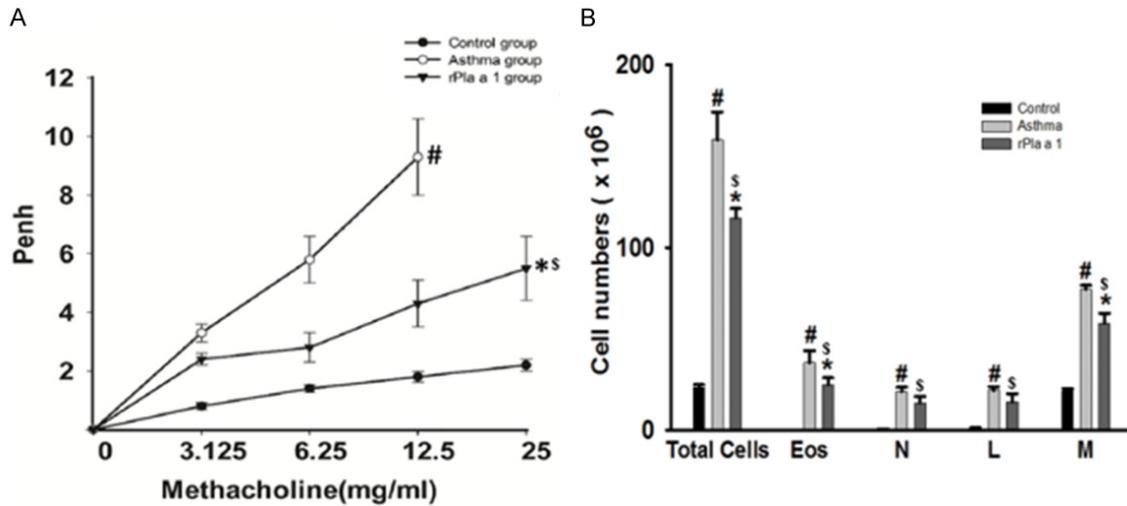


Figure 4. A. Penh response of mice with no challenge and treatment (control), *P. acerifolia* sensitization (asthma) and rPlA a 1 treatment after *P. acerifolia* sensitization (rPlA a 1). A Dose-dependent elevation in Penh in response to Mch was found in sensitized mice. The treatment of rPlA a 1 lowered significantly the bronchial responsiveness compared to asthma group. Error bars represent standard error of mean (n=10 samples). B. The number and classification of inflammatory cells in BALF. The total number and all kinds of inflammatory cells were significantly increased in the sensitized mice compared to control. The total number, eosinophil and macrophage were significantly decreased after the treatment of rPlA a 1. EOS: eosinophil; N: neutrophil; L: lymphocyte; M: macrophage. Error bars represent standard error of mean (n=5 samples). Symbol (#) indicates significance between asthma and control group (P<0.05); Symbol (*) indicates significant differences between rPlA a 1 and asthma group (P<0.05); Symbol (\$) indicates significant differences between rPlA a 1 and control group (P<0.05).

influencing the composition of inflammatory cells in BALF. As shown in **Figure 4B**, the total number and all kinds of inflammatory cells were significantly increased in asthma group compared to control (P<0.05). The total number, eosinophil and macrophage were significantly decreased after the treatment of rPlA a 1 (P<0.05). However, the decline of neutrophil and lymphocyte in BALF were not notable.

Repetitive injection of rPlA a 1 benefits the production of sIgG

To investigate the mechanism that repetitive injection of rPlA a 1 decreased the airway inflammation and hyperresponsiveness in *P. acerifolia* sensitized experimental mice model, we measured the amount of sIgE and sIgG in the blood. Serum samples from the mice were obtained on Day 64 and levels of sIgE and sIgG were measured by using the antibody specific ELISA. Levels of sIgE in asthma group were significantly higher than that in the control group (**Figure 5A**, P<0.05). Treatment with rPlA a 1 had no significant effect on sIgE levels. On the other hand, the levels of sIgG in asthma group

were not significantly elevated compared to that in the control group. Nevertheless, sIgG in rPlA a 1 group was significantly elevated compared to that in the asthma group (**Figure 5B**, P<0.05).

Repetitive injection of rPlA a 1 increases IL-10 and decreases IL-13, IL-17

Given the reduction of inflammation by repetitive injection of rPlA a 1, we next looked at the levels of anti-inflammatory cytokine (IL-10) and pro-inflammatory cytokines (IL-13 and IL-17) in the spleen. Protein levels of IL-10 in spleen tissue homogenate from rPlA a 1 group were significantly elevated compared to asthma group (**Figure 5C**, P<0.05), but the change between asthma and control group was not significantly. Conversely, the levels of pro-inflammatory cytokines, IL-13 and IL-17 in asthma group were enhanced significantly compared to control group, but the increase was significantly inhibited in rPlA a 1 group (**Figure 5D** and **5E**, P<0.05). This further demonstrates repetitive administration of rPlA a 1 has the role of anti-inflammation.

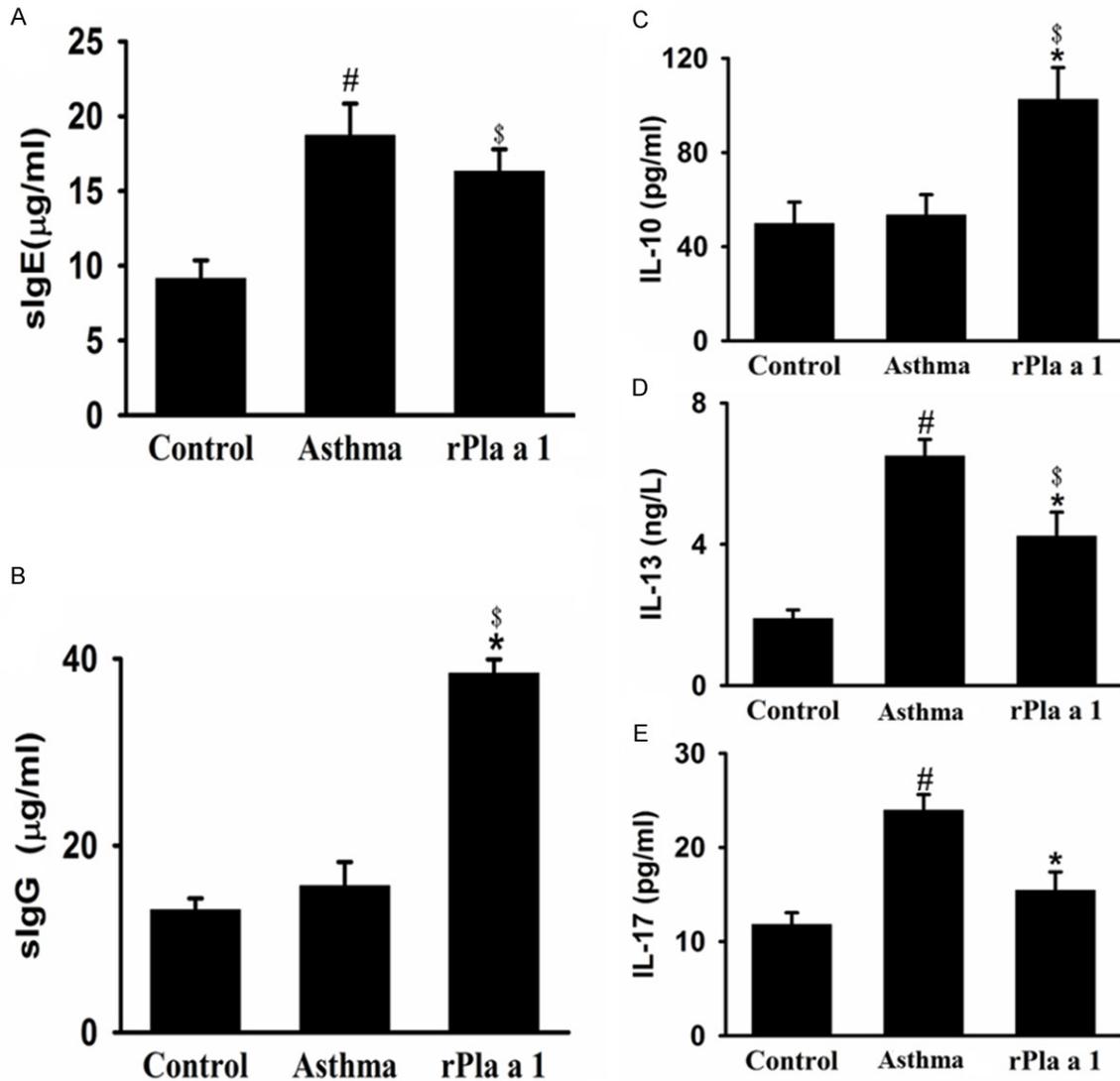


Figure 5. (A) sIgE levels in the serum from asthma group were elevated significantly compared to control group, but no significant effect of rPla a 1 injection on sIgE. (B) Repetitive injection of rPla a 1 significantly increased sIgG levels in the serum, but this effect was not significant between asthma and control group. (C) Intraperitoneal administration of rPla a 1 significantly increased IL-10 levels in the spleen tissue homogenate, but no significant difference between asthma and control group was found. (D) Intraperitoneal administration of rPla a 1 significantly decreased IL-13 and IL-17 (E) in the spleen tissue homogenate, which were significant elevated in asthma group compared to control. Error bars represent standard error of mean (n=10 samples). Symbol (#) indicates significance between asthma and control group (P<0.05); Symbol (*) indicates significant differences between (rPla a 1) and asthma group (P<0.05); Symbol (\$) indicates significant differences between (rPla a 1) and control group (P<0.05).

Discussion

Allergies related to *P. acerifolia* have become a significant clinical problem in recent years because the population in many counties showed positive SPT test to pollen and positive rate was up to 56% [2, 17, 18]. Specific immunotherapy (SIT) has become the first choice of treatment by injecting the elevated amounts of

allergen extracts so as to induce hyporesponsiveness to respective allergens [19]. One important drawback is the risk of anaphylactic-reactions. Therefore, recombinant allergen has been recommended to reduce side effects and enhance treatment efficacy [20]. Although the relative quantity of *P. acerifolia* component (Pla a 1) is very low (<0.5%), it is a major allergen responsible for allergic responses to *P. acerifo-*

lia [21]. It is therefore essential that using rPlA a 1 improves and standardizes future diagnosis and therapy related to this allergy. In our study, repetitive administration of rPlA a 1 attenuated the airway inflammation, bronchial hyperresponsiveness and mucus production caused by *P. acerifolia* sensitization. Further study showed repetitive administration of rPlA a 1 may promote the expression of IL-10 and FoxP3 in the lungs, increase the levels of sIgG, and inhibit the rise of IL-13 and IL-17 in the spleen, which are possible mechanisms to produce the immunological tolerance.

The chronic airway inflammation and hyperresponsiveness are two important features of asthma. We simulated the two notable characteristics through sensitizing and challenging the mice with crude *P. acerifolia* extract. Afterwards, we proved repetitive injection of rPlA a 1 reduced the airway inflammation and hyperresponsiveness in *P. acerifolia* sensitized experimental mice model. The data showed intraperitoneal application of rPlA a 1 was an effective and helpful method for allergic asthma model. However, asthma animal models are not same as human asthma. For example, the airway inflammations produced in one month are different from the chronic process in human asthma. So its safety and efficiency need be studied further.

Now specific IgG may work by blocking antibodies and competing with IgE for allergen binding, thus impeding IgE-dependent activation of basophils and mast cells. Therefore, IgG is considered to act as a protective antibody in response to specific immunotherapy (SIT). Our study showed sIgG was elevated notably, but no significant decline of sIgE was found in rPlA a 1 group. It is possible that the improvement of airway inflammation in the experimental mice model relies on the production of sIgG, not on the reduction of sIgE.

On the other hand, IL-13 and IL-17 are cytokines to mount AHR and goblet cell metaplasia, and cause direct contraction of airway smooth muscle cells in the absence of neutrophilic inflammation in mice and humans [19, 22, 23]. Our study showed IL-13 and IL-17 levels in rPlA a 1 group were significantly lowered compared to asthma group, which were also possibly responsible for the improvement of airway inflamma-

tion and hyperresponsiveness in experimental mice after the treatment of rPlA a 1.

For many years, it is assumed that inflammatory responses in asthma develop due to a deficiency in natural or induced regulatory T cells. Adoptive-transfer studies of mice have proven that Treg cells can inhibit asthmatic features through IL-10 [4]. As a master regulator for Treg development and function, the fork head/winged helix transcription factor FoxP3 is currently considered as the most specific Treg marker [22, 23]. It has been shown in both animals and humans that development of immune abnormalities such as severe allergic inflammation occur in the absence of FoxP3 [24]. Our results demonstrated the treatment elevated significantly the expression of IL-10 and FoxP3 mRNA in the lungs, which was consistent with IL-10 in spleen tissue homogenate in mice. Therefore, repetitive administration of rPlA a 1 may induce the development of Treg cells from the lungs and peripheral blood in the experimental mice, which is one of possible mechanisms to improve the airway inflammation and hyperresponsiveness.

In summary, our study presented that repetitive injection of rPlA a 1 attenuates the airway inflammation and mucus production and reduces the airway hyperresponsiveness. The improvements are possibly depended on the production of sIgG, the decline of IL-13 and IL-17. Each of these factors may serve as an impetus for further research on the use of recombinant protein in management of human allergic asthma.

Acknowledgements

This study was supported by funds from the Health Department of Shaanxi Provincial Government (2012D76).

Disclosure of conflict of interest

None.

Authors' contribution

Conception and design: WSY, SXZ and LMX. Provision of study materials or patients: WSY, WGZ, WYY and XXM. Data analysis and interpretation: WSY. Manuscript writing: All authors. Final approval of manuscript: All authors.

rPla a 1, airway inflammation and hyperresponsiveness

Address correspondence to: Dr. Manxiang Li, Department of Respiratory Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, No.277, West Yanta Road, Xi'an 710061, Shaanxi, PR China. Tel: 86-15129312682; E-mail: Manxiangli02@126.com

References

- [1] Wuthrich B, Schindler C, Leuenberger P and Ackermann-Liebrich U. Prevalence of atopy and pollinosis in the adult population of Switzerland (SAPALDIA study). Swiss study on air pollution and lung diseases in adults. *Int Arch Allergy Immunol* 1995; 106: 149-156.
- [2] Subiza J, Cabrera M, Valdivieso R, Subiza JL, Jerez M, Jimenez JA, Narganes MJ and Subiza E. Seasonal asthma caused by airborne *Platanus* pollen. *Clin Exp Allergy* 1994; 24: 1123-1129.
- [3] Alcazar P, Carinanos P, De Castro C, Guerra F, Moreno C, Dominguez-Vilches E and Galan C. Airborne plane-tree (*Platanus hispanica*) pollen distribution in the city of Cordoba, Southwestern Spain, and possible implications on pollen allergy. *J Investig Allergol Clin Immunol* 2004; 14: 238-243.
- [4] Liu Y, Sun X, Wang G, Tao A, Wu Y, Li M, Shi H and Xie M. Expression, purification and identification of Pla a1 in a codon-optimized *Platanus* pollen allergen. *Mol Med Rep* 2015; 12: 2197-202.
- [5] Enrique E, Cistero-Bahima A, Bartolome B, Alonso R, San Miguel-Moncin MM, Bartra J and Martinez A. *Platanus acerifolia* pollinosis and food allergy. *Allergy* 2002; 57: 351-356.
- [6] Arilla MC, Ibarrola I, Mir A, Monteseirin J, Conde J, Martinez A and Asturias JA. Development of a sandwich-type ELISA for measuring Pla a 1, the major allergen of *Platanus acerifolia* pollen. *Int Arch Allergy Immunol* 2005; 138: 127-133.
- [7] Ibarrola I, Arilla MC, Martinez A and Asturias JA. Identification of a polygalacturonase as a major allergen (Pla a 2) from *Platanus acerifolia* pollen. *J Allergy Clin Immunol* 2004; 113: 1185-1191.
- [8] Lauer I, Miguel-Moncin MS, Abel T, Foetisch K, Hartz C, Fortunato D, Cistero-Bahima A, Vieths S and Scheurer S. Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid-transfer protein, Pru p 3. *Clin Exp Allergy* 2007; 37: 261-269.
- [9] Lowrey JL, Savage ND, Palliser D, Corsin-Jimenez M, Forsyth LM, Hall G, Lindey S, Stewart GA, Tan KA, Hoyne GF and Lamb JR. Induction of tolerance via the respiratory mucosa. *Int Arch Allergy Immunol* 1998; 116: 93-102.
- [10] Macaubas C, DeKruyff RH and Umetsu DT. Respiratory tolerance in the protection against asthma. *Curr Drug Targets Inflamm Allergy* 2003; 2: 175-186.
- [11] Garcia-Ortega P, Martinez J, Martinez A, Palacios R, Belmonte J and Richart C. *Mercurialis annua* pollen: a new source of allergic sensitization and respiratory disease. *J Allergy Clin Immunol* 1992; 89: 987-993.
- [12] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-254.
- [13] Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG and Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997; 156: 766-775.
- [14] Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J and Koki AT. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* 2000; 89: 2637-2645.
- [15] Wiedermann U, Jahn-Schmid B, Fritsch R, Bauer L, Renz H, Kraft D and Ebner C. Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: cholera toxin induces a Th1-like response to Bet v 1, the major birch pollen allergen. *Clin Exp Immunol* 1998; 111: 144-151.
- [16] Jahn-Schmid B, Wiedermann U, Bohle B, Repa A, Kraft D and Ebner C. Oligodeoxynucleotides containing CpG motifs modulate the allergic TH2 response of BALB/c mice to Bet v 1, the major birch pollen allergen. *J Allergy Clin Immunol* 1999; 104: 1015-1023.
- [17] Bousquet J, Cour P, Guerin B and Michel FB. Allergy in the Mediterranean area. I. Pollen counts and pollinosis of Montpellier. *Clin Allergy* 1984; 14: 249-258.
- [18] Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM and Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; 282: 2261-2263.
- [19] Norman PS. Immunotherapy: 1999-2004. *J Allergy Clin Immunol* 2004; 113: 1013-1023; quiz 1024.
- [20] Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D and Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999; 29: 896-904.
- [21] Asturias JA, Ibarrola I, Bartolome B, Ojeda I, Malet A and Martinez A. Purification and characterization of Pla a 1, a major allergen from

rPl_a 1, airway inflammation and hyperresponsiveness

- Platanus acerifolia pollen. *Allergy* 2002; 57: 221-227.
- [22] Fontenot JD, Gavin MA and Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; 4: 330-336.
- [23] Khattri R, Cox T, Yasayko SA and Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 2003; 4: 337-342.
- [24] Chatila TA. Role of regulatory T cells in human diseases. *J Allergy Clin Immunol* 2005; 116: 949-959; quiz 960.