

ORIGINAL ARTICLE

The effect of astragaloside IV on JAK2-STAT6 signalling pathway in mouse model of ovalbumin-induced asthma

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Funding information

This work was supported by grant from Start-up Foundation of Tong De Hospital (No. 1705378).

Abstract

Asthma is a chronic inflammatory lung disease of the airway; the incidence and prevalence of asthma remain high worldwide. Astragaloside IV (AS-IV) is the main active constituent of *Astragalus membranaceus*. Accumulating evidence suggests that AS-IV possesses anti-inflammatory and anti-asthmatic ability, but the potential molecular mechanism is required to further clarify. In this study, the anti-asthmatic effects of AS-IV on mice with ovalbumin (OVA)-induced allergic inflammation were analysed. We analysed airway hyperresponsiveness (AHR), numbers of inflammatory cells, inflammation situation in lung tissue and cytokines level in bronchoalveolar lavage fluid (BALF) between OVA-induced mice with and without AS-IV treatment. Moreover, we explored the possible signalling pathway behind the anti-asthmatic effects. Our results revealed that AS-IV treatment ameliorates airway inflammation and AHR in an OVA-induced asthma model. Besides, AS-IV treatment inhibits the interleukin (IL)-4, -5 and -13 production, and further study indicated that AS-IV treatment downregulates the expression level of p-JAK2/p-STAT6 proteins. Taken together, the present study suggested that the inhibitory effects of AS-IV on asthma therapy are at least partially involved in inhibiting the JAK2/STAT6 signalling pathway.

KEYWORDS

airway hyperresponsiveness, *Astragalus membranaceus*, Chinese herb, cytokines, inflammation

1 | INTRODUCTION

Asthma is a complex inflammatory disease in the lungs which is characterized by infiltration of the bronchial mucosa by mast cells, lymphocytes and eosinophils, goblet cell hyperplasia and increased level of blood immunoglobulin E (IgE) (Frieri, 2007). Despite the widespread use of inhaled corticosteroids and bronchodilators (β_2 -agonists), the incidence and prevalence of asthma are still high (Chung et al., 2015). Therefore, it is imperative to identify the mechanisms underlying asthma and explore new therapies. Astragaloside IV (AS-IV) is the main active constituent of *Astragalus membranaceus*, a Chinese herb that is traditionally used to prevent airway inflammation in chronic asthma patients. Accumulated evidences indicated that AS-IV possess a broad range of pharmacological properties,

including immunomodulatory effects, as well as anti-inflammatory, antiviral and antitumor activities (McCulloch et al., 2006). Previous studies showed that AS-IV treatment markedly inhibited airway inflammation and airway hyperresponsiveness which could remodel asthma (Jin et al., 2017; Li, Hou, Xu, Liu, & Tu, 2017; Qiu et al., 2014). Although some studies explored the possible signalling mechanisms of AS-IV treatment in asthma therapy, the related information is still few (Jin et al., 2017; Qiu et al., 2014).

Cytokines such as interleukin (IL)-4, -5 and -13 secreted from T helper 2 (Th2) cells are involved in the development of asthma (Hou et al., 2017). IL-4 and -13 prompt the production of IgE, and IL-5 can stimulate proliferation, differentiation and activation of eosinophil. Gradually, immune responses lead to an aggregation and infiltration in airway and lung tissues (Lopez et al., 1988; Sanderson,

1992; Simon, Braathen, & Simon, 2015). JAK/STAT pathway is one of the principal signalling pathways for a wide range of cytokines. The JAK/STAT signalling pathway consists of the tyrosine kinase JAK family and the transcription factor STAT family, and is an important cytokine signal transduction pathway (Kiu & Nicholson, 2012; Schindler & Darnell, 1995). Many cytokine-specific JAKs and STATs have been confirmed, and it has been known that IL-5 is specific to JAK2 while IL-4 and IL-13 are specific to STAT6 (Cai, Cai, Luo, Chen, & Zhang, 2015; Neubauer et al., 1998). Studies have shown that the inflammatory mediators (especially IL-13) synthesized by Th2 cells are upstream stimulators for JAK/STAT6 pathway activation. Nevertheless, it is still unknown whether AS-IV has a potential ability to affect JAK-STAT signalling pathway in asthma therapy. To further clarify the molecular mechanism of AS-IV, the present study is the first time to elucidate the effect of AS-IV treatment on JAK-STAT signalling pathway in mouse model of ovalbumin-induced asthma. In this study, our results provide more understandings about the possible molecular mechanism of AS-IV treatment in asthma therapy.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Astragaloside IV (AS-IV, purity >98%) was purchased from Ronghe (Ronghe). Ovalbumin (OVA, purity >98%, Grade V) and methacholine (purity >98%) were purchased from Sigma (Sigma Chemical).

2.2 | Animals

Male and female BALB/c mice, 4 to 5 weeks old and weighing 18–22 g, were used. The animals were housed by 2–3 mice/cage under a standard 12-hr light/dark cycle at $23 \pm 1^\circ\text{C}$. Free access to water and food was provided to animals during the experiments. All animal experiments were carried out in strict adherence to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Tong De Hospital.

2.3 | Murine model of ovalbumin induced airway inflammation

The sensitization and challenge protocols were employed as previously described (Leigh et al., 2002, 2004), with minor modifications. Briefly, each mouse in the OVA and AS-IV treatment groups was sensitized by intraperitoneal injection of 100 μg OVA (grade V; Sigma) conjugated to 400 μg aluminium hydroxide (Sigma) in a total volume of 0.1 ml on days 0 and 7. One week after the last sensitization, mice were challenged with OVA (1.5%, w/v, in PBS) through the airway for 30 min using an ultrasonic nebulizer (NE-U11B; Omron), which was continued for 4 weeks. Meanwhile, mice in the AS-IV treatment groups were administered intragastrically with AS-IV at doses of 50 or 100 mg/kg daily for 4 weeks 30 min

before inhaling OVA. The control group was given an intragastrically injection of 0.2 ml PBS and inhaled PBS. Thirty min before inhaling PBS, the mice in the control group were administered intragastrically with 1% carboxymethylcellulose in a volume of 10 ml/kg.

2.4 | Leucocyte counts in bronchoalveolar lavage fluid

Twenty-four hours after the last OVA challenge, animals were sacrificed and a total of 1 ml bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 400 g at 4°C for 10 min; the supernatants were carefully removed and stored at -80°C for ELISA. The total leucocyte cell number, neutrophils, lymphocyte, monocyte and eosinophils in BALF were counted in a haematology counter (Beckman Coulter LH 750).

2.5 | Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured 24 hr after ovalbumin challenge. The technique for measuring non-invasive respiratory function was previously described (Jin et al., 2017). We used the whole-body plethysmography (Buxco's unrestrained WBP systems) to measure the pulmonary function and record the net (or sum) of nasal and thoracic flows. Actual flows were calculated in the software considering the temperature, humidity and pressure. After a stable baseline was achieved, mice were given aerosolized PBS or various concentrations of methacholine (6.25, 12.5, 25 or 50 mg/ml) via a jet nebulizer in the chamber. The enhanced pause (Penh) index of airway hyperreactivity was used as an indicator of changes in airway resistance.

2.6 | Histological analysis of lung tissue

Lung tissues from the mice were harvested and embedded in paraffin. The paraffin blocks were serially sectioned at 8 μm thickness and stained with haematoxylin and eosin (H&E) to assess the degree of inflammatory cell infiltration. For quantification of pathological disorder, a grade pattern ranging from 0 to 3 was used as previously described. Increasing grade was directly related to asthma severity. The following grading was applied: Grade 0 (absent): lack of infiltration of inflammatory cells and normal distributions of goblet cells in lung tissue; Grade 1 (mild): slight infiltration of inflammatory cell and goblet cell metaplasia; Grade 2 (moderate): moderate infiltration of inflammatory cells and goblet cell metaplasia; and Grade 3 (severe): strong inflammatory cell infiltrations and goblet cell metaplasia.

2.7 | The measurement of cytokines

Twenty-four hours after the final OVA challenge, BALF was harvested from the mice. Levels of interleukin (IL)-4, -5, -6, -13 in the supernatants of BALF were measured using ELISA kits (Thermo Fisher Scientific), following the manufacturer instructions.

2.8 | Western blot

The lung tissues were homogenized in ice-cold RIPA lysis buffer (Applygen Technologies) containing a protease inhibitor cocktail (including protease and phosphatase inhibitor). The lysate was centrifuged at 15,000 g for 15 min at 4°C and quantified with BCA method. Proteins (25 µg) were separated in a 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% non-fat milk for 1 hr at room temperature and probed with specific primary antibodies for p-JAK2/JAK2 (1:200), p-STAT6/STAT6 (1:500) and GAPDH (1:2,000) (Abcam) at 4°C overnight. After three times washing with TBST, the membranes were incubated in the corresponding secondary antibody for 1 hr at room temperature and detected by Western Blot Imaging System (Clinx Science Instruments). Densitometry of bands from Western blots was done by Image J software (Wayne Rasband, National Institutes of Health), and the density of proteins relative to JAK2 and STAT6 was measured.

2.9 | Statistical analysis

The data are expressed as the mean with the standard error mean (SEM). All data were analysed using Prism 7.0 (Graphpad) software. Statistical significance was determined using ANOVA to compare independent mean values, followed by multiple comparisons by Dunnett's test. *P* value of <0.05 was considered indicative of a statistically significant difference.

3 | RESULTS

3.1 | Astragaloside IV treatment decreased the levels of inflammatory cells in bronchoalveolar lavage fluid

To investigate the effects of AS-IV on the recruitment of inflammatory cells, total numbers of leucocytes and its differential count in BALF were determined. Compared with the control group, sensitization and challenge with ovalbumin (OVA) resulted in a markedly increase in the total leucocytes, neutrophils, lymphocytes, monocytes and eosinophils counts in BALF (Figure 1). When OVA-sensitized and -challenged mice were treated with AS-IV, the counts of total leucocytes, neutrophils, lymphocytes and eosinophils were significantly downregulated, compared to the model group. (Figure 1, *p* < 0.05).

3.2 | AS-IV treatment attenuated airway hyperresponsiveness to methacholine

To evaluate the effects of AS-IV on OVA-induced AHR, the airway responsiveness to aerosolized PBS or methacholine was assessed within 24 hr after the final challenge. The enhanced pause (Penh) index was measured as an indicator of bronchial responsiveness to inhaled methacholine. Only mild changes in Penh were observed in

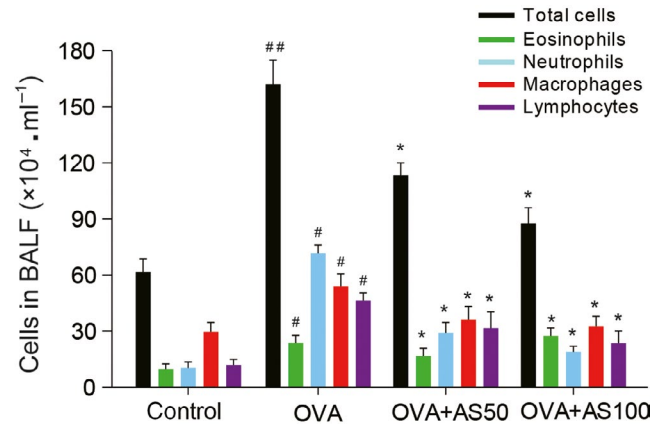


FIGURE 1 Effects of astragaloside IV (AS-IV) treatment on the recruitment of inflammatory cells in bronchoalveolar lavage fluid (BALF). Cell amounts were assessed by direct counting under a light microscope in at least five squares of a haemocytometer after exclusion of dead cells stained. Data were expressed as mean $\pm$ SEM (*n* = 6). The significance of the differences between groups was determined by one-way ANOVA, followed by post hoc Dunnett's tests. [#]*p* < 0.05 and ^{##}*p* < 0.01 compared with control group; ^{*}*p* < 0.05 and ^{**}*p* < 0.01 compared with OVA group

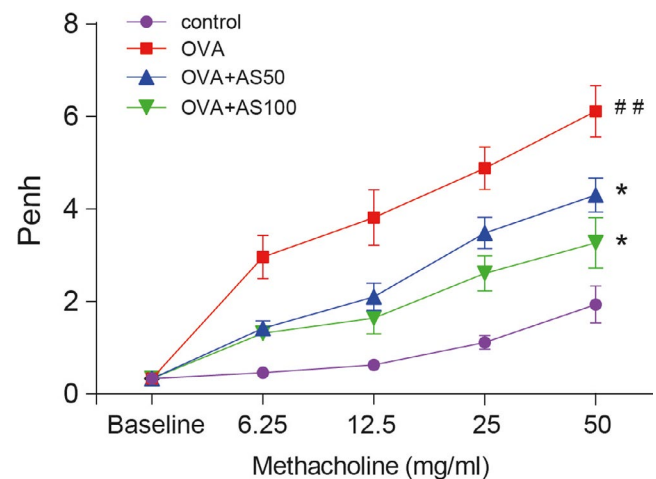


FIGURE 2 Airway responsiveness to aerosolized methacholine was evaluated by Buxco's whole-body barometric plethysmography in awake, unrestrained mice. The mice were nebulized with PBS followed by increasing doses (6.25 to 50 mg/ml) of methacholine. The enhanced pause (Penh) index of airway hyperreactivity was used as an indicator of changes in airway resistance. Data were expressed as mean $\pm$ SEM (*n* = 6). The significance of the differences between groups was determined by one-way ANOVA, followed by post hoc Dunnett's tests. [#]*p* < 0.05 and ^{##}*p* < 0.01 compared with control group; ^{*}*p* < 0.05 and ^{**}*p* < 0.01 compared with OVA group

saline control mice; however, upon OVA re-exposure, the AHR significantly increased (Figure 2). Both 50 and 100 mg/kg AS-IV had inhibitory effects on AHR provoked by methacholine at 6.25 to 50 mg/ml (Figure 2).

3.3 | AS-IV attenuated the inflammatory cell infiltration in allergic mice

H&E staining was used to assess the effects of AS-IV on airway inflammation of the lung sections (Figure 3a). The quantification of peribronchiolar and perivascular inflammatory cell infiltration was determined using an inflammatory score respectively (Figure 3b). Compared to the control mice, OVA-induced mice showed a significantly increasing number and marked infiltration of inflammatory cells into perivascular and peribronchial regions (Figure 3b). AS-IV treatment significantly attenuated the inflammatory cell infiltration in both the peribronchial and perivascular regions compared with the model group ($p < 0.05$, Figure 3b). The model mice showed a significantly increase in total inflammatory scores, compared to control

mice. However, AS-IV treatment was able to attenuate this increase significantly ($p < 0.05$, Figure 3b).

3.4 | AS-IV reduced inflammatory cytokine levels in BALF

Airway inflammation in asthma is characterized by an imbalance of Th1/Th2 cytokines. To determine whether AS-IV could modulate this imbalance by influencing the secretion of cytokines into BALF, interleukin (IL)-4, -5, -6 and -13 levels were measured 24 hr after the final challenge. As shown in Figure 4, levels of IL-4, IL-5 and IL-13 in BALF were significantly upregulated (Figure 4a–d). However, administration of AS-IV was able to significantly inhibit the upregulation of IL-4, IL-5, IL-6 and IL-13 but not IL-6 in OVA-induced mice (Figure 4a–d).

3.5 | AS-IV treatment inhibited the p-JAK2 and p-STAT6 expressions in lungs of allergic mice

JAK2/STAT6 signalling pathway has previously been shown to play an important role in the progression of asthma (Ding et al., 2018; Péron et al., 2011). To explore whether the effects of AS-IV on asthma were associated with JAK2/STAT6 pathway, the p-JAK2/JAK2, p-STAT6/STAT6 protein expressions in lungs were analysed by Western blot. When mice were sensitized and challenged by OVA, the expression level of p-JAK2 and p-STAT6 was dominantly increased without significant changes in total JAK/STAT proteins meanwhile, compared to control group (Figure 5a, b). By contrast, AS-IV treatment was able to markedly decrease the protein expressions of p-JAK2 and p-STAT6 in OVA-induced mice (Figure 5a, b).

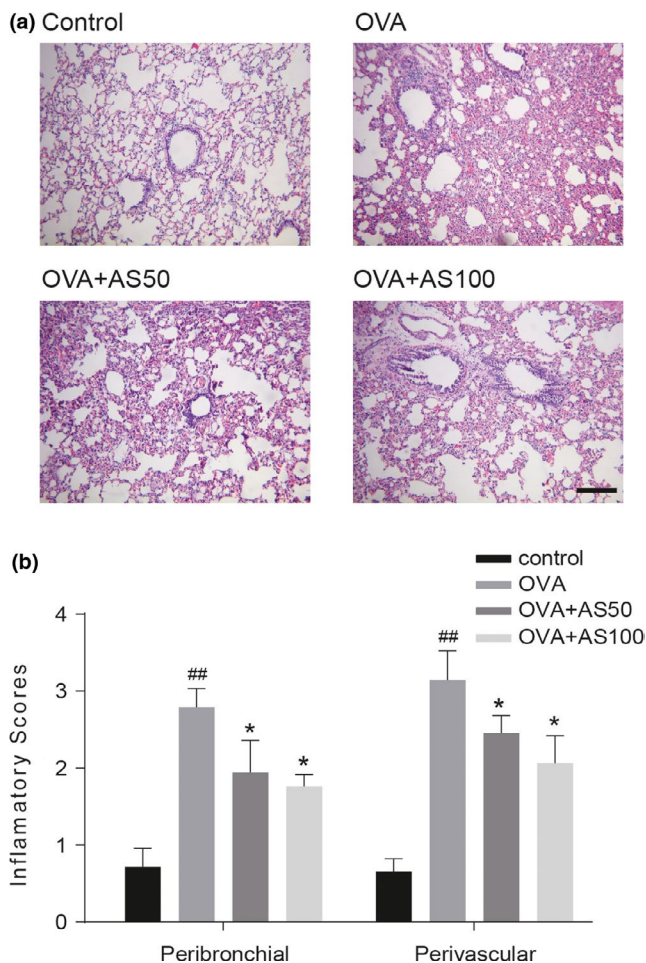


FIGURE 3 Effects of AS-IV treatment on the inflammatory cell infiltration in allergic mice. (a) Pathological changes in lung tissue were determined by haematoxylin and eosin (H&E) staining (magnified $\times 100$), scale bar = 50 μ m. (b) The scores of inflammatory cells infiltration in HE-stained lung sections. Data were expressed as mean $\pm$ SEM ($n = 6$); the significance of the differences between groups was determined by one-way ANOVA, followed by post hoc Dunnett's tests. ## $p < 0.05$ and ### $p < 0.01$ compared with control group; * $p < 0.05$ and ** $p < 0.01$ compared with OVA group

4 | DISCUSSION

Asthma is a serious, sometimes life-threatening, chronic airway inflammation disease that affects million individuals worldwide (Qiu et al., 2014). Over the past decade, several studies have shown that both augmented T helper (Th) 2 responses and impaired Th1 responses, together with Th2-type cytokines, IL-4, -5 and -13, play a critical role in inflammation of asthma (Lloyd & Saglani, 2013; Woodruff et al., 2009). Cytokines, IL-4, -5 and -13 secreted by Th2 are involved in the progression of asthma (Richter et al., 2001; Truyen et al., 2006; Zhou, Zhang, Hu, Zhu, & Yin, 2008). In our mouse model of asthma, we found the Th2-related cytokines, IL-4, -5 and -13 were significantly increased and stimulated, respectively, after ovalbumin (OVA) sensitization and challenge (Figure 4). Besides, infiltration and levels of inflammatory cells and AHR were also significantly upregulated in OVA-induced mice (Figure 2). These phenomena are corresponding to mouse model of asthma in previous studies (Jin et al., 2017; Qiu et al., 2014).

Astragalus membranaceus, a traditional Chinese medicinal herb, has been widely used in asthma remission to prevent asthma. Astragaloside IV (AS-IV), an important derivative of *A. membranaceus*,

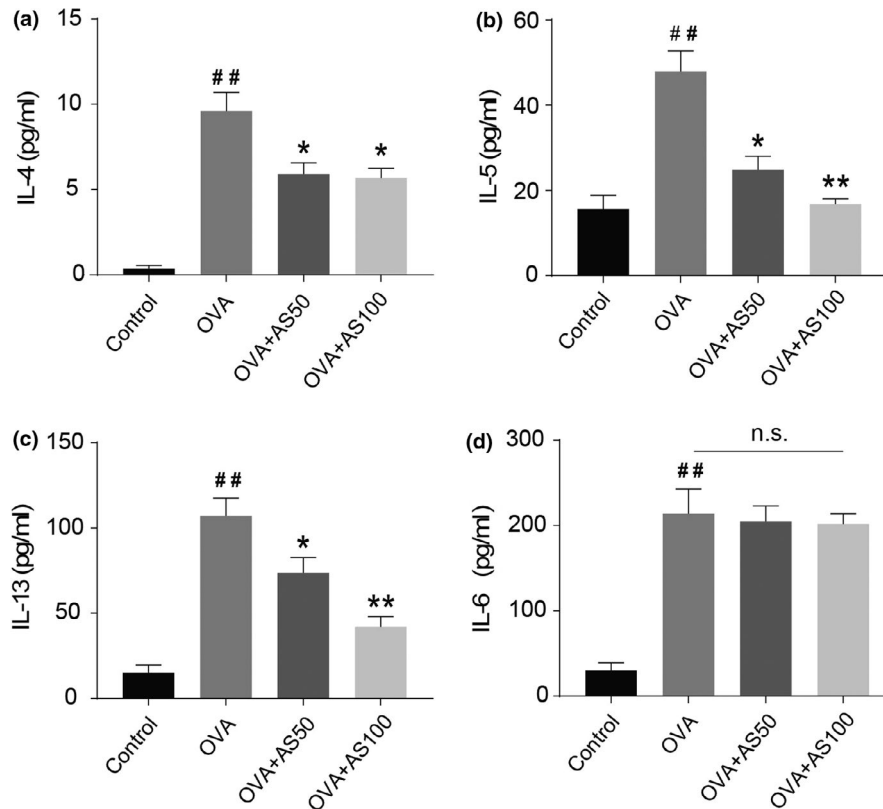


FIGURE 4 Effects of AS-IV treatment on cytokines secretion in BALF in allergic mice. Secreted levels of the interleukin (IL)-4, -5 and -13 were measured by ELISA. The data are expressed as the mean $\pm$ SEM ($n = 6$); the significance of the differences between groups was determined by one-way ANOVA, followed by post hoc Dunnett's tests. [#] $p < 0.05$ and ^{##} $p < 0.01$ compared with control group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ compared with OVA group

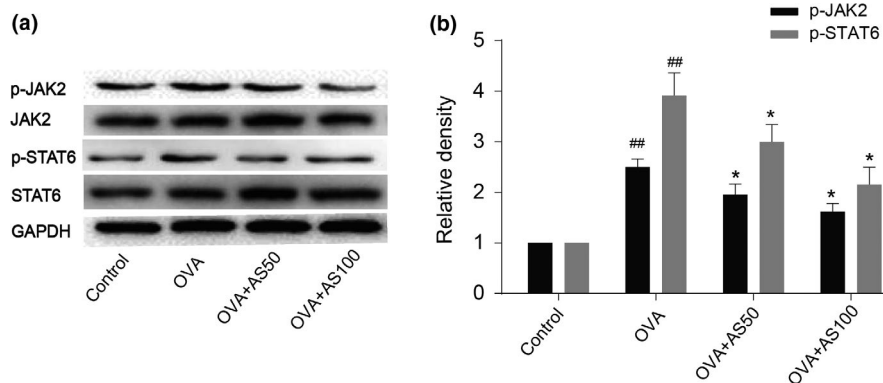


FIGURE 5 Effects of AS-IV treatment on JAK/STAT signalling proteins in lung tissues of allergic mice. (a) The expression of JAK/STAT signalling proteins in lung homogenates. The protein expressions were detected by Western blotting with specific antibodies. (b) The relative protein expression of JAK/STAT signalling proteins in lung homogenates. The protein expression of GAPDH was used to normalize expressions of target proteins. The data are expressed as the mean $\pm$ SEM ($n = 6$); the significance of the differences between groups was determined by one-way ANOVA, followed by post hoc Dunnett's tests. [#] $p < 0.05$ and ^{##} $p < 0.01$ compared with versus control group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ compared with OVA group

could alleviate airway inflammation when it was applied early in asthma. AS-IV is able to inhibit the inflammatory response and provide the protective effect on the respiratory system (Brouns & De Deyn, 2009; Huang, Tan, Chen, & Deng, 2017; Jin, Yang, & Li, 2010). Besides, some studies showed AS-IV attenuates the eosinophilic

infiltration in ovalbumin-induced asthma (Du et al., 2008; Gu, Jiang, Wang, Du, & Cai, 2012; Huang, Tang, Wang, & Song, 2014). Previous study used 20–40 mg/kg doses of astragaloside IV (AS-IV) daily to treat OVA-induced mouse model of asthma and had different degree levels to inhibit asthma syndrome (Jin et al., 2017). Cytokine levels,

IL-4 and -5, in BALF of mouse model and airway inflammation in the lung tissue were more dominantly affected by 40 mg/kg dose daily of AS-IV. In contrast, IL-13 was not regulated by AS-IV treatment (Jin et al., 2017). On the other hand, 20 mg/kg dose daily of AS-IV only had effects on IL-4 and -5 levels in BALF (Jin et al., 2017). In the present study, we tried a higher dose, 100 mg/kg dose daily, to test whether higher dose of AS-IV can further affect the progression of asthma. In OVA-induced mice, we found either 50 or 100 mg/kg dose of AS-IV had a similar inhibitory effect on the counts of inflammatory cells in BALF and inflammation in lung tissue (Figure 1). In addition, the present results revealed that either 50 or 100 mg/kg dose daily significantly downregulated cytokine levels of IL-4, -5 and -13 in BALF. Moreover, 100 mg/kg dose daily had more dominant effects on the inhibition of cytokine levels (Figure 4). Compared to the previous study, the present results implied a higher dose of AS-IV can further suppress the development of asthma.

In this study, we found that IL-4, -5 and -13, the Th2-related cytokine levels, were significantly decreased in the OVA-induced mice with AS-IV treatment comparing with the non-treatment control, which could exhibit effects of AS-IV in asthma therapy again. JAK-STAT signalling has been demonstrated to play an important role in T-cell proliferation and differentiation (Kiu & Nicholson, 2012; Schindler & Darnell, 1995). Previous studies indicated Th2-related cytokines IL-4, 5 and 13 are separately related to the activation of JAK2 and STAT6 signalling pathway (Cai et al., 2015; Neubauer et al., 1998). Previous studies have shown that the inflammatory mediators (especially IL-13) synthesized by Th2 cells are upstream stimulators for JAK/STAT6 pathway activation and could induce phosphorylation of STAT6 (Kiu & Nicholson, 2012; O'Shea & Plenge, 2012). Furthermore, p-STAT6 is involved in goblet cell metaplasia and leads to excessive mucus secretion (O'Shea & Plenge, 2012). The JAK2/STAT6 pathway plays an important role in airway hyper-reactivity and mucus overproduction (Perkins et al., 2011). Here in results, we found a significant increase of p-JAK2 expression in our OVA-induced asthma model, and AS-IV pre-treatment significantly decreased the levels of p-JAK2. As such, we measured the change in STAT6 expression in each group. We found that p-STAT6 expression was significantly increased after OVA, and AS-IV pre-treatment inhibited the expression of p-STAT6. These results suggested that JAK2/STAT6 pathway is one of the possible targets, in which AS-IV mediated the allergic inflammatory response. AS-IV was also widely used to treat ischaemic diseases. In previous study, AS-IV was shown to be a vital regulator of NO and angiogenesis through the JAK2/STAT6 pathways (Wang, Xu, Chen, Yang, & Chen, 2013). Therefore, AS-IV is suggested to have the potential to modulate JAK-STAT signalling pathway. Previous study also reported that AS-IV could inhibit mTORC1 signalling pathway in mouse model of asthma (Jin et al., 2017) and suppresses GATA-3 and increases T-bet expressions in asthmatic reactions both in vivo and in vitro (Qiu et al., 2014). Therefore, we cannot exclude these possible mechanisms.

The present study suggested that AS-IV attenuated airway inflammation and asthma syndrome at least partially from inhibiting the JAK2/STAT6 signalling pathway. This finding provides us a new

insight into the molecular mechanism of AS-IV in asthma therapy. In future study, in vitro and clinical experimental analysis could be necessary to reinforce the experimental findings.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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How to cite this article: Yang X, Wang F. The effect of astragaloside IV on JAK2-STAT6 signalling pathway in mouse model of ovalbumin-induced asthma. *J Anim Physiol Anim Nutr*. 2019;00:1–7. <https://doi.org/10.1111/jpn.13114>