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# $CD4^+CD25^+Foxp3^+$ 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.



## 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 $H_2O$ 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-β1 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 ± 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 μm) 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 ($\mu m^2/\mu m$) (collagen area on the basal membrane of airway/the perimeter of the basement membrane of bronchioles).

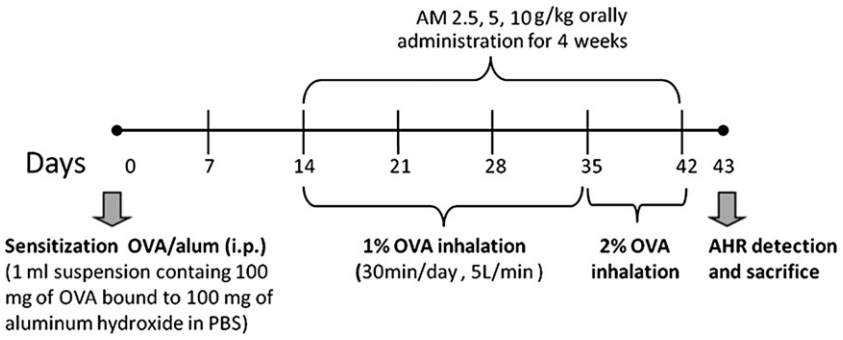


**Fig. 1.** Experimental protocol. Rats were sensitized and challenged by OVA as described in the Materials and methods section.