



CD4⁺CD25⁺Foxp3⁺ T cells contribute to the antiasthmatic effects of *Astragalus membranaceus* extract in a rat model of asthma

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ABSTRACT

Astragalus membranaceus (AM), a traditional Chinese medicinal herb, has been widely used for centuries to treat asthma in China. Previous studies demonstrated that AM had inhibitory effects on airway hyperresponsiveness, inflammation and airway remodeling in murine models of asthma. However, it remained unclear whether the beneficial effects of AM on asthma were associated with CD4⁺CD25⁺Foxp3⁺ Treg cells; this issue is the focus of the present work. An asthma model was established in Sprague–Dawley (SD) rats that were sensitized and challenged with ovalbumin. Bronchoalveolar lavage fluid (BALF) was assessed for inflammatory cell counts and cytokine levels. Airway hyperresponsiveness was detected by direct airway resistance analysis. Lung tissues were examined for cell infiltration, mucus hypersecretion and airway remodeling. CD4⁺CD25⁺Foxp3⁺ Treg cells in the BALF and Foxp3 mRNA expression in lung tissues were examined. The oral administration of AM significantly reduced airway hyperresponsiveness to aerosolized methacholine and inhibited eosinophil counts and reduced IL-4, IL-5 and IL-13 levels and increased INF- γ levels in the BALF. Histological studies showed that AM markedly decreased inflammatory infiltration, mucus secretion and collagen deposition in the lung tissues. Notably, AM significantly increased population of CD4⁺CD25⁺Foxp3⁺ Treg cells and promoted Foxp3⁺ mRNA expression in a rat model of asthma. Together, these results suggest that the antiasthmatic effects of AM are at least partially associated with CD4⁺CD25⁺Foxp3⁺ Tregs.

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1. Introduction

Bronchial asthma is a chronic airway disease that is typically characterized by chronic airway inflammation, bronchial hyperresponsiveness and airway remodeling [1]. Over the last 15 years, T helper 2 (TH2) cells have emerged as critical in the pathogenesis of asthma. TH2 cell-derived cytokines have been implicated in eosinophil accumulation, mucus hypersecretion, AHR development, and lung remodeling [2,3]. However, mechanisms of asthma beyond inappropriate TH2 responses are beginning to be appreciated. Recently, regulatory T cells (Tregs) were found to play a key role in the maintenance of immune homeostasis. These cells are capable of inhibiting the proliferation and function of conventional effector T cells in a cell contact-dependent fashion either directly or by acting on antigen-presenting cells [4]. The best-characterized Tregs are CD4⁺ T cells, which also express the alpha chain of the IL-2 receptor (CD25). The forkhead family

transcription factor Foxp3 has been regarded as not only a faithful marker of Tregs but also a critical component for Treg development and function [5]. Several lines of evidence suggest that Tregs are intimately involved in asthma pathogenesis. It was shown that TH2-mediated airway inflammation in asthma may be due to defective suppression by Tregs [6]. Furthermore, the levels of CD4⁺CD25^{hi} Tregs and Foxp3 mRNA expression were found to be significantly lower in asthmatic children [7]. Therefore, Tregs may be an attractive therapeutic target for the treatment of asthma.

Astragalus membranaceus (AM), which is a traditional Chinese medicinal herb, has a long history of medicinal use for asthma treatment in China. It is well known to increase metabolism and stimulate tissue regeneration, and it is used to treat colds and allergies, digestive problems and fatigue in Traditional Chinese Medicine [8,9]. The major active constituents of AM include flavonoids, saponins, polysaccharides and amino acids [10]. Recently, AM and its constituents were confirmed to have extensive pharmacological functions, including immunomodulatory, anti-inflammatory, anti-fibrosis, anti-neoplastic, and anti-diabetic properties [11–14]. Moreover, evidence has indicated that AM or combined with other herbs can improve airway inflammation, hyperresponsiveness and remodeling in murine models of asthma

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[15–17]. Data regarding the effects of AM on Tregs is scarce. A recent study demonstrated that AM could prolong allograft survival via upregulating the CD4⁺CD25⁺ Tregs ratio and promoting Foxp3 expression in an allografted mouse model [18]. Nevertheless, the mechanisms through which AM exerts its antiasthmatic effects have not been fully elucidated. In the present study, we aimed to confirm the involvement of CD4⁺CD25⁺Foxp3⁺ Tregs in the antiasthmatic effects of AM in a rat model of asthma.

2. Materials and methods

2.1. Reagents

A. membranaceus was purchased from Shanghai KangQiao Herbal Co. (Shanghai, China), and its extract was prepared as follows: 1.2 kg dried Radix Astragali was soaked in 7.2 L water for 2 h and extracted twice with H₂O at 100 °C. After filtration, the decoction was diluted in distilled water to a concentration of 1 g/ml (each ml of solution contained 1 g crude drug of Radix Astragali). Ovalbumin (OVA), aluminum hydroxide, and methacholine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone sodium phosphate injection (5 mg/ml) was provided by Xianju Medicine Co. (Zhejiang, China). ELISA kits for IL-4, IL-5, and IL-13 were obtained from Xitang Biological Pharmaceutical Co. (Shanghai, China), and INF- γ and TGF- β 1 were provided by BioLegend Inc. (San Diego, USA). FITC-labeled anti-rat CD4, APC-labeled anti-rat CD25, and PE-labeled anti-rat Foxp3 were procured from eBioscience Co. (San Diego, USA). The reverse transcription system kit was supplied by MBI Fermentas (Vilnius, Lithuania).

2.2. Animals

Male specific-pathogen-free Sprague–Dawley (SD) rats weighing 200–250 g were purchased from Shanghai SLAC Co. (Shanghai, China). Animals were housed in stainless steel cages in a temperature-controlled environment (22 $\pm$ 2 °C) on a 12-h light–dark schedule (lights on from 6:00 am to 6:00 pm) and given free access to water and rodent chow. The animals were acclimatized for at least 7 days before use in experiments. Sixty rats were randomly divided into six groups as follows: a control group (10 rats), an OVA control group (10 rats), a DEX (0.5 mg/kg) treatment group (10 rats), and three AM (2.5, 5.0, or 10.0 g/kg) treatment groups (10 rats per group). The animal study protocols were approved by the Institutional Animal Care and Use Committee of Fudan University.

2.3. Sensitization and treatment

As previously described, a rat model of asthma was developed by OVA sensitization and inhalation [19]. Briefly, on day 0, rats were

systemically sensitized with an intraperitoneal injection of 1 ml alum-precipitated Ag in PBS which contained 100 mg of OVA mixed with 100 mg aluminum hydroxide. Two weeks after the sensitization, the rats were exposed to aerosolized OVA for 30 min/day each day for 4 weeks. The concentration of OVA was increased from 1% for 3 weeks to 2% for 1 week to maintain the asthma condition. From days 14 to 42, the OVA-sensitized rats in the treatment groups were given AM or DEX orally once per day 1 h before OVA inhalation, at doses of 2.5, 5.0 and 10.0 g/kg/day for AM and 0.5 mg/kg/day for DEX. The rats in the normal group received with the same volume of PBS instead. The protocol for the sensitization, challenge and drug administration is summarized in Fig. 1.

2.4. Measurement of airway hyperresponsiveness (AHR)

AHR was evaluated by a Buxco's modular and invasive system (Buxco Electronics Inc., NY, USA). Changes in airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were measured directly as described by Amdur and Mead [20]. Briefly, each anesthetized rat was tracheostomized and intubated with an appropriate cannula, and then laid supine inside the body plethysmograph chamber connected to the ventilator. After a stable baseline airway pressure (<5% variation over 2.5 min) was achieved, the rats were given aerosolized PBS or various concentrations of methacholine (3.125, 6.25, 12.5, or 25 mg/ml) via a jet nebulizer into the head chamber. Minimum values for R_L and C_{dyn} were calculated in response to increasing concentrations of inhaled methacholine and expressed as a percent change from the baseline value, as in previous studies [21].

2.5. Histological analysis

After BALF was isolated, lung tissue slices were inflated with 10% neutral-buffered formalin. Thin sections (3–4 μ m) were cut from blocks and stained with hematoxylin–eosin (H&E), periodic acid Schiff (PAS) and Masson's Trichrome (M-T). Image-Pro Plus software was used for histopathological analysis. The severity of inflammatory cell infiltration in the lung was evaluated with a 5 point scoring system: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; and 4, a ring of cells > 4 cells deep. PAS staining was used to evaluate the levels of airway mucus expression; at least 6 bronchioles were counted in each slide. The results were expressed as the area of airway epithelium staining positive for mucus/the perimeter of the basement membrane of the bronchioles. M-T stain was used to detect collagen deposition in the lung tissues. The peribronchiolar area stained with M-T was calculated by counting at least 6 bronchioles in each slide. The result was expressed as Wac/Pbm (μ m²/ μ m) (collagen area on the basal membrane of airway/the perimeter of the basement membrane of bronchioles).

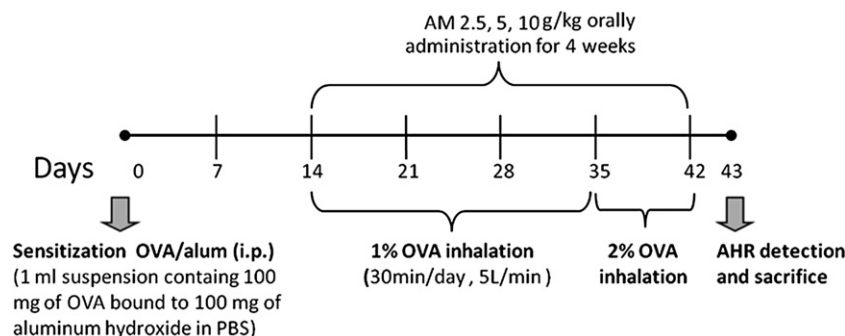


Fig. 1. Experimental protocol. Rats were sensitized and challenged by OVA as described in the Materials and methods section.

2.6. Preparation and analysis of bronchoalveolar lavage fluids (BALF)

Immediately following the assessment of AHR, BALF was obtained by inserting a tracheal tube and lavaging the lung two times with 4 ml sterilized normal saline containing 2% bovine serum albumin (BSA). The collected lavage fluid was centrifuged at $500\times g$ at 4°C for 10 min. The supernatants were harvested and stored at -80°C for measurements of cytokine production. The pellets were resuspended in 1 ml PBS for flow cytometric analysis or cell count and classification. Total cell counts were determined by a hemocytometer. Differential cell counts were performed after staining by Wright–Giemsa. At least 400 cells/slide were counted. Interleukin (INF- γ , IL-4, IL-5, IL-13 and TGF- β) levels in the BALF supernatant were analyzed by ELISA according to the manufacturer's instructions.

2.7. Flow cytometric analysis

Cells from the BALF were analyzed for $\text{CD4}^{+}\text{CD25}^{+}\text{Foxp3}^{+}$ expression using a Treg cell staining reagent according to the manufacturer's instructions. Briefly, prepared cells (1×10^6) were washed by centrifugation in flow cytometry staining buffer. Then, cells were stained with FITC-labeled anti-CD4 and APC-labeled anti-CD25 antibodies in staining buffer for 30 min at 4°C . Next, cells were fixed and permeabilized in a fixation/permeabilization solution for 30 min and subsequently stained with $0.5\text{ }\mu\text{g}$ of anti-rat Foxp3 PE. Finally, the cells were resuspended in flow cytometry staining buffer and analyzed by flow cytometry using a FACSCalibur instrument with CellQuest software (BD Biosciences, Mountain View, CA, USA).

2.8. Quantitative real-time RT-PCR

Total RNA was isolated from the lung tissues using a kit from GBCBio-Tech (Guangzhou, China), and the quality of the RNA was subsequently evaluated by measuring the ratio of the absorbance at 260/280 nm. For reverse transcription, the First Strand cDNA Synthesis Kit was used. For PCR amplification, the following mouse-specific sense and antisense primers were used: Foxp3, 5'-CCC TCA AAG TTA CAA TCC TG-3' (forward) and 5'-GGG TTA GTG GCA AGT GAT A-3' (reverse); GAPDH, 5'-GGG GAG CCA AAA GGG TCA TC-3' (forward) and 5'-GAC GCC TGC TTC ACC ACC TTC TTG-3' (reverse). The amplification was performed in a Line-Gene FQD-33A instrument (Bioer Inc., Hangzhou, China) under the following conditions: initial denaturation at 94°C for 10 min, 40 cycles of amplification at 94°C for 20 s, annealing at 53°C for 25 s, and then extension at 72°C for 30 s.

2.9. Statistical analysis

The data are expressed as the means $\pm$ standard error (S.E.M.). SPSS 16 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The significance of the differences was determined by one-way ANOVA followed by post hoc Dunnett's tests. P values less than 0.05 were considered to indicate significant differences from the OVA control.

3. Results

3.1. The inhibitory effect of AM on AHR to methacholine

To evaluate the effect of AM on OVA-induced AHR, the airway responsiveness to aerosolized PBS or methacholine was assessed within

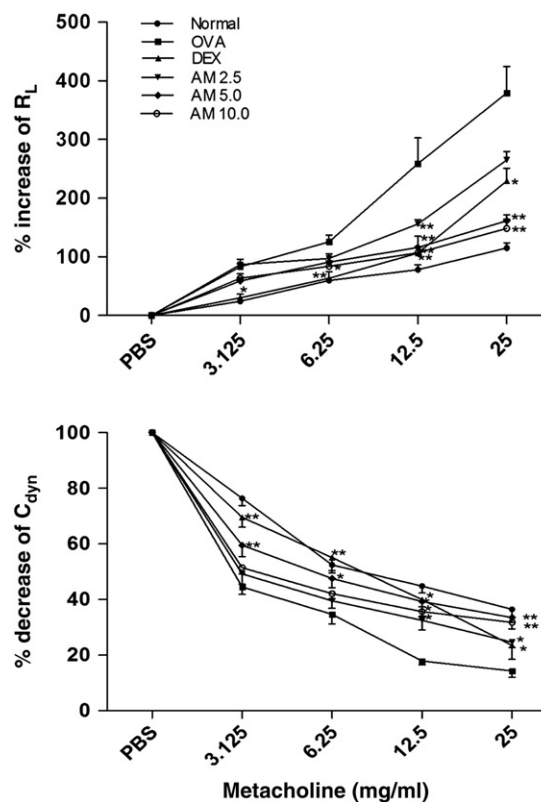


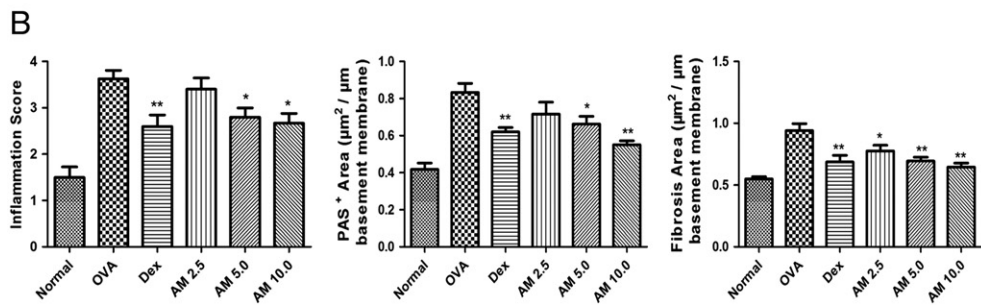
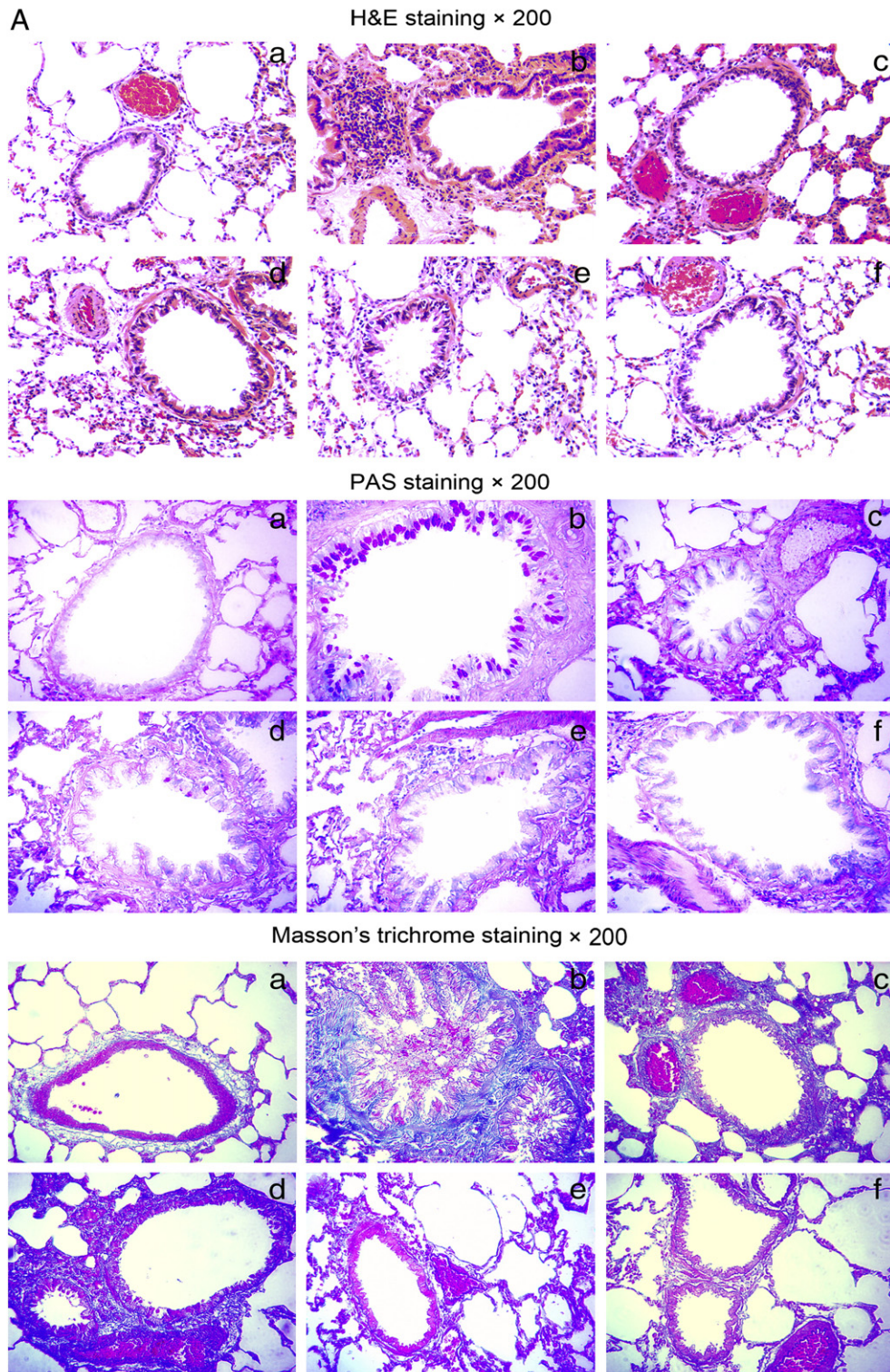
Fig. 2. Airway responsiveness to aerosolized methacholine was evaluated by a Buxco's modular and invasive system, as described in the [Materials and methods](#) section. The rats were laid in a supine position inside the body plethysmograph chamber and were nebulized with PBS followed by increasing doses (3.125 to 25 mg/ml) of methacholine. The data are expressed as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ versus the OVA group.

24 h after the final challenge. Only mild changes in airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were observed in normal rats. However, there was a significant enhancement of airway responsiveness in OVA-exposed rats, with an obvious increase in R_L and C_{dyn} compared to control rats (Fig. 2). Oral administration of 2.5, 5.0 or 10.0 g/kg AM significantly reduced the airway responsiveness provoked by methacholine. DEX also had a significant inhibitory effect on the reduction of AHR. The effects of 5.0 and 10.0 g/kg AM on the reduction of AHR was similar to that of DEX (Fig. 2).

3.2. The inhibitory effect of AM on inflammatory cells, collagen deposition and mucus production in lung tissues

To demonstrate the inhibitory effects of AM on inflammation in the lung tissue, pulmonary pathology was observed by hematoxylin and eosin (H&E) staining. As shown in Fig. 3, a significant infiltration of inflammatory cells into peribronchiolar and perivascular connective tissues was observed in OVA-exposed rats. In contrast, AM treatment remarkably attenuated airway inflammation at the dose of 5.0 and 10.0 g/kg. Periodic acid Schiff (PAS) and Masson's Trichrome (M-T) staining were conducted to evaluate the effect of AM on mucus production and collagen deposition in lung tissues. The OVA-exposed rats exhibited a marked increase of peribronchial collagen deposition and mucus hypersecretion within the bronchi in the lungs. In contrast, the rats in the 5.0 and 10.0 g/kg AM treatment

Fig. 3. The effect of AM on airway inflammation, mucus production and collagen deposition in the lung tissue. (A) Lung tissue slices were fixed, embedded, sectioned at $3-4\text{ }\mu\text{m}$ and stained with H&E, periodic acid-Schiff (PAS) and Masson's Trichrome (M-T). (B) Graphs represent the inflammation score, PAS-positive area and fibrosis area in the bronchial basement membrane. The results are expressed as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ versus the OVA group. Representative pictures are shown for each group: a: normal; b: OVA; c: DEX; d: AM 2.5 g/kg group; e: AM 5.0 g/kg group; and f: AM 10.0 g/kg group.



groups exhibited significantly reduced mucus production and collagen deposition, similar to those found in the DEX group. It is noteworthy that 2.5 g/kg AM also inhibited collagen deposition but not airway inflammation and mucus production.

3.3. The inhibitory effect of AM on inflammatory cells in BALF

BALF was recovered to determine the effects of AM on the accumulation of inflammatory cells in the airway. The total number of cells in the BALF was counted, and different cell types were distinguished using morphological criteria. As shown in Fig. 4, the total numbers of leukocytes, eosinophils, lymphocytes, macrophages and neutrophils in the BALF of OVA-exposed rats were significantly increased compared to the normal control rats. A significant reduction of infiltration of inflammatory cells into airways was observed after oral treatment with 5.0 or 10.0 g/kg AM. Moreover, the classification of these inflammatory cells indicated that the oral administration of AM reduced the numbers of eosinophils, lymphocytes, macrophages and neutrophils in a dose-dependent manner. DEX (0.5 mg/kg) also had a marked effect on the inhibition of total leukocytes in airways. The inhibitory effect of AM at 10.0 g/kg on inflammatory cells was similar to that of 0.5 mg/kg DEX (Fig. 4).

3.4. The inhibitory effect of AM on cytokines in BALF

Airway inflammation in asthma is characterized by an imbalance of TH1/TH2 cytokines. Thus, to determine whether AM could modulate this imbalance, levels of INF- γ , IL-4, IL-5, and IL-13 were measured by ELISA. As shown in Fig. 5, OVA-exposed rats presented significantly elevated levels of IL-4, IL-5, IL-13, as well as reduced INF- γ in the BALF in comparison to normal rats. In contrast, the oral administration of AM obviously and dose-dependently inhibited the up-regulation of TH2 cytokines, while increase TH1 cytokine in the BALF. The effect of 10 g/kg AM was comparable to that of 0.5 mg/kg DEX on TH2 cytokines. However, unlike AM, DEX had no significant effect on INF- γ levels. As TGF- β plays an important role in airway remodeling, we also measured TGF- β 1 level in BALF. As shown in Fig. 5, TGF- β 1 was found remarkably increased in the model group. However, AM significantly inhibited TGF- β 1 in BALF in a dose-dependent manner.

3.5. The effect of AM on the CD4⁺CD25⁺Foxp3⁺ Treg population in BALF cells

CD4⁺CD25⁺Foxp3⁺ Treg cells play a significant role in regulation of asthma, and the induction of allergen-specific Tregs has become one appealing strategy for asthma therapy. Thus, we determined the relative proportion of Treg cells in the BALF after oral treatment with AM. Our study showed that AM significantly increased the number of CD4⁺CD25⁺Foxp3⁺ Treg cells in a dose-dependent manner (Fig. 6). Notably, the oral administration of 10.0 g/kg AM significantly enhanced the frequency of Treg cells by up to 17.9% compared to the OVA-exposed group. Consistently, there was a great increase in the expression of Foxp3 mRNA in lung tissues after treatment with 10.0 g/kg AM (Fig. 6).

4. Discussion

In the present study, we investigated the effects of AM on AHR, airway inflammation and remodeling in a rat model of asthma. In this rat model, antigen sensitization and repeated challenge led to significant AHR, inflammatory infiltration, collagen deposition and mucus secretion in lung tissues, as well as an increase in the level of IL-4, IL-5, IL-13 and TGF- β 1 and down-regulation of INF- γ in the bronchoalveolar lavage fluids. These changes were markedly reversed by the oral administration of AM. Notably, AM significantly increased

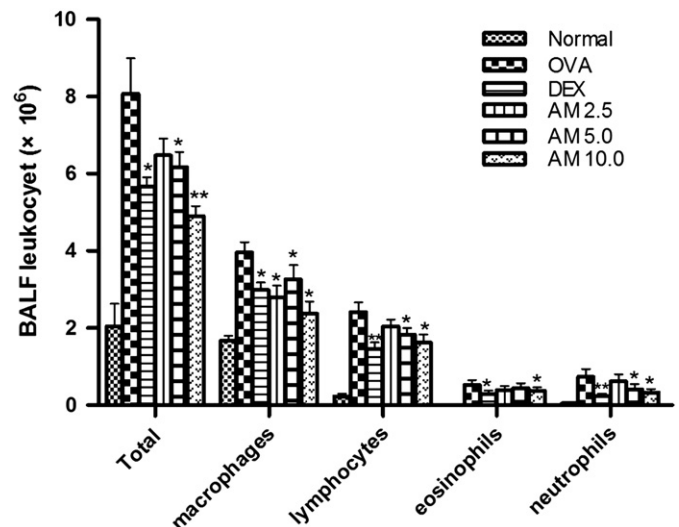


Fig. 4. The effects of AM on inflammatory cell levels in the BALF. Rats were treated as described in Fig. 1, and the BALF was harvested 24 h after the last OVA challenge. The total numbers of inflammatory cells in the BALF were counted, and cell classification was performed on a minimum of 400 cells to classify macrophages, lymphocytes, eosinophils, and neutrophils. The data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ versus the OVA group.

the population of CD4⁺CD25⁺Foxp3⁺ Treg cells and promoted Foxp3 expression.

Previous studies have shown that AM could inhibit airway inflammation and AHR by modulating the balance of TH1/TH2 cytokines in the BALF, as well as by reducing eosinophilic airway infiltration in a mouse model of asthma [22]. However, relevant data are still scarce and have mainly been obtained from mouse models. To further understand the antiasthmatic mechanism of AM, a unique rat model of asthma was used in our study. Moreover, an invasive measurement was applied to more directly and accurately assess AHR by determining changes in pulmonary resistance (R_L) and dynamic compliance (C_{dyn}). This rat model displayed characteristics typical of asthma, as illustrated above, and we confirmed the inhibitory effects of AM on airway inflammation, AHR and airway remodeling. In line with the published data, AM was also found to suppress the expression of TH2 cytokines including IL-4, IL-5 and IL-13, and increased TH1 cytokine like INF- γ . It seems that regulation of TH1/TH2 cytokine imbalance may contribute to the antiasthmatic effects of AM. Nevertheless, the pathogenetic mechanism of bronchial asthma is extremely complex, and treatments specifically targeting these TH2-type mediators had little effect on asthma or failed to progress to clinical trials [23]. Moreover, in human studies of airway remodeling, only anti-IL-5 has thus far proven to be useful for targeted interventions for airway remodeling [24]. Considering the broad immunomodulatory function of AM, we speculated that other mechanisms might be involved in the antiasthmatic effects of AM.

Recently, the crucial immunoregulatory effect of Tregs has been suggested to suppress conventional CD4 T cells-mediated immune responses. Moreover, their roles in asthma pathogenesis are increasingly recognized [25,26]. However, it was still unknown whether Tregs were involved in the protective effect of AM on asthma. To test this hypothesis, we analyzed the CD4⁺CD25⁺Foxp3⁺ Tregs in the BALF, which should reliably reflect their effects on airway inflammation. AM was found to significantly increase the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs in the BALF compared with the OVA control. Moreover, AM treatment significantly enhanced the expression of Foxp3 mRNA at 10.0 g/kg, but not at 5.0 or 2.5 g/kg, which may at least partly explain why the greatest antiasthmatic effects of AM are observed at

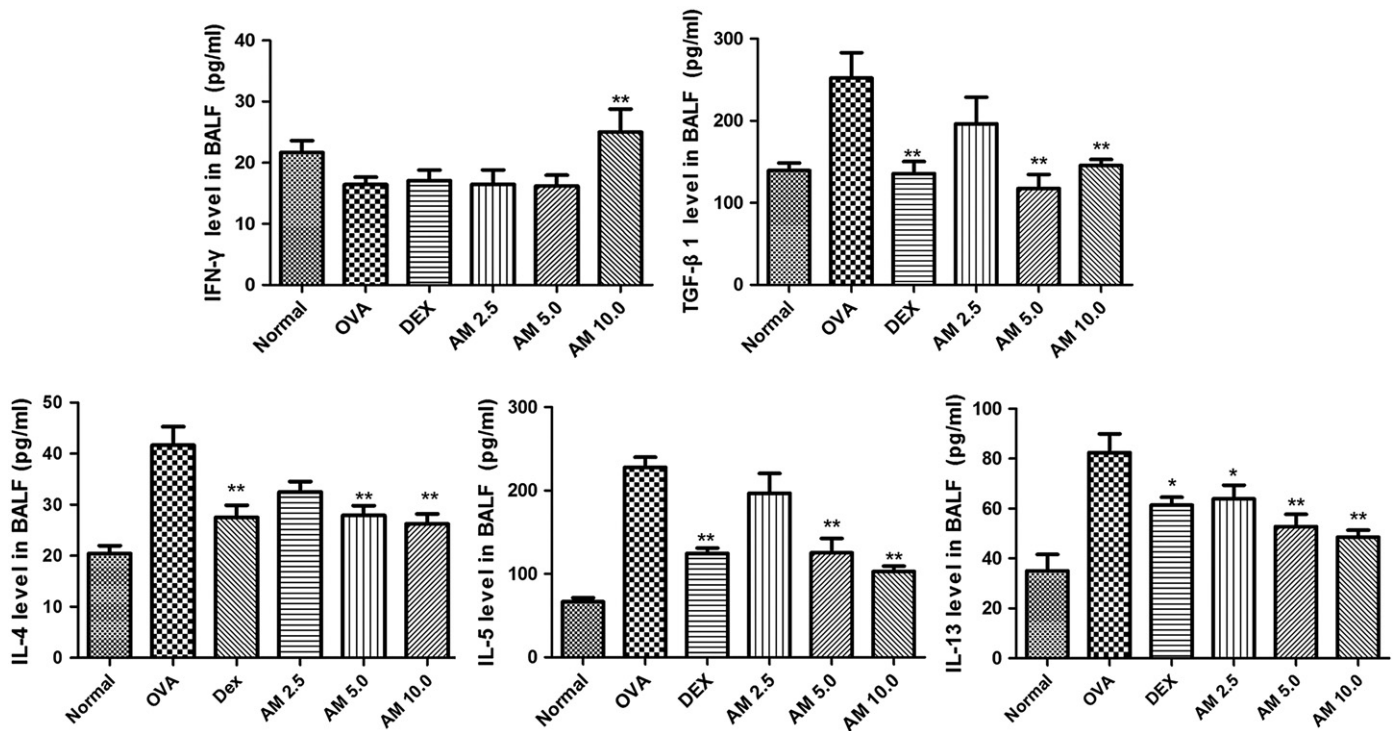


Fig. 5. The effect of AM on cytokine secretion in a rat model of asthma. Secreted levels of the cytokines INF- γ , IL-4, IL-5, IL-13, and TGF- β were measured by ELISA. The data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ versus the OVA group.

10.0 g/kg. In line with our study, Qu LL et al. demonstrated that AM could up-regulate the Treg ratio and promote Foxp3 expression to prolong allograft survival [18]. However, *Astragalus* polysaccharides (APS), a major constituent in AM, was surprisingly found to inhibit CD4⁺CD25⁺ Treg cells in the tumor microenvironment, and thus exert anti-tumor effects [27]. It is possible that AM possesses functions of bidirectional regulation, which may be associated with its complex constituents including flavonoids, saponins, polysaccharides and amino acids. Besides, the effects of AM and APS may be different, which is more possible when used in different dosage. Nevertheless, further studies are still needed to illustrate their contradictory effects on Tregs in different diseases.

As supporting evidence for the current study, it has been suggested that Tregs have an arsenal of regulatory mechanisms at their disposal to suppress TH2 cells, including inhibitory cytokines, cytotoxicity, metabolic disruption, and augmented APC function [28]. Therefore, the observed reduction in airway inflammation may be a result of inhibitory effects on IL-4, IL-5, and IL-13, which are secondary to CD4⁺CD25⁺Foxp3⁺ Treg enhancement. While TH2 cytokines are widely reported to be involved in the induction of AHR, as illustrated above, the mechanisms by which they mediate AHR remain unclear. Recent studies have suggested that the regulation of AHR is not mediated by TH2 cytokines alone, e.g., a direct association between Tregs and the suppression of AHR in asthma has been demonstrated. Burchell et al. found that AHR was suppressed by airway CD4⁺CD25⁺Foxp3⁺ Tregs, potentially via a paracrine induction of TGF- β 1 in the airways [29]. Kearley et al. also reported that the transfer of CD4⁺CD25⁺ Tregs could suppress AHR and Th2 inflammation in a mouse model [30]. Therefore, the enhancement of CD4⁺CD25⁺Foxp3⁺ Tregs by oral administration of AM in our study may be associated with the reduction of AHR.

Our results demonstrated a dramatic reduction in collagen deposition and mucus secretion in AM-treated rats compared with untreated controls. Astragaloside IV, a major active constituent of AM, has also been demonstrated to markedly inhibit airway structure

changes in a mouse model [31,32]. TGF- β was considered as a key in airway remodeling processes, and several previous studies have suggested that AM could improve airway remodeling by inhibiting the TGF- β /Smad signaling pathway in a mouse model of asthma [17]. In line with this observation, we found TGF- β 1 level in BALF was also significantly inhibited by AM administration. Thus, the inhibitory effect of AM on TGF- β 1 may be associated with improvement in airway remodeling. However, the mechanisms by which AM prevents airway remodeling are still unclear. The therapeutic implications of Tregs in asthmatic airway remodeling are just beginning to be appreciated. Recently, studies have demonstrated that the transfer of CD4⁺CD25⁺ regulatory T cells can prevent airway remodeling by reducing mucus hypersecretion, collagen deposition and/or angiogenesis [33,34]. Therefore, the enhancement of CD4⁺CD25⁺Foxp3⁺ Tregs may contribute to the favorable effects of AM on airway remodeling.

Unfortunately, our results do not clearly establish whether CD4⁺CD25⁺Foxp3⁺ T cells directly contribute to the antiasthmatic effects of AM. Notwithstanding this limitation, this study does suggest that AM can regulate the activity of CD4⁺CD25⁺Foxp3⁺ Treg cells in the BALF. Considering the strong evidence implicating Tregs in the control of allergic diseases [35,36], our results suggest that AM can stimulate CD4⁺CD25⁺Foxp3⁺ Tregs to regulate asthma. Moreover, a direct relationship could be shown by experiments investigating changes in the antiasthmatic effects of AM after Treg cell depletion or in vivo transfer. Further studies with more appropriate empirical approaches are still needed.

AM is a relatively safe Chinese herbal medicine especially when used in general dosage. However, toxicity or side effects of AM are still not fully understood. A recent study demonstrated that the safety dosage of AM could be as high as 39.9 g/kg for rats, while no distinct toxicity and side effects were observed after 3 months treatment in rats [37]. However, side effects in patients have been reported when AM is inappropriately or excessively used, including

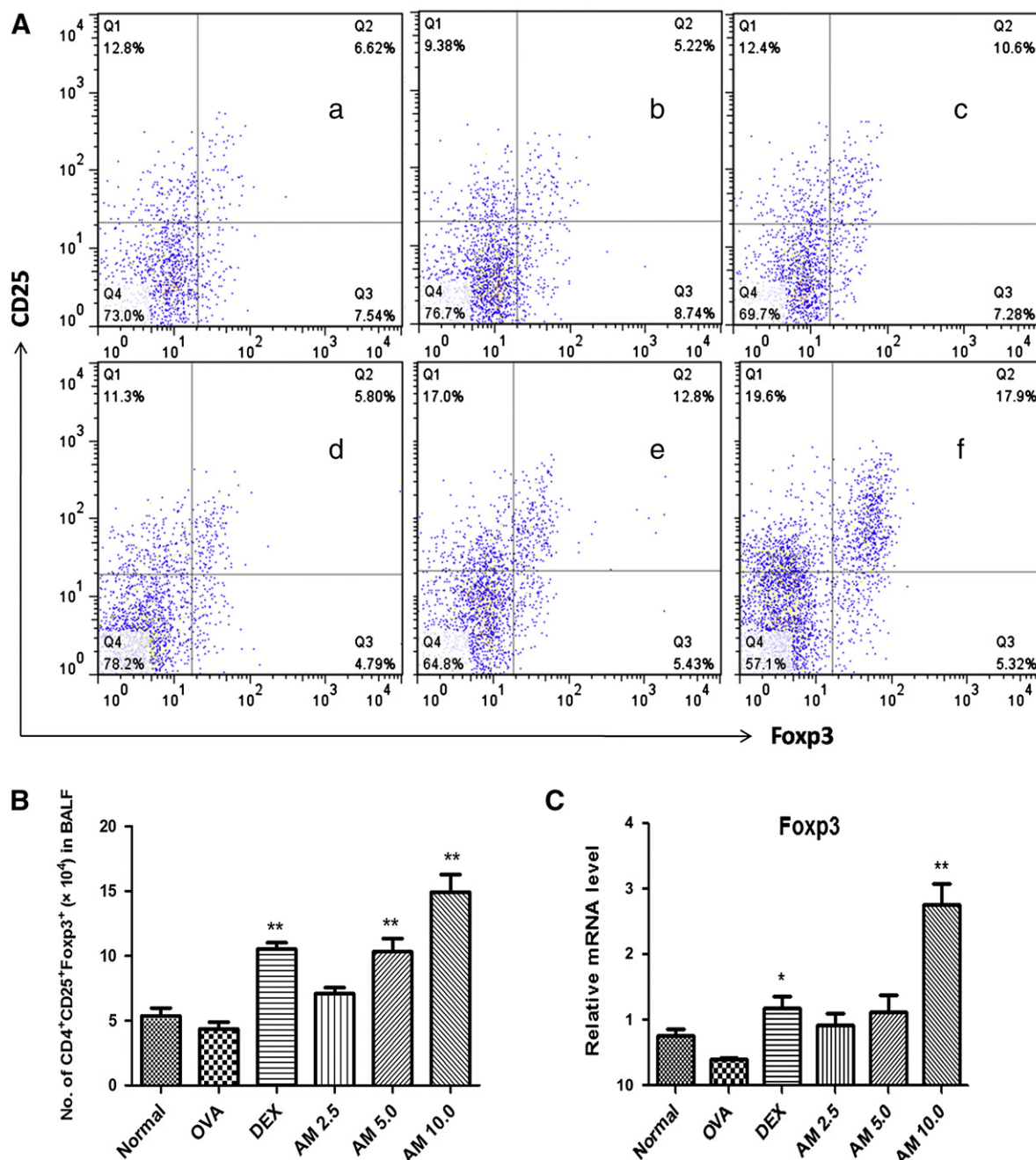


Fig. 6. The effect of oral AM treatment on the CD4⁺CD25⁺Foxp3⁺ Treg population of BALF cells in a rat model. (A) Representative plots from a single rat in each group. Numbers inside the histograms indicate the percentage of cells. (B) Population of CD4⁺CD25⁺Foxp3⁺ Treg cells in each group. (C) The mRNA expression of Foxp3 in lung tissues was analyzed by real-time RT-PCR. The results are expressed as the mean ± S.E.M. **P* < 0.05, ***P* < 0.01 versus the OVA group. Representative images are shown for each group: a: normal; b: OVA; c: DEX; d: AM 2.5 g/kg group; e: AM 5.0 g/kg group; and f: AM 10.0 g/kg group.

skin allergy, elevation of blood pressure, agrypnia, headache and gastrointestinal discomfort. Thus, it is meaningful to investigate therapeutic effects of critical components in AM in near future which will help reduce the dosage of AM and potential side-effects.

In summary, the present study demonstrates that AM can effectively attenuate airway inflammation, inhibit release of TH2 cytokines, and improve lung function and airway remodeling in a rat model of asthma. Importantly, the population of CD4⁺CD25⁺Foxp3⁺ Treg cells and Foxp3 expression are significantly increased by AM administration. These findings support AM as a potent agent for asthma prevention and treatment and demonstrate that CD4⁺CD25⁺Foxp3⁺ Tregs may contribute to the antiasthmatic effect of AM.

Conflict of interest

None of the authors has any conflicts of interest.

Acknowledgments

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