# Effects of Astragaloside IV on IFN-Gamma Level and Prolonged Airway Dysfunction in a Murine Model of Chronic Asthma

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**Key words**
- astragaloside IV
- asthma
- ovalbumin
- IFN-gamma
- chronic asthma

## Abstract

Astragaloside IV (AST) is the main active constituent of Radix Astragali, a Chinese herb traditionally used to prevent asthma attack from chronic asthma patients. Its efficacy and action mechanisms in asthma attack prevention remain nonetheless to be further explored. In this study, chronic asthma was induced exposing ovalbumin (OVA) sensitized mice to repeated OVA challenges twice every two weeks for 12 weeks. Mice were treated with AST for 4 weeks just after the final challenge. In this murine model of chronic asthma, the airway dysfunction and remodeling remained severe and was accompanied with suppression of the IFN-gamma level in the bronchoalveolar lavage fluid (BALF) even four weeks after the final challenge, indicating that the airway structural changes continued to develop even after interruption of OVA challenges. However, after AST treatment, the airway hyperresponsiveness was sharply relieved, accompanied by the reduction of collagen deposition and mucus production, meanwhile the inflammatory cells were decreased but the IFN-gamma level increased in BALF. In conclusion, AST could prevent the development of chronic asthma, thus reducing asthma attacks. Our results indicated that it should be used as a supplementary therapy on preventing asthma attacks from chronic asthma patients.

**Supporting information** available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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## Introduction

Asthma is characterized by inflammation, reversible airway obstruction, and increased airway responsiveness to various stimuli [1]. The public health burden of chronic asthma has increased over the past two decades [2]. So far the recommended treatment protocol by the Global Initiation for Asthma (GINA) on child's chronic asthma was the inhalation of glucocorticoids combined with $\beta 2$ receptor agonists or leukotriene receptor antagonists. Results from many studies have shown that inhaled glucocorticosteroids benefit patients with chronic asthma by decreasing airway inflammation, improving lung function, lessening symptoms and airway hyperresponsiveness [3, 4], but a large scale of clinical trial published in the New England Journal of Medicine (NEJM) recently indicated that in preschool children at high risk for asthma, a period of two years of inhaled-corticosteroid therapy did not change the development of asthma symptoms or lung function during a third, treatment-free year [5]. The results indicated that the inhalation of corticosteroid did not further prevent asthma attacks, which might be due to the failing improvement of the structural changes of airway wall.

In China, chronic asthma patients always supplemented herbal medicines to help prevent an asthma attack, and Radix Astragali is one of the most widely prescribed herbal medicines with this purpose. Its main active constituent is believed to be astragaloside IV (AST). AST is a natural saponin which has been used for quality control of Radix Astragali in the 2010 edition of Pharmacopoeia of the People's Republic of China.

AST has been proven to possess varied pharmacological activities. Many investigators have confirmed the neuron-, cardio-, and gastro-protective effects of AST. The mechanisms undermined might be ascribed to the prevention of depression in SR $Ca^{2+}$ handling and sarcoplasmic reticulum $Ca^{2+}$ transport, the upregulation of SOD-1 content, the antioxidant properties, and the regulation of tight junctional proteins in the endothelial cells of the blood-brain barrier [6–18]. Besides,

AST also has a benefit in the treatment of diabetes mellitus and hepatic fibrosis [19–21]; the protective mechanisms of AST on hepatic fibrosis might be due to its inhibition of liver collagen synthesis and hepatic stellate cell proliferation [21].

Apart from these, AST has also exhibited many activities related to asthma. For example, it possesses anti-inflammatory activity via inhibition of the NF-kappa B pathway, it can have an influence upon the immune system and has been used to treat asthma [22–25]. However, although Radix Astragali has been used to prevent asthma attacks for hundreds of years, the efficacy and the action mechanisms of AST, its main active component, on this process remained unexplored. The aim of this study was to investigate the efficacy and understand the mechanisms of AST in preventing asthma attacks.

Chronic asthma can be induced in ovalbumin (OVA) sensitized mice by exposing them to repeated OVA challenges twice every two weeks for 12 weeks [26]. Airway hyperresponsiveness and airway remodeling are the two important characters of chronic asthma, meanwhile Th1 and Th2 cytokines also play important roles in repeated asthma attack. Surprisingly, in this murine model of chronic asthma, the airway hyperresponsiveness and remodeling remained severe, accompanied with suppression of the IFN-$\gamma$ level in BALF even four weeks after the final challenge. The pathological progression of chronic asthma in mice was similar to the chronic asthma in patients who had repeated asthma attacks. In order to illustrate the efficacy and action mechanisms of AST in preventing asthma attacks, we established this mice model of chronic asthma, on which the effects of AST on airway hyperresponsiveness, airway remodeling, and Th1, Th2 cytokine levels were observed. Glucocorticoids, $\beta$2 receptor agonist, and leukotriene receptor antagonist are the recommended asthma treatment protocols by GINA. In this study, AST was administered orally and might influence cytokine levels of chronic asthmatic mice, so we chose montelukast, a leukotriene receptor antagonist as a positive control to compare the effects of AST in preventing asthma attacks, as glucocorticoids were administered per inhalation and $\beta$2 receptor agonists might have no effects on cytokines levels of chronic asthmatic mice.

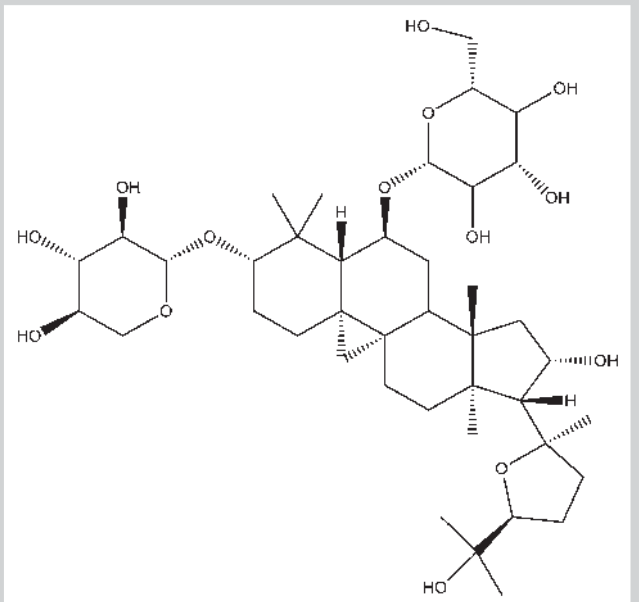


**Fig. 1** Chemical structure of AST.

## Materials and Methods

### Chemicals

AST (purity > 98% by HPLC method) (**Fig. 1**) was purchased from J & K. Montelukast (montelukast sodium salt, purity > 98%) was purchased from Cayman Chemical Company.

### Animals

Female Balb/c mice purchased from Slac Inc., aged 8 to 10 weeks, were housed in environmentally controlled, specific pathogen-free conditions for 1 week prior to the study and for the duration of the experiments. All animal studies were approved by the Animal Research Committee of the Nanjing University of Chinese Medicine.

### Sensitization and challenge

The sensitization and challenge protocol was employed as described previously [26–28], with small modifications. Briefly, each mouse was sensitized by i.p. injection of 80 µg OVA conjugated to 1 mg aluminum potassium sulfate (Sigma) in a total volume of 0.2 mL on days 1 and 11 and aeroallergen inhaled OVA (5 mg/mL) on day 11. Sensitized mice were exposed to six 2-day periods of aeroallergen challenges (OVA, 5 mg/mL), each separated by 12 days (from day 19 to day 90). Control mice were subjected to the same sensitization protocol but received aeroallergen saline challenges. Twenty-four hours after the final exposure to allergen, mice were randomly divided into four groups: AST mice groups were administered AST intragastrically at doses of 50 or 150 mg/kg daily for four weeks; montelukast group mice were administered montelukast 2.6 mg/kg for four weeks [29]. In OVA and control groups, mice were administered the same volume of solvents. Twenty-four hours after the final administration, the following outcome measurements were made: (i) *in vivo* airway responsiveness to intravenous methacholine (MCh; Sigma); (ii) total and differential cell counts in bronchoalveolar lavage (BAL) fluids; (iii) IL-4 and IFN-$\gamma$ levels in BALF supernatants; and (iv) airway morphometry (**Fig. 2**). For aeroallergen sensiti-

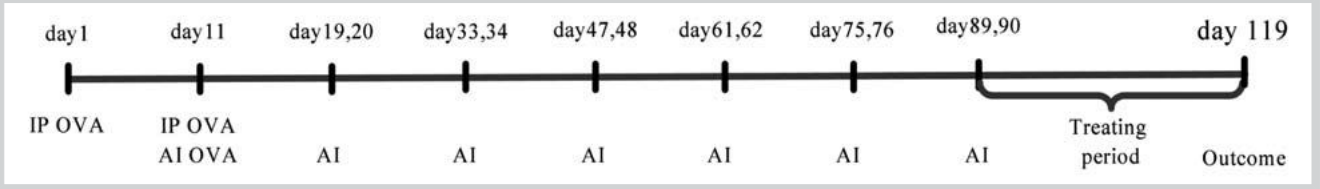


**Fig. 2** Study protocol. Sensitization, challenge, and drug administration protocol used in our study. IP: intraperitoneal injection, AI: aeroallergen inhaling.

zations and challenges, mice were placed in a Plexiglas box (22 cm × 22 cm × 50 cm) and subjected to aeroallergen OVA with a ultrasonic atomizer (S-888E; Daofen).

### Airway responsiveness

Airway responsiveness was measured based on the response of lung resistance ($R_L$) to saline and increasing (10, 30, 100, and 300 µg/kg) intravenous doses of methacholine (MCh; Sigma). $R_L$ was measured with AniRes 2005 animal lung function analysis systems (SYNOL High-Tech). The mice were anesthetized by i.p. injection of pentobarbital sodium (60 mg/kg); a plastic cannula was then inserted into the trachea for mechanical ventilation and measurement of airway pressure, air flow rate, and tidal volume. A 27-gauge needle was punctured into the caudal vein for drug administration. The mice were then placed in a whole-body plethysmography chamber and ventilated mechanically at a rate of 90 breaths per minute with a tidal volume of 5 ~ 6 mL/kg. After reaching a stable airway pressure recording, MCh was administered intravenously in progressive doses (10, 33, 100, 300 µg/kg). After each dose, the data were recorded for 5 minutes, and the $R_L$ was calculated by the AniRes 2005 animal lung function analysis systems.

### Bronchoalveolar lavage

Following the airway responsiveness measurement, the BALF was acquired as described previously [30]. Briefly, 0.6 mL of ice-cold PBS was instilled into the mice lung through a tracheal cannula, followed by gentle aspiration and repeated two additional times. Fluids from all three lavages was pooled and centrifuged at 1000 g for 10 minutes, the supernatant of BALF was collected and frozen at − 70 °C for IL-4 and IFN-γ determination and the cell pellet was resuspended with 0.5 mL PBS. The total inflammatory cells in BALF were counted with a hemocytometer. Differential cell counts were conducted by cytospin techniques and Fast Wright-Giemsa Stain (Jiancheng); at least 200 cells per slide were counted [31].

IL-4 and IFN-γ levels in BALF were evaluated using a commercially available ELISA kit (Keygene).

### Lung histology and morphometry

Lungs of mice were removed from the chest cavity and fixed by injection of 4% buffered paraformaldehyde (1.0 mL) into the tracheal cannula at a pressure of 20 cm $H_2O$ and immersed in paraformaldehyde for at least 24 hours. Three 5-µm thick sections were cut. One section was stained with hematoxylin and eosin for histological assessment using light microscopy, and the remaining sections were stained with Masson's Trichrome, to demonstrate the presence of collagen deposition, and with periodic acid Schiff (PAS) to demonstrate the presence of mucin within goblet cells. Histological analyses were performed by a pathologist blinded to the groups. The positive peribronchiole areas stained with Masson's Trichrome were quantified with a light microscopy hybrid to an Image-Pro Plus image analysis system (Media Cybernetics). For sections stained with Masson's Trichrome as described previously, the region of interest was a 20-µm band immediately beneath the epithelium. Thereafter the results were expressed as the area of Trichrome staining in proportion to the total area of interest by counting at least 6 bronchioles in each slide [32]. To evaluate the level of mucus expression in the airway, the number of PAS-positive epithelial cells (goblet cells) in individual bronchioles was counted as described [33]; at least 6 bronchioles were counted in each slide. Results were expressed as the percentage of PAS-positive cells per bronchiole.

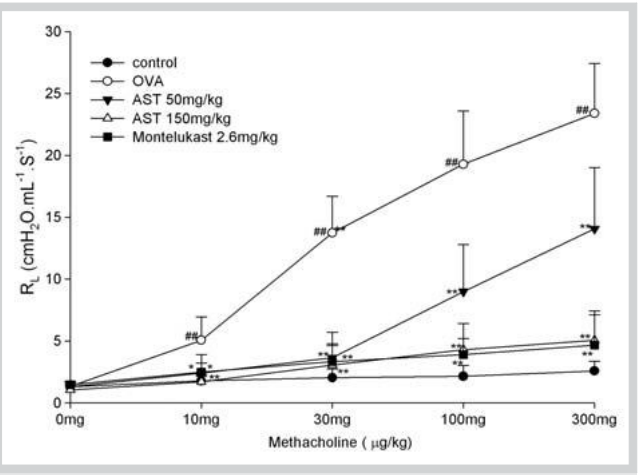


**Fig. 3** AST decreased the airway hyperresponsiveness in asthmatic mice. Values are expressed as mean ± SEM; six animals were assessed per group. Significant differences between OVA and control group are shown as ## $p < 0.05$; between OVA and AST or montelukast treated groups are shown as * $p < 0.05$, ** $p < 0.01$.

### Statistical analysis

Reported values are expressed as means and SEM. Comparisons were made using one-way analysis of variance (ANOVA), followed by S-N-K tests for comparing all pairs of groups. All comparisons were two-tailed, and p values ($p < 0.05$) were considered to be significant.

### Supporting information

Data on hematoxylin and eosin (HE), Masson's Trichrome, and PAS stained sections of the airway wall in asthmatic mice are available as Supporting Information.

## Results

There were no significant differences in baseline airway resistance among the four groups. The $R_L$ in the OVA group was obviously increased in a dose-dependent manner by MCh administration and slightly increased in the control group. AST 50 and 150 mg/kg and montelukast 2.6 mg/kg could sharply decrease the $R_L$ values compared with the OVA group ($p < 0.05$, $p < 0.01$) (**Fig. 3**).

In the control group, few inflammatory cells were observed in BALF, but in the OVA group, the inflammatory cells in BALF were obviously increased ($p < 0.01$). AST 150 mg/kg and montelukast 2.6 mg/kg significantly decreased the number of eosinophils, neutrophils, lymphocytes, and total inflammatory cells in BALF ($p < 0.05$, $p < 0.01$), while AST 50 mg/kg only decreased neutrophils, lymphocytes and total inflammatory cells but not eosinophils in BALF (**Fig. 4**).

We examined the IL-4 and IFN-γ levels in the supernatant of BALF and found that the IL-4 level showed no differences among control and OVA groups, but the IFN-γ level in the OVA group was dramatically decreased ($p < 0.05$). AST 50 and 150 mg/kg significantly increased the IFN-γ level in BALF and subsequently raised the IFN-γ/IL-4 ratio ($p < 0.05$, $p < 0.01$). Unlike AST, montelukast

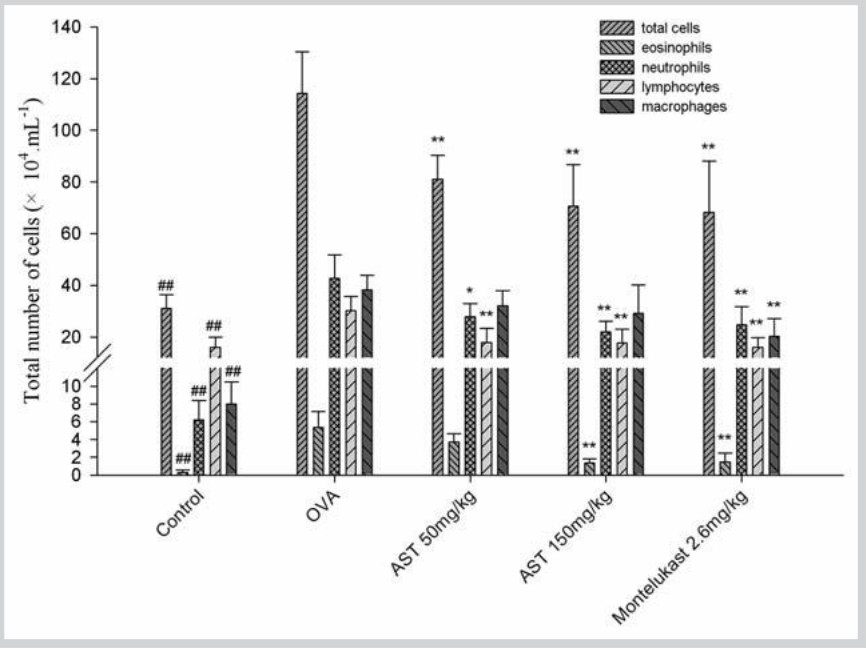


**Fig. 4** Total inflammatory cells and cell differentiation in BALF asthmatic mice. Values are expressed as mean ± SEM; six animals were assessed per group. Significant differences between OVA and control group are shown as $^{\#\#}$ $p < 0.01$; between OVA and AST or montelukast treated groups are shown as $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

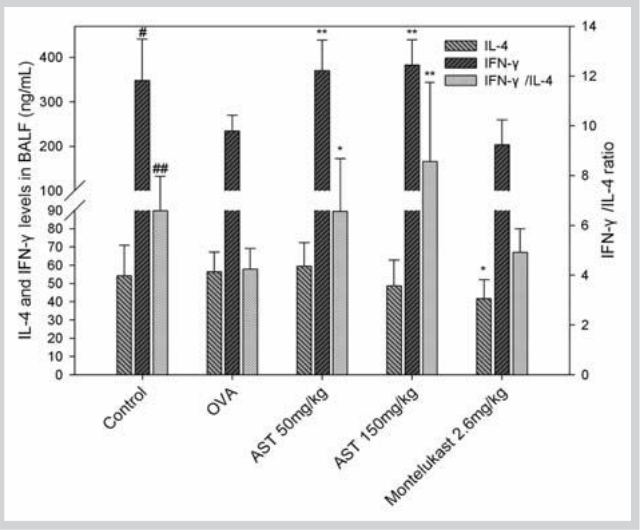


**Fig. 5** IL-4 and IFN-γ levels in BALF asthmatic mice. Values are expressed as mean ± SEM; six animals were assessed per group. Significant differences between OVA and control group are shown as $^{\#\#}$ $p < 0.01$; between OVA and AST or montelukast treated groups are shown as $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

exhibited no influence on the IFN-γ level, which could only slightly reduce the IL-4 level in BALF, thus it could not raise the IFN-γ/IL-4 ratio (**Fig. 5**).

A small eosinophil infiltration was detected in the control mice lung by hematoxylin and eosin staining. Sensitization and challenge with OVA resulted in a little amount of eosinophil infiltration and thickening of the airway wall. AST and montelukast completely inhibited eosinophil infiltration, and the diameters of the airway wall were back to its normal condition (**Fig. 1S**).

Collagen deposition is the main pathological change of asthmatic bronchus. In our study, OVA group mice developed universal collagen deposition throughout the lung interstitium around the airway wall, whereas mice in the control group developed slight collagen deposition ($p < 0.05$, $p < 0.01$) (**Fig. 6**, **1S**). Treatment with AST and montelukast caused a sharp reduction of collagen deposition in the lung interstitium around the airway wall.

Mucin production is another character of asthmatic bronchus. We observed the mucin production by PAS staining. The number of goblet cells staining positively with PAS for mucin was significantly increased in the OVA mice group compared with the control group mice ($p < 0.01$). AST and montelukast sharply decreased the presence of mucin compared with the OVA group ($p < 0.05$, $p < 0.01$) (**Fig. 6**, **1S**).

## Discussion

In this study, we confirmed that mice had developed prolonged airway hyperresponsiveness and signs of airway remodeling in response to chronic allergen exposure. However, we also demonstrated that in this model of mild chronic asthma, the IFN-gamma level in BALF was dramatically decreased without the IL-4 level increasing, indicating that in this stage of asthma, the inflammation of the airway was alleviated, which was also confirmed by the small amount of airway eosinophil infiltration.

It is well known that Th1 and Th2 cytokines play an important role in prolonged airway hyperresponsiveness in addition to airway remodeling. Although many studies confirmed that Th2 cytokine contributes to the initial pathology of asthma, there were more and more evidences indicating that Th1 cytokine might contribute to the prolonged airway hyperresponsiveness (AHR) [34]. Truyen et al. measured the mRNA levels of Th1 and Th2 cytokines in induced sputum and confirmed the predominance of Th2 cytokine in asthma. The IL-5 level reflected infiltration and hyperreactivity, whereas the IFN-γ level indicated asthma severity [35]. Meanwhile several studies have proved that the increase of local lung Th1 immune response with the subsequent increase of the Th1/Th2 cytokine secretion ratio might attenuate symptoms of asthma [36–40]. Herbert et al. found that dexamethasone, a glucocorticoid, could inhibit both Th1 and Th2 type cytokines; and roflumilast, a selective phosphodiesterase-4 in-

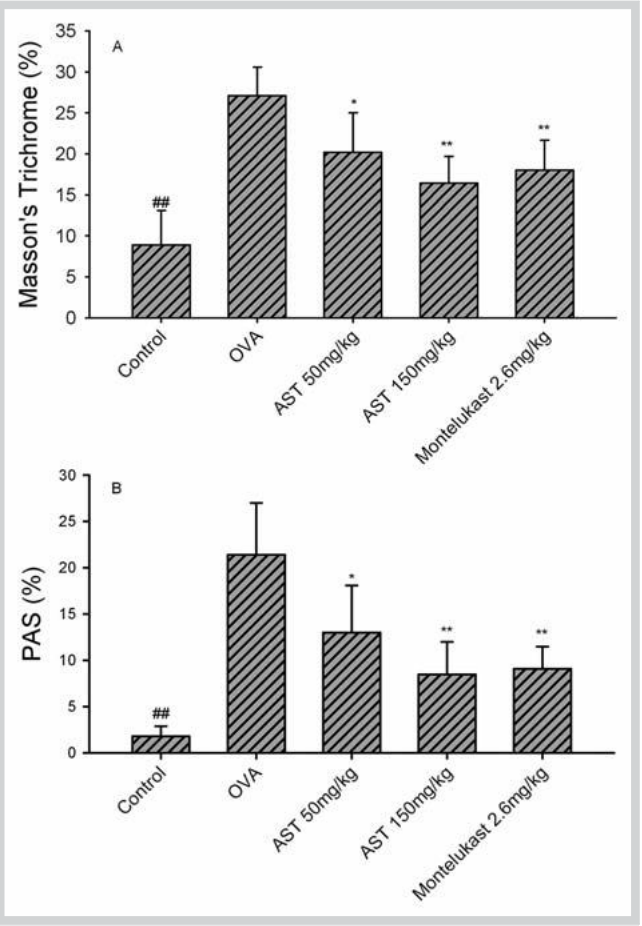


**Fig. 6** Morphometric changes of airway in asthmatic mice. **A** Staining as assessed using morphometry for collagen deposition (Masson's Trichrome) in the 20 µm region beneath epithelium of the airway wall in mice. **B** Staining as assessed using mucin in the epithelial portion of the airway wall in mice. Values are expressed as mean ± SEM; six animals were assessed per group. Significant differences between OVA and control group are shown as $^{\#\#}$ $p < 0.01$; between OVA and AST or montelukast treated groups are shown as $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

hibitor, selectively inhibited a subset of pro-inflammatory cytokines [41]. In our study, montelukast, a leukotriene receptor antagonist, reduced the IL-4 levels in BALF but had no effect on the IFN-γ level and the Th1/Th2 ratio. Unlike montelukast, AST, a natural compound from Chinese herbal medicines, could increase the IFN-γ level and the Th1 cytokine and subsequently enhance the Th1/Th2 ratio, which might be the main mechanism of AST on reducing airway hyperresponsiveness.

Subepithelial fibrosis is an important characteristic feature of the asthmatic bronchus. It appears to consist of a plexiform deposition of collagen I and III, tenascin, and fibronectin proteins that are mainly produced by activated myofibroblasts [42]. Many investigators reported the structural airway changes following chronic allergen exposure, but in those studies, the dysfunction and remodeling of the airway was observed immediately after the final allergen exposure. In our study, mice developed universal collagen deposition even 4 weeks after the final allergen exposure, which might contribute to the prolonged airway dysfunction. AST and montelukast could sharply reduce collagen deposition, the main feature of airway remodeling, and thus ameliorate sustained airway hyperresponsiveness.

Goblet cell hyperplasia and mucus hyperproduction are also important features of airway remodeling in chronic asthma and contribute substantially to morbidity and mortality. Goblet cells are the leading source of mucin glycoproteins, which are the main constituents of airway mucus and the major determinants of its viscoelastic and adhesive properties [43]. It has been reported that the Th1 cytokine IFN-γ inhibited airway mucus production induced by Th2 and non-Th2 inflammatory responses [44]. Interestingly, AST not only inhibited mucin production, but increased the IFN-γ level in BALF as well, suggesting that the reduction of mucin production by AST might be attributed to the increase of the IFN-γ level. Meanwhile, montelukast also caused the reduction of mucin production without increasing the IFN-γ level in BALF, suggesting that the effects of montelukast on mucin production were not associated to Th1 cytokines. They might be associated with the suppression of Th2 cytokines and the powerful anti-inflammatory activity.

Compared with montelukast, AST exhibited similar activities on airway hyperresponsiveness and airway remodeling. In addition, AST increased the IFN-γ level and the Th1/Th2 ratio, while montelukast had no effect on the IFN-γ level and the Th1/Th2 ratio but reduced the IL-4 level. In comparison to the control mice, AST adjusted the cytokines to their normal levels, suggesting that AST might be more suitable on preventing the development of chronic asthma than montelukast.

In conclusion, AST could prevent the development of chronic asthma, thus reducing asthma attacks. The mechanism of AST on preventing asthma attacks might be the reduction of airway hyperresponsiveness, which was probably due to the decrease of collagen deposition and mucus production of the airway wall and the increasing of the IFN-gamma level. Our results suggest that AST could serve as a supplementary therapy on preventing asthma attacks in chronic asthma patients.

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X. Y. and S. S. contributed equally to this work. The authors would like to thank Prof. Su Ning for his assistance with Masson's Trichrome and PAS staining. This work was financially supported by grant # 30772822 from the National Natural Science Foundation of China.

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