



Protostemonine attenuates alternatively activated macrophage and DRA-induced asthmatic inflammation

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ABSTRACT

Asthma is one of the most common pulmonary diseases that threatens human life because of lack of effective medicines. Protostemonine (PSN), an active alkaloid extracted from the roots of *Stemona sessilifolia*, has anti-inflammatory effects on acute lung injury and acute liver failure. However, it has not been defined whether PSN alleviates asthmatic inflammation. Here, we reported that PSN inhibits pulmonary eosinophil infiltration, goblet cell hyperplasia, mucus secretion, IgE and Th2 cytokine (IL-4, IL-5, IL-13 and IL-33) production by using DRA (dust mites, ragweed and aspergillus)-induced murine asthma model. Moreover, PSN also attenuated the expression of Arginase-1 (Arg-1), Ym-1 and Fizz-1, markers of AAM (alternatively activated macrophage) polarization, in lung tissues. In addition, PSN attenuated IL-4-induced expression of Arg-1, Ym-1 and Fizz-1 in bone marrow derived macrophages (BMDMs). Treatment with PSN decreased IL-4-induced STAT6 phosphorylation, KLF4 and IRF4 expression in BMDMs. Collectively, our results indicated that PSN ameliorates AAM polarization and asthmatic inflammation and might be a potential agent for treating asthma.

1. Introduction

Asthma is a common heterogeneous pulmonary disease with a complex pathophysiology, that resulting in recurrent, reversible bronchial obstruction along with cough, wheezing and shortness of breath. It is the 14th most important chronic disease in the world and affects up to 300 million people from infancy to senior persons [1–3] and leads to about 0.3 million people die every year [4–6]. The economic costs of asthma are very high because of limited treatment for targeting the asthmatic symptoms in the clinic. Therefore, it is importance of developing novel medicines in asthma treatment.

Allergic asthma is inappropriate Th2-mediated responses to inhaled allergens and affects most of asthmatic patients. In response to allergens, injured endothelial and epithelial cells release alarmins, such as the cytokines interleukin (IL)-25 and IL-33, which results in abnormal

immunoglobulin E (IgE) production, mucus hypersecretion and airway hyperresponsiveness (AHR) [7,8] and finally leads to a progressive lack of lung function. Th2 cytokines (IL-4, IL-5 and IL-13) have been shown to play a crucial role in the pathogenesis of allergic asthma [9–12]. In addition, eosinophils, mast cells, macrophages and neutrophils are also involved in the formation of asthmatic inflammation [13–16].

Macrophage is a type of tissue-resident innate immune cells, that constitutes the most abundant immune response cells in the lung (approximately 70% of the immune response cells) [17] and plays a critical role in the allergic airway inflammation [18,19]. Th2 cytokines such as IL-4 and IL-13 lead to alternative activated macrophages (AAMs) polarization, which, in turn, can promote asthmatic inflammation [6,20–22]. Thus, restricting AAM polarization is a potential strategy for improving asthma control in clinic.

Chinese herbal medicine has been widely used treating diseases

Abbreviations: AAM, alternatively activated macrophage; AHR, airway hyperresponsiveness; Arg-1, arginase-1; BALF, bronchoalveolar; BMDM, bone marrow derived macrophage; COPD, chronic obstructive pulmonary disease; DRA, dust mites, ragweed and aspergillus; Eos, eosinophil; Fizz-1, found in inflammatory zone 1; H&E, hematoxylin&eosin; IgE, immunoglobulin E; IL-4, interleukin-4; iNOS, inducible nitric oxide synthase; IRF4, interferon regulatory factor-4; KLF4, Kruppel-like factor 4; Mac, macrophage; TNF- α , tumor necrosis factor; PAS, Periodic acid-Schiff; STAT6, signal transducer and activator of transcription 6; Ym-1, chitinase3-like 3

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[23–25]. *Stemona sessilifolia*, known as “Baibu” in Chinese, has been applied in traditional Chinese medicine for centuries to control respiratory disorders, including pulmonary tuberculosis and bronchitis, and it has also been used as insecticide to prevent agricultural pests, domestic insects, and parasites [26–28]. Protostemonine (PSN), an active alkaloid of *stemona sessilifolia*, has been found to protect against LPS/galactosamine-induced liver injury in mice [29]. In our previous study, we found that PSN has anti-inflammatory effects on LPS-induced acute lung injury in mice [30]. However, it has not been clarified whether PSN has benefits in asthmatic inflammation.

In this study, treatment of PSN attenuated triple allergens (dust mite, ragweed, and aspergillus, DRA)-induced IgE generation, IL-4, IL-5, IL-13 and IL-33 production, eosinophils infiltration and mucus hypersecretion. Moreover, PSN alleviated DRA-induced asthmatic inflammation may through inhibition of AAM polarization with reduction of STAT6 phosphorylation, and attenuation of KLF4 and IRF4 expression. Hence, our results indicated that PSN may be a potential therapeutic agent for the treatment of asthma.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (6–8 weeks old, 20 ± 3 g, Specific pathogen Free) were purchased from Slac Laboratory Animal Corporation (Shanghai, China), 5 mice were kept in one cage, kept in a climate-controlled room (25 °C, 55% humidity and 12-h light/darkness cycle), and fed with a standard laboratory diet and acclimated for at least 2 weeks before use. This research adhered to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1996). All experimental protocols described in this study were approved by Animal Care and Use Committee of Shanghai Jiao Tong University.

2.2. Allergens, compound and reagents

Triple allergens (DRA) include extracts of dust mite (Greer Laboratories, Lenoir, NC, USA), ragweed (Greer Laboratories, Lenoir, NC, USA), and aspergillus fumigates (Greer Laboratories, Lenoir, NC, USA). Aluminum (Inject Alum; Thermo Fisher Scientific, Waltham, MA, USA) was used as adjuvant. Quantities of allergens for intraperitoneal (200 µl) per mouse were used as follows: dust mite (5 µg), ragweed (50 µg), and aspergillus fumigates (5 µg). Quantities of allergens for intranasal injection (50 µl) were used as follows: dust mite (8.3 µg), ragweed (83.4 µg), and aspergillus fumigates (8.3 µg) for the *in vivo* asthma model, that was referred to our previous study [20]. PSN (Protostemonine, (5Z)-4-Methoxy-3-methyl-5furan-2(5H)-one, $C_{23}H_{31}NO_6$; MW, 417.50; purity > 98%) was purchased from Baoji Herbest Bio-Tech Co., Ltd. (Baoji, China). Bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime Biotechnology (Shanghai, China). IgE ELISA kits were obtained from eBioscience (San Diego, CA, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The primers used in this study were synthesized by HuaGene Biotech Co., Ltd (Shanghai, China).

2.3. Murine asthma model

Mice were randomly divided into three groups: control group (PBS + vehicle), DRA group (DRA + vehicle) and PSN treated group (DRA + PSN). Each group contained five mice. PSN was dissolved in a solvent (polyoxyethylene hydrogenated castor oil: ethanol: 0.9% NaCl = 1:1:8). On day 0 (D0), mice were sensitized by an intraperitoneal (i.p.) injection of 200 µl alum-precipitated antigens containing triple allergen mix (60 µg of DRA) in PBS vehicle. Five days later, mice were given a second intraperitoneal booster of the same dose of allergens in alum adjuvant (D5). On days 12–14, mice were exposure

to DRA (100 µg) combination (50 µl) or PBS (50 µl) as control by intranasal injection (i.n.) after anesthesia with pentobarbital sodium. PSN was given an i.p. injection on days 12–14, 1 h prior to DRA administration. Then mice were sacrificed by humane CO₂ suffocation on Day 15, and peripheral blood, bronchoalveolar lavage (BAL) fluid and lung tissues were collected for further analysis [6,20].

2.4. Collection of blood and bronchoalveolar lavage fluid (BALF)

Mice were anesthetized on the D15, blood was collected and centrifuged at 4 °C (3000 rpm) for 15 min, and the serum was stored at –80 °C for measurement of IgE. The lungs were lavaged three times with 0.6 mL PBS via a tracheal catheter and BAL fluid was centrifuged at 4 °C. The cell-free supernatant was harvested for total protein analysis using the BCA protein assay kit (Beyotime, Shanghai, China). Remaining cell pellets were re-suspended in PBS for total cell counts, the rate of eosinophils and macrophages using hemacytometer and flow cytometry assay.

2.4.1. Analysis of IgE

Plasma collected from mice as described above were used for detecting the level of total IgE with the enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

2.4.2. Flow cytometry assay

Cells collected from BAL fluid were incubated with Fc blocking anti-mouse CD16/32 antibody (BD Bioscience, San Jose, CA, USA) followed by PE-conjugated anti-Siglec F (BD Bioscience, San Jose, CA, USA) and APC-conjugated anti-CD11c (Biolegend, San Diego, CA, USA) antibodies. Cells were analyzed on flow cytometry (LSRFortessa TM X-20, BD Bioscience, San Jose, CA, USA) and data were analyzed with FlowJo software.

2.5. Histopathology

Lung tissues (left lobe) were fixed by 4% paraformaldehyde, embedded in paraffin and cut into 5-µm thick sections in a microtome (RM2235, Leica Biosystems, Wetzlar, Germany). Sections were stained with hematoxylin and eosin (H&E) for analysis of inflammatory cell infiltration, and Periodic acid-Schiff (PAS) for analysis of inflammatory goblet cells, then images were captured by a microscope (RX51, Olympus Optical Co. Ltd., Tokyo, Japan).

2.6. RNA isolation, reverse transcription and quantitative PCR

Frozen lungs (one of right upper lobe) were homogenized and total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was prepared by ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and amplified by Real-time PCR on StepOne Plus (Thermo Fisher Scientific, Waltham, MA, USA) with primer sets for Muc5ac (forward 5'-CTCCGCTTAGTCAATAACCAACC-3'; reverse 5'-GGAACCTCGTTGGATTTTGGACTG-3'), IL-4 (forward 5'-GGTCTCAACCCCAGCTAGT-3'; reverse 5'-GCCGATGATCTCTCTCAAGTGAT-3'), IL-5 (forward 5'-TCAGGGGCTAGACATACTGAAG-3'; reverse 5'-CCAAGGAACCTCTGCAGGTAAT-3'), IL-13 (forward 5'-CTACAGCTCCCTGGTCTCT-3'; reverse 5'-TTGCTCAGCTCCTCAATAAG-3'), IL-33 (forward 5'-ATTCTTGGCTTACGATGTTGTG-3'; reverse 5'-TCTGAAGTCGTGAGTGTGATT-3'), Arg-1 (forward 5'-CAATGAAGAGCTGGCTGGTGT-3'; reverse 5'-GTGTGAGCATCCACCCAAATG-3'), Ym-1 (forward, 5'-GGGCA TACCTTTATCCTGAG-3'; reverse, 5'-CCACTGAAGTCATCCATGTC-3'), Fizz-1 (forward 5'-GAGACCATAGAGATTATCGTGGA-3'; reverse 5'-CACACCCAGTAGCAGTCATC-3') and GAPDH (forward 5'-TGCGACTTCAACAGCAACTC-3'; reverse 5'-CTTGCTCAGTGCTCTTGCTG-3').

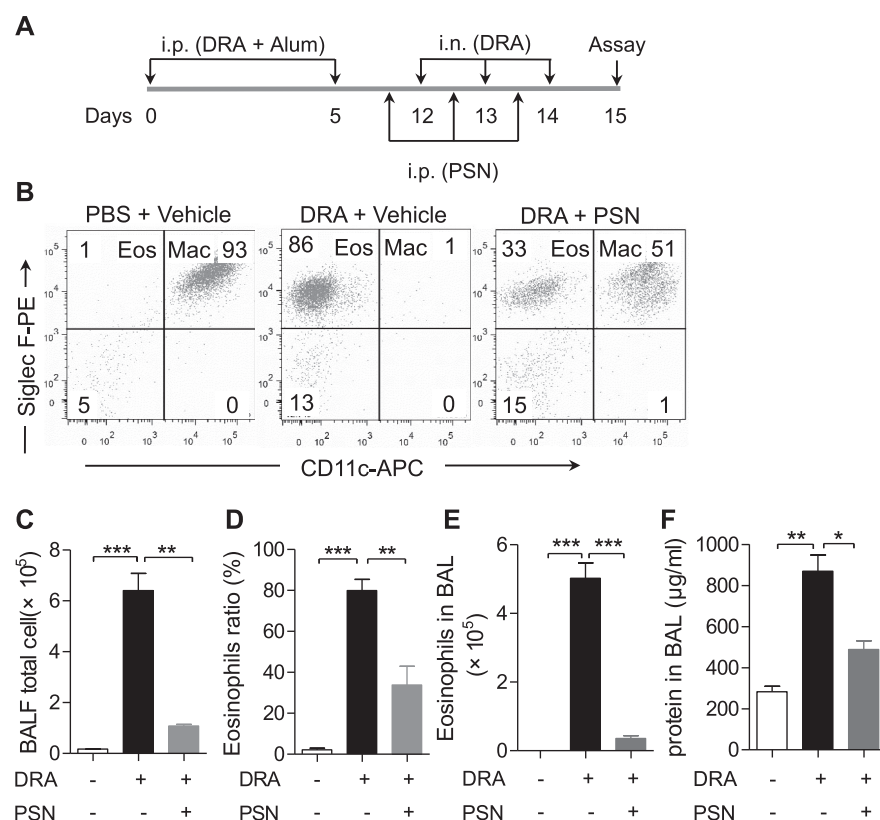


Fig. 1. PSN alleviates DRA-induced asthmatic inflammation. (A) The schematic timeline of DRA-induced asthma model. Mice were sensitized (i.p.) with DRA on Days 0 and 5, and challenged (i.n.) with DRA on Days 12, 13 and 14. On day 15, mice were sacrificed and analyzed. To determine whether PSN has therapeutic effect on asthma, we performed intraperitoneal injection with PSN (20 mg/kg) 1 h prior to DRA challenge on days 12, 13 and 14. (B) BAL fluid were collected and centrifuged, cell pellets were suspended and stained for identifying macrophages (Mac, Siglec F⁺CD11c⁺) and eosinophils (Eos, Siglec F⁺CD11c⁺) by flow cytometry. Total infiltrated cells (C), eosinophil ratio (D) and eosinophils (E) in BAL fluid were counted based on total BAL cell number and percentage of eosinophil. (F) BAL fluid were centrifuged, and total protein concentrations in supernatant were detected. Values represent means $\pm$ SEM, $n = 5$ mice each group, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ between the indicated groups.

2.7. Preparation of BMDMs and treatment

Femoral and tibia bone marrow was isolated from male C57BL/6 mice, and cultured in DMEM media supplemented with antibiotics (100 units/ml of penicillin and 100 $\mu\text{g/ml}$ streptomycin), 10% FBS and 10% culture supernatant of L929 cell for 7 days (37 $^{\circ}\text{C}$, 5% CO_2) as our previous study [30]. Cells were treated with indicated concentrations of PSN (dissolved in DMSO for cell treatment) according to experiment requirements, 1% DMSO was used as the solvent control.

2.8. Cytotoxicity assay

MTT assay was performed to detect the cytotoxicity of PSN on BMDMs. Briefly, BMDMs were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with PSN at different concentrations (0, 1, 10, 30 and 100 μM) or vehicle (1% DMSO) for 24 h. After 24 h, 20 μl MTT (5 mg/ml) were added into each well for incubating for another 4 h at 37 $^{\circ}\text{C}$, after which the medium was removed before 100 μl of DMSO was added and mixed well. Finally, the OD value was measured by a microplate reader (Flex Station 3; Molecular devices, California, USA) at 490 nm. Inhibition ratio (%) was calculated by the formula: Inhibition ratio (%) = $(1 - \text{absorbance of treated group} / \text{absorbance of control group}) \times 100\%$. Control groups were treated with 1% DMSO.

2.9. Western blotting analysis

The lung tissues were homogenized and lysed with RIPA Lysis Buffer (Genstar BioSolutions, Beijing, China) containing protease inhibitor cocktail (MedChem Express, Monmouth Junction, NJ, USA). Proteins were subjected to immunoblot using antibodies against Muc5ac (Abnova, Taipei, Taiwan) and β -actin (Cell Signaling Technology, Danvers, MA, USA). BMDMs were plated on 6-well plates (1.0×10^6 cells per well) and incubated overnight (37 $^{\circ}\text{C}$, 5% CO_2). Then cells were pretreated with different dose of PSN (0, 3, 10, 30 and 100 μM) for 0.5 h before

challenged with 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ, USA) for indicated times. Cells were then collected and lysed in loading buffer (175 mM Tris-HCl, 100 mM DTT, 4.0% SDS, 7.5% Glycerinum and 0.2% Bromophenol blue in ddH₂O). The cell lysate was analyzed by immunoblot using antibodies against Arg-1 (Santa Cruz, CA, USA), Ym-1 (Stemcell Technologies, Vancouver, BC, Canada), Fizz-1 (Abcam, Cambridge, MA, UK), p-STAT6, STAT6, IRF4 and KLF4 (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat IgG antibodies were supplied by Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Quantification of western blots was performed with ImageJ software (NIH).

2.10. Statistical analysis

Data are presented as means $\pm$ SEM obtained from at least three independent tests. The one-way ANOVA and the post test of Dunnett's were performed using Prism 5 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. PSN inhibits the permeability of the lung tissue and the accumulation of inflammatory cells

To determine whether PSN has therapeutic effects on asthma, we used DRA-induced murine asthma model and measured asthmatic inflammation. On day 15, after the last challenge with DRA for 24 h, the lungs were lavaged and the bronchoalveolar lavage fluid (BALF) was collected (Fig. 1A). After centrifugation, cell pellets were suspended in PBS, based on flow cytometry assay, we found that DRA challenge led to eosinophil (Siglec-F⁺CD11c⁺) infiltration of $79.94\% \pm 5.435\%$ in BALF compared to control group (PBS + vehicle) that was composed of $2.197\% \pm 0.7044\%$ of eosinophil accumulation and $85.80\% \pm 4.839\%$ of alveolar macrophages (Siglec-F⁺CD11c⁺)

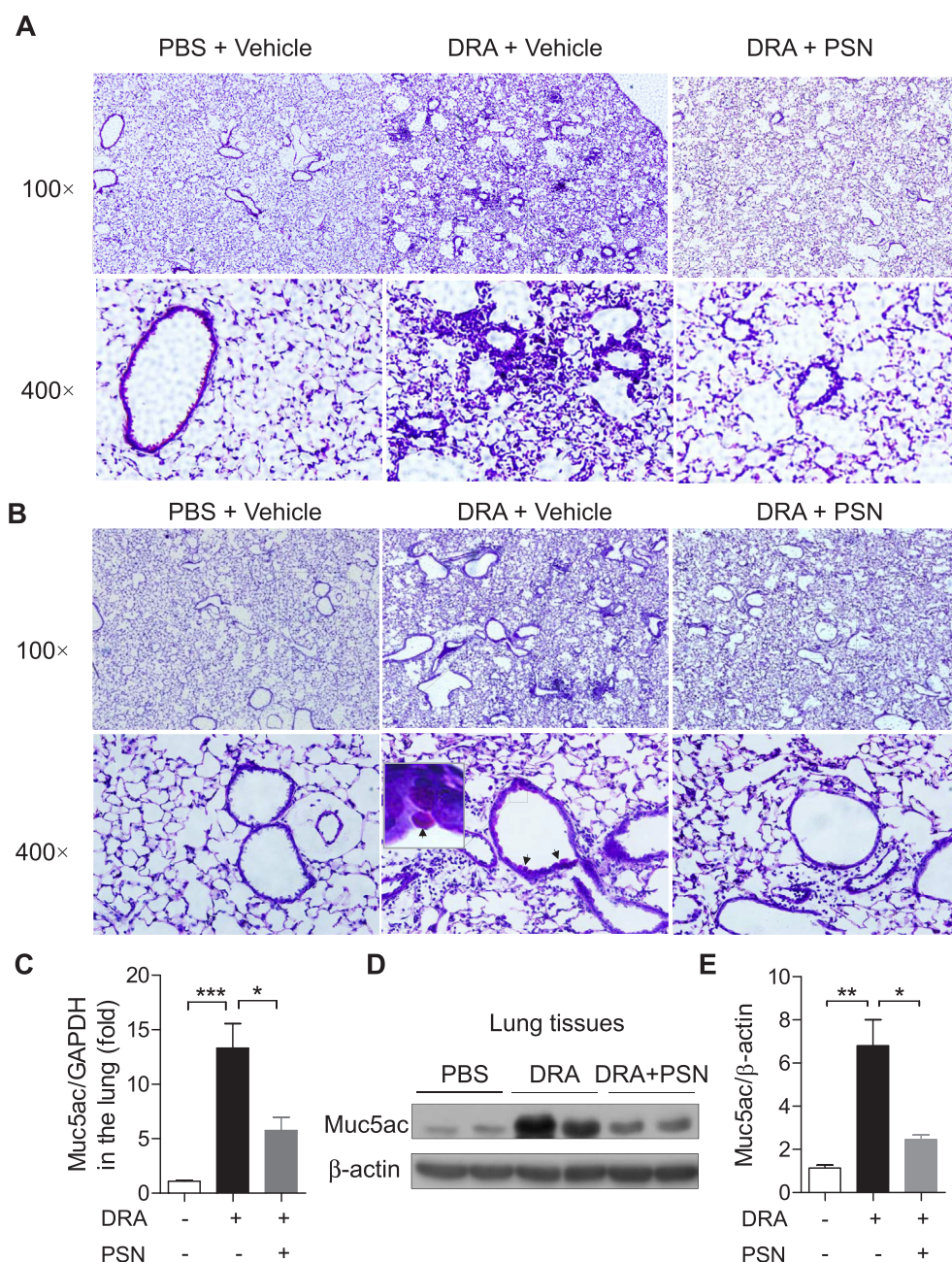


Fig. 2. PSN attenuates the pathological changes in DRA-induced asthma. Lung sections were excised, fixed and processed for histological analysis. (A) H&E staining was performed to evaluate DRA-induced asthmatic inflammation in response to vehicle and PSN treatment. (B) PAS staining was performed to assess goblet cell hyperplasia in DRA-induced asthmatic inflammation. Black arrowheads pointed out PAS-positive goblet cells. (C) The total RNAs were isolated from lung homogenates and the mRNA expression levels for Muc5ac was quantified using real-time PCR. (D) Muc5ac expression was measured by Western blot in the lungs. (E) The ratio of Muc5ac/β-actin was measured by densitometry. Values were presented as means ± SEM, $n \geq 4$. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

(Fig. 1B). After treatment with PSN, the ratio of eosinophil accumulation was reduced to $33.83\% \pm 9.099\%$ (Fig. 1B). In addition, total inflammatory cell infiltration in BALF was markedly increased in DRA-challenged mice. Treatment with PSN reduced inflammatory cell infiltration (Fig. 1C) and eosinophil accumulation (Fig. 1D and E). Furthermore, the total protein concentrations in BALF were significantly raised in DRA-challenged mice and treatment of PSN decreased protein concentrations, indicating that treatment of PSN alleviates the pulmonary permeability (Fig. 1F). Therefore, these results suggested that PSN alleviates DRA-induced asthmatic inflammation and reduces the pulmonary injury.

3.2. PSN ameliorates pathologic injury in DRA-induced asthma

Lung histological changes were used to assess the protective effects of PSN on DRA-induced asthma. As shown in Fig. 2, the lung of DRA-challenged mice revealed allergic airway inflammation, including inflammatory cell infiltration and airway epithelial thickening. Treatment

of PSN attenuated DRA-induced pathological injury (Fig. 2A). PAS staining was used to evaluate airway hypersecretion of mucus and goblet cell hyperplasia (Fig. 2B). DRA-challenged mice showed the increase of goblet cell hyperplasia in the bronchioles. Treatment of PSN markedly ameliorated DRA-induced mucus hypersecretion and goblet cell hyperplasia (Fig. 2B). Furthermore, we detected the expression of the major airway mucin Muc5ac in the lung tissues as well (Fig. 2C–E). The secretion of Muc5ac was increased in DRA-challenged mice, however, the mRNA (Fig. 3A) and protein (Fig. 3B and C) level of Muc5ac was notably suppressed after treatment with PSN (20 mg/kg). These results demonstrated that PSN attenuates DRA-induced mucus hypersecretion and goblet cell hyperplasia.

3.3. PSN inhibits DRA-induced IgE generation and type 2 cytokine production

Since asthma has been considered as the Th2-mediated pulmonary disorders [1], in which the production of IgE, IL-4, IL-5, IL-13 and IL-33

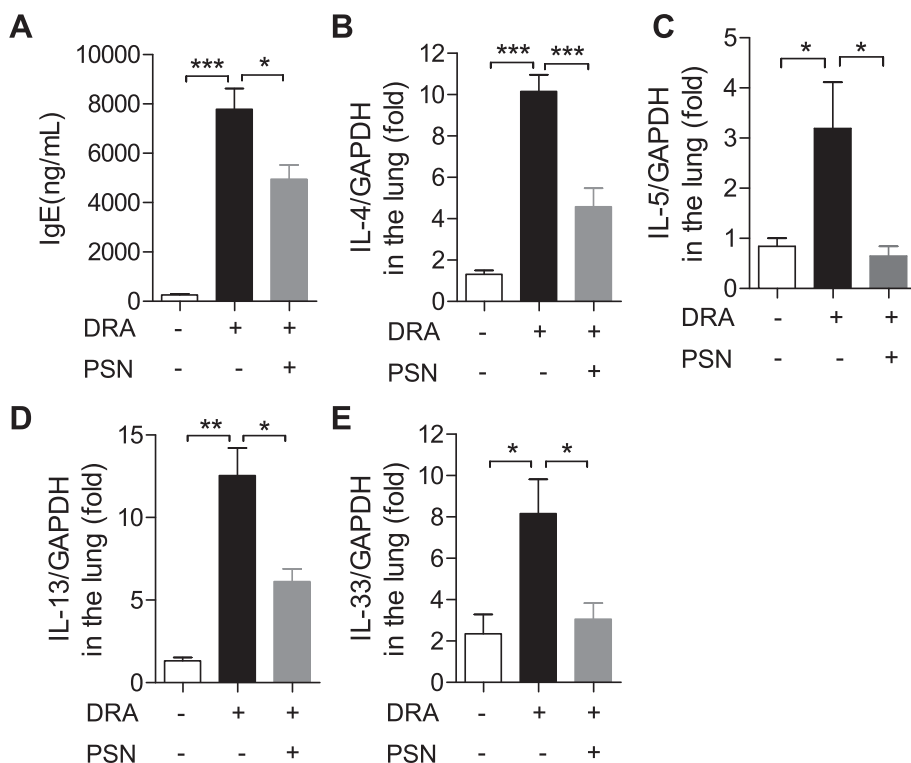


Fig. 3. PSN treatment inhibits IgE generation in serum and IL-4, IL-5, IL-13 and IL-33 production in lung tissues. (A) The concentration of IgE in serum was measured by ELISA. The mRNA levels of IL-4 (B), IL-5 (C), IL-13 (D) and IL-33 (E) were detected by real-time PCR. Values were presented as means $\pm$ SEM ($n = 5$ mice for each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared between the indicated two groups.

is increased, we measured total IgE in serum and mRNA level of IL-4, IL-5, IL-13 and IL-33 in lung tissues. As shown in Fig. 4, the production of serum IgE (Fig. 3A), IL-4 (Fig. 3B), IL-5 (Fig. 3C), IL-13 (Fig. 3D) and IL-33 (Fig. 3E) were markedly increased in DRA-challenged mice. However, the level of IgE, IL-4, IL-5, IL-13 and IL-33 was attenuated after administration of PSN (20 mg/kg). These results suggested that PSN alleviates asthmatic inflammation and decreases type 2 cytokine secretion.

3.4. PSN attenuates AAM polarization in asthmatic inflammation

Type 2 cytokine IL-4 can induce AAM polarization that plays a critical role in modulation of asthma [20,31–33]. Thus, we investigated whether PSN has effect on AAM polarization *in vivo*. Lung tissues were isolated and collected, then we detected the expression of AAM markers Arg-1 (Fig. 4A), Ym-1 (Fig. 4B) and Fizz-1 (Fig. 4C). In response to DRA, murine lung tissues displayed 70-fold of Arg-1, 6-fold of Ym-1 and 30-fold of Fizz1 production, compared to PBS treatment. PSN dramatically inhibited the expression of these genes in which Arg-1, Ym-1 and Fizz-1

were decreased to 10-fold, 2-fold and 12-fold respectively (Fig. 4). These data suggested that PSN attenuates DRA-induced AAM polarization *in vivo*.

3.5. PSN inhibits AAM polarization *in vitro*

As described above, PSN inhibited the expression of AAM markers in murine asthma model, then we investigated whether PSN inhibits AAM polarization *in vitro*. BMDMs were isolated from C57BL/6 mice, and the potential cytotoxicity of PSN were measured by MTT assay after incubating with PSN for 24 h. The results showed that PSN displayed no cellular toxicity against BMDMs (Fig. 5A). BMDMs were preincubated with PSN (0, 3, 10, 30 and 100 μ M) for 30 min, then stimulated with 10 ng/mL of IL-4 for 24 h, the expression of Arg-1, Ym-1 and Fizz-1 (Fig. 5B) were measured by western blot assay. PSN treatment attenuated IL-4-induced the expression of Arg-1 (Fig. 5C), Ym-1 (Fig. 5D) and Fizz-1 (Fig. 5E). These data indicated that PSN also alleviates IL-4-induced AAM polarization.

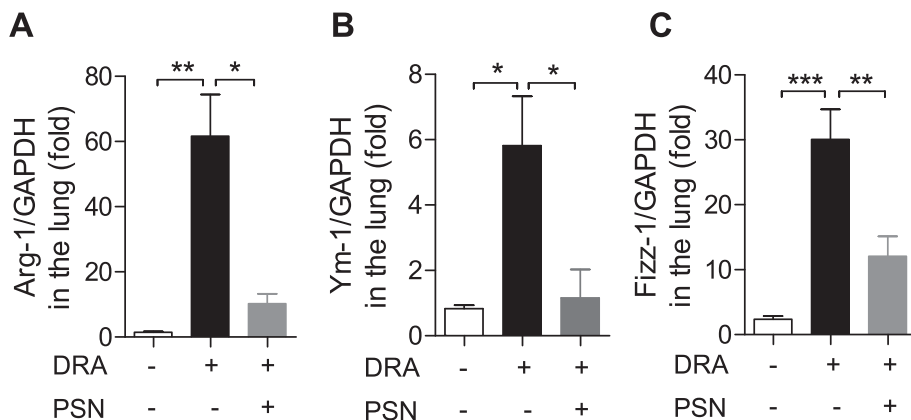


Fig. 4. PSN attenuates AAM polarization in DRA-induced allergic asthma. Lung tissues were collected 24 h after the last DRA challenge. The total RNAs were separated from lung homogenates, and supernatant was stored at -80°C . The mRNA expression levels for Arg-1 (A), Ym-1 (B) and Fizz-1 (C) were quantified using real-time PCR. Values were presented as means $\pm$ SEM, $n = 5$. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ between the indicated two groups.

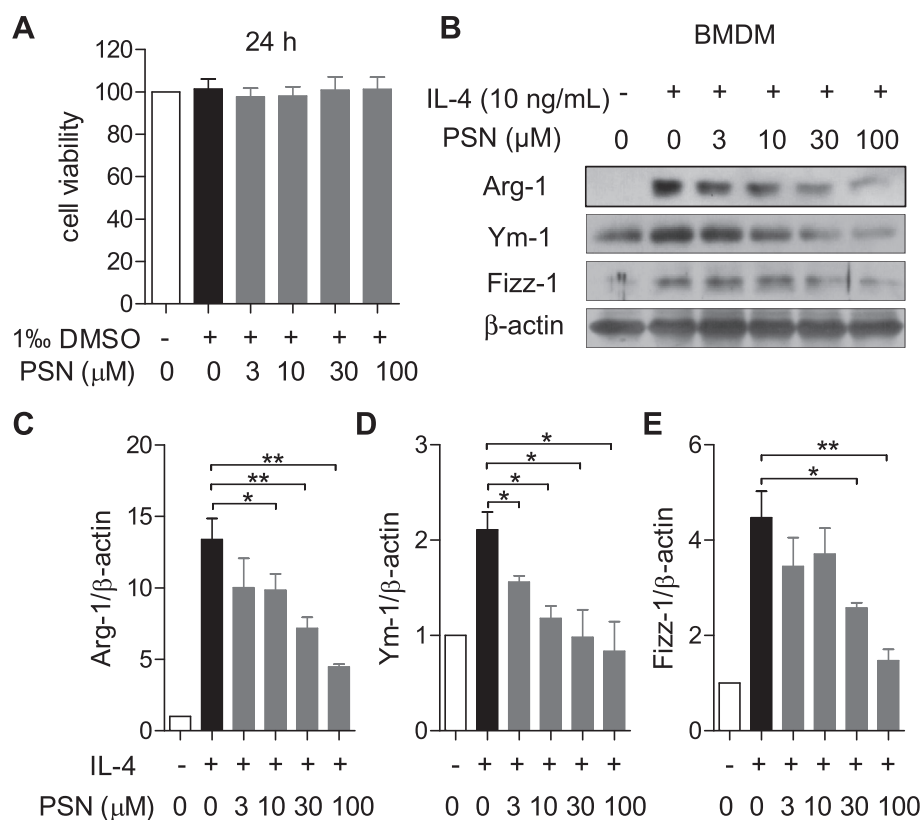


Fig. 5. PSN reduces IL-4-mediated AAM polarization *in vitro*. (A) BMDMs were treated with PSN at 0, 3, 10, 30 and 100 μM for 24 h. And cytotoxicity of PSN on BMDMs were detected by MTT analysis. (B) BMDMs from C57BL/6 mice were pre-incubated with PSN (0, 3, 10, 30 and 100 μM) for 30 min and stimulated with IL-4 (10 ng/ml) for 24 h. The protein expression of Arg-1, Ym-1, Fizz-1 and β-actin were identified by western blot. The ratios of Arg-1/β-actin (C), Ym-1/β-actin (D) and Fizz-1/β-actin (E) were quantified using ImageJ software. Statistical analysis was performed, $n = 3$, * $P < 0.05$, ** $P < 0.01$, compared with the group merely stimulated with IL-4.

3.6. PSN reduces the phosphorylation of STAT6 and the expression of KLF4 and IRF4 in response to IL-4 treatment

Transcription factors such as STAT6, Kruppel-like factor 4 (KLF4) and interferon regulatory factor (IRF)-4 play an essential role in AAM polarization [34–40]. To evaluate whether PSN modulates AAM polarization through regulating these transcription factors, we pre-incubated BMDMs with various doses (0, 3, 10, 30 and 100 μM) of PSN for 30 min, then stimulated them with 10 ng/ml of IL-4 for indicated times. As shown in Fig. 6, IL-4 challenge induced phosphor-STAT6 and the expression of KLF4 and IRF4. PSN significantly inhibited STAT6 activation (Fig. 6A and B). Moreover, the increased expression of KLF4 (Fig. 6C and D) and IRF4 (Fig. 6E and F) was remarkably reduced by PSN with dose-dependent manner. These results suggested that PSN attenuates AAM polarization by suppressing the activation of STAT6 and expression of KLF4 and IRF4.

4. Discussion

Asthma is one of the most common pulmonary diseases that threaten the life of adults and children in all ages [41]. Moreover, the prevalence of asthma has been remarkably increasing over the last three decades [7]. The cost of the disease is estimated to exceed \$ 18 billion annually in the USA [1]. In clinic, an inhaled corticosteroid and a short-or long-acting β₂-adrenergic agonist are the most effective medicine for controlling asthma. However, in some patients, refractory asthma resists to corticosteroid treatment [1,7]. Therefore, novel effective therapeutic agents are needed to be developed for curing asthma.

PSN is a natural compound isolated from *Stemona sessilifolia*, which has been used as a traditional Chinese medicine and Korean herbal formula [42]. As an alkaloid, PSN is considered to be one of the active components isolated from the roots of *S. sessilifolia*. PSN has been reported to attenuate inflammatory responses. Zhuo Cheng et al. demonstrated that PSN can inhibit the production of pro-inflammatory

cytokines (e.g. TNF-α, IL-1β and IL-6) and have anti-inflammatory effect in LPS/galactosamine-induced liver injury by upregulating HO-1 expression, reducing reactive oxygen species (ROS) production and enhancing malondialdehyde (MDA) levels [29]. Our previous study demonstrate that PSN protects against LPS-induced acute lung injury by inhibiting iNOS expression and suppressing MAPK and PI3K/AKT signaling pathway [29,30]. In consideration of the function of *S. sessilifolia* on respiratory disorders therapy, we wondered whether PSN has effects on a common respiratory disease, asthma. In the present study, we investigated the effect of PSN on DRA-induced asthmatic inflammation. Since IgE is involved in allergic inflammation and leads to asthma, targeting IgE and blocking its function may alleviate some diseases [43–46]. Furthermore, Th2 cytokines including IL-4, IL-5, IL-13 and IL-33 also play critical roles in allergic asthma, targeting these cytokines may exert antiasthmatic effects. It is worth mentioning that Nucala, a IL-5 monoclonal antibody, has been approved by FDA for the treatment of severe eosinophilic asthma. Our study proved that PSN inhibits the eosinophil accumulation and downregulates the production of total IgE, IL-4, IL-5, IL-13 and IL-33 in DRA-induced asthmatic inflammation. In addition, PSN reduced inflammatory cell infiltration and mucus hypersecretion, as well as increased pulmonary permeability. These findings indicated that PSN might be a potential anti-allergic agent for asthma treatment.

Innate immunity plays an important role in asthma and macrophages are critical innate immune cells in the lung tissues [47,48]. Accumulating studies demonstrate that increased AAM polarization and activation are observed in asthma [19,21,47], that suggesting macrophages play a critical role in allergic asthma. AAM polarization is correlated with recruitment of eosinophils to the lung tissue and peritoneal cavity, that contributing to allergic lung inflammation [49–52]. Furthermore, asthma patients have more AAMs in their lungs, suggesting a critical role for AAM polarization in human asthma [21,52–54]. Taken together, these results from allergic asthma models in mouse and human demonstrated that interference of AAM polarization may attenuate asthma. Therefore, alleviation of AAM

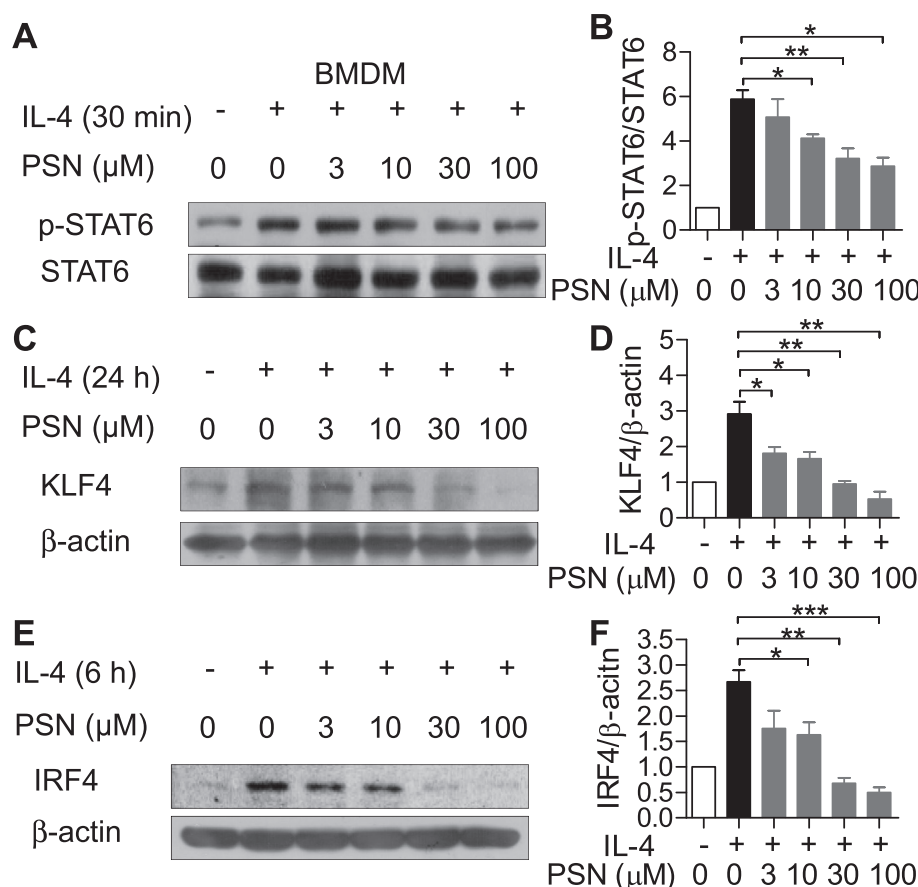


Fig. 6. PSN inhibits the phosphorylation of STAT6 and the expression of KLF4 and IRF4 in response to IL-4 treatment. BMDMs from C57BL/6 mice were treated with PSN at 0, 3, 10, 30 and 100 μ M for 30 min, then stimulated with 10 ng/ml IL-4 for indicated times. Cell lysates were collected for western blot analysis. (A) The phosphorylation of STAT6 and total STAT6 were detected 30 min after IL-4 stimulation. The expression of KLF4 (C) and IRF4 (E) were measured, β -actin used as loading control. Densitometric analysis of the ratios of phosphorylated STAT6/total STAT6 (B), KLF4/ β -actin (D) and IRF4/ β -actin (F) were analyzed by ImageJ software. Statistical analysis was performed, $n = 3$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the group stimulated with IL-4.

polarization might be an effective therapies for asthmatic inflammation.

AAM polarization is associated with the expression of Arg-1, Ym-1 and Fizz-1, which is elicited by Th2 cytokine including IL-4 and IL-13 through a signal transducer and activator of transcription 6 (STAT6)-mediated transcriptional program [55,56]. In addition, as the downstream of STAT6, the transcription factors KLF4 and IRF4 play a critical role in modulation of IL-4-induced macrophage polarization [37,39,57,58]. And previous findings suggest that STAT6, KLF4 and IRF4 may coordinately regulate AAM polarization [35,40]. Targeting STAT6, KLF4 or IRF4 could attenuates several diseases, such as cancer, asthma and chronic obstructive pulmonary disease (COPD) [59–63]. Inhibitors of STAT6 can also reduce allergic asthma such as AS1517499 (leflunomide) and 18 Gly, the major component of Glycyrrhizae Radix [64,65]. In this study, we found that PSN reduces IL-4 induced STAT6 activation and KLF4 or IRF4 expression. Therefore, PSN could ameliorate immune diseases such as allergic asthma via inhibition of STAT6, KLF4 and IRF4.

The prevalence of asthma is rapidly increasing and leads to a substantial global health and economic burden [1,3,66]. Although many strategies have been developed to target asthma, there is still no cure for asthma. Therefore, it is urgent to find effective anti-asthma agent and an appropriate murine model. Ovalbumin (OVA)-induced asthmatic inflammation is classic murine model for studying asthma [10,67–69]. However, OVA-induced asthma model is lack of the impressive asthmatic airway remodeling and chronic lung changes that are identified in human [6,70]. Asthma in clinic is usually initiated by a complex mix of environmental, immunological and host genetic factors [71]. In most cases, house dust mite, ragweed and aspergillus are common environmental causes for human asthma. In this study, we used DRA instead of OVA to induce murine asthma model, in which the pathological process of DRA-induced asthma is more similar with clinic

patient [6,20]. Moreover, several studies showed that compounds, such as IMD-4690 and picoside II, can alleviate house dust mite-induced allergic asthma [72,73] by regulating angiogenesis and airway remodeling via downregulation of the transcription factor GATA3 and Th2-related cytokines. In this study, we found that PSN also exhibited protective effect against allergic asthma. However, the mechanism might be that PSN inhibits AAM polarization via inhibition of the phosphorylation of STAT6 and expression of KLF4 and IRF4, which in turn ameliorating DRA-induced asthmatic inflammation. Therefore, PSN may become a new agent for curing asthma.

Collectively, our study confirmed that PSN attenuates DRA-induced allergic asthma and AAM polarization through inhibition of IL-4-induced STAT6 phosphorylation and KLF4/IRF4 expression. Although DRA-induced murine model closely mimics asthma, IL-4 cannot completely drive the complex progress of asthma, including mucus secretion, vascular remodeling, airway smooth muscle hyperplasia and hypertrophy. As an alarmin, IL-33 is secreted by epithelial cells, innate lymphocytes, macrophages or other immune cells and plays as a booster in several chronic inflammation diseases, including idiopathic pulmonary fibrosis (IPF), systemic sclerosis and asthma [74–78]. Since IL-33 production is reduced by treatment with PSN in DRA-induced mice, suggesting that PSN may have therapeutic effects on these chronic inflammation diseases. Therefore, it is required to perform *in vitro* and *in vivo* experiments to determine how PSN modulates IL-33 production.

In this study, we have proved that PSN has a potential therapeutic effect on DRA-induced murine asthmatic inflammation, in which PSN inhibits AAM polarization via attenuating IL-4-mediated signaling pathway. Given AAM polarization is involved in various diseases, such as cancer, obesity, diabetes and atherosclerosis, we predicted that PSN might be a candidate for curing asthma and other chronic diseases.

Conflict of interest

The authors declare no conflicts of interest.

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