

Protective Effects of Astragaloside IV against Ovalbumin-Induced Lung Inflammation Are Regulated/Mediated by T-bet/GATA-3

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Key Words

Asthma · T-bet · GATA-3 · Astragaloside IV

Abstract

Bronchial asthma is characterized by chronic lung inflammation, airway hyperresponsiveness, and airway remodelling. Astragaloside IV (3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl-cycloastragenol, AST), the primary pure saponin isolated from the root of *Astragalus membranaceus*, is an effective compound with distinct pharmacological effects including anti-inflammation, immunoregulation, and antifibrosis. However, the effect of AST on asthma remains unclear. In the present study, in the murine model of asthma, the airway hyperresponsiveness was relieved after treatment with AST, accompanied by a reduction of inflammatory cells. In addition, the levels of IL-4 and IL-5 decreased, while the IFN-γ level increased, in bronchoalveolar lavage fluid. The compound also significantly inhibited the synthesis of GATA-3-encoding mRNA and protein in addition to increasing the synthesis of T-bet-encoding mRNA and protein in both lung tissues and CD4⁺ T cells. Our findings indicate that AST treatment inhibits ovalbumin-induced airway inflammation by modulating the key master switches GATA-3 and T-bet, which results in committing T helper cells to a Th1 phenotype.

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Introduction

The prevalence of asthma has significantly increased since the 1970s; it was estimated that about 300 million people were affected worldwide in 2010 [1]. It is thought that the T cell response is critical for the development of the disease – in particular, the development of allergen-specific Th2 cells and their resultant cytokine products, IL-4, IL-5, and IL-13, which have effects on cells of the lung. Consequently, shifting T cells towards a Th0 or Th1 phenotype may present a therapeutic strategy for the treatment of asthma [2, 3].

Astragalus membranaceus Bunge, a perennial belonging to the Fabaceae family, delivers an important herbal drug in traditional Chinese medicine, which has been widely used for centuries to treat asthma in China. Astragaloside IV (3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl-cycloastragenol, AST), the primary pure saponin isolated from the root of *A. membranaceus*, is an effective compound with distinct pharmacological effects including anti-inflammation, immunoregulation [4, 5], and antifibrosis [6, 7]. Our previous study showed that AST treatment markedly inhibited airway inflammation and airway hyperresponsiveness and exhibited profound immunoregulatory activities both in vitro and in vivo [8]. Moreover, AST could prevent the development of chronic asthma and in-

crease the IFN- γ level in bronchoalveolar lavage fluid (BALF) [9]. However, it is unclear whether AST treatment might mediate antiallergic responses via the regulation of Th1 and Th2 responses. We undertook the present study to investigate the critical mechanisms of the protective effects of AST against allergic inflammation.

Materials and Methods

Chemicals

AST (purity >98%) was purchased from Nanjing Zelang Medical Technology Co. Ltd. It was suspended in a 1% carboxymethylcellulose solution.

Animals and Groups

Male and female BALB/c mice (age 6–8 weeks) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All animals were maintained in specific pathogen-free conditions, had free access to food and water, and were routinely serologically screened for the relevant respiratory pathogens. The animals were then randomly assigned to four groups of 8 mice each. The two AST treatment groups were given AST intragastrically at doses of 50 or 150 mg/kg daily for 4 weeks. In the ovalbumin (OVA) group, OVA was given to each mouse. The mice left untreated served as controls.

Sensitization and Airway Challenge

The sensitization and challenge protocols were employed as previously described [10–12], with minor modifications. Briefly, each mouse in the OVA and AST treatment groups was sensitized by intraperitoneal injection of 100 μ g OVA (grade V; Sigma, St. Louis, Mo., USA) conjugated to 400 μ g 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 Co., Tokyo, Japan), which was continued for 4 weeks. Mice in the AST treatment groups were administered intragastrically with AST at doses of 50 or 150 mg/kg daily for 4 weeks 30 min before the OVA challenge. The control group were given an intraperitoneal injection of 0.2 ml PBS and inhaled PBS. At 30 min before inhaling PBS, the mice in the control group were administered intragastrically with 1% carboxymethylcellulose in a volume of 10 ml/kg.

Measurement of Airway Responsiveness to Acetylcholine Chloride

For the measurement of airway responsiveness to acetylcholine chloride, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg) 24 h after the final challenge. A plastic tube with an internal diameter of 2 mm was inserted into the trachea via tracheotomy for mechanical ventilation, and a 27-gauge needle was inserted into the caudal vein. The mice were placed in a whole-body plethysmograph and then mechanically ventilated with an animal ventilator (AniRes2003; Beijing SYNOL High-Tech Co. Ltd., China) with a tidal volume of 5–6 ml/kg and a frequency of 90–120 breaths/min. The baseline airway expiration resistance (R_e) was continuously measured and recorded for the subsequent analysis. The mice were then injected with saline, and

1 min later, acetylcholine chloride was injected at a series of doses (10, 30, 90, and 270 μ g/kg) for 1 min each until the R_e increased to twice that of the baseline level.

BALF and Accumulation of Inflammatory Cells

Following the measurement of airway responsiveness, 0.5 ml PBS was instilled into lungs, and BALF was obtained by 3 aspirations (total volume 1.5 ml) via tracheal cannulation. Each aliquot of BALF was centrifuged (1,000 g, 4°C, 10 min), and the supernatant was collected and stored at –70°C. The total amounts of the inflammatory cells were assessed by counting cells on a haemocytometer with at least 5 squares after exclusion of dead cells stained with trypan blue. The cell pellets were washed 3 times with PBS, resuspended in 1.0 ml PBS and 100 μ l BALF, and then placed on a slide and centrifuged (200 g, 4°C, 10 min) for fixation using cyto-spin techniques and fast Wright-Giemsa staining; then, the amounts were estimated by counting at least 200 cells per slide.

Histopathological Examinations of Lung Tissues

Following bronchoalveolar lavage, the left lungs were fixed by immersion in 10% (v/v) neutral buffered formalin, trimmed, and embedded in paraffin. They were then cut into 5- μ m sections and stained with H&E solution (haematoxylin: Sigma MHS-16; eosin: Sigma HT110-1-32). Histological analyses were performed, and eosinophils in the lungs and the area of airway smooth muscle (ASM)/bronchial internal perimeter (P_i) were quantified using an Olympus BX40F4 microscope and an FR-988 image processing system (Smart Scape 2002; China). Five high-power fields in the area of peripheral bronchi were randomly selected for quantification. Counts are given as eosinophils per square millimetre.

Measurement of Th1 and Th2 Cytokines in BALF

ELISA was performed according to the manufacturer's instructions. IFN- γ , IL-4, and IL-5 levels in the BALF were determined using specific murine IFN- γ , IL-4, and IL-5 ELISA kits (R&D, USA).

CD4+ T Cell Isolation, Culture, and Flow Cytometry

Twenty mice received an intraperitoneal injection of 100 μ g OVA emulsified in 400 μ g aluminium hydroxide in a total volume of 0.1 ml on days 0 and 7. Seven days after the second immunization, spleens were excised aseptically from the mice after cervical dislocation and placed in RPMI medium. The spleens were coarsely chopped, and single-cell suspensions were prepared by dispersing the tissues through a 75- μ m nylon tissue strainer. The cells were washed in RPMI medium, followed by lysis of erythrocytes with ammonium chloride reagent (BD Pharmingen, San Diego, Calif., USA). CD4+ T cells were isolated from splenocytes by positive selection using the MACS CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Germany). In brief, CD4+ T cells were separated by passing the cell suspension over a magnetically activated cell sorter LS column held in the MACS magnetic separator (Miltenyi Biotec). The isolation of CD4+ T cells is achieved by depletion of magnetically labelled cells. The final composition of the purified CD4+ T cells was determined by flow-cytometric analysis with CD4+ T cell enrichment of 70–90%.

Purified CD4+ T cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mmol/l L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 μ g/ml streptomycin (Life Technologies) at a density of 1×10^6 /ml (6 ml/well) in 6-well culture plates. CD4+ T cells were cultured

with PMA (25 ng/ml). The CD4⁺ T cells were cultured with OVA (100 µg/ml), except in the control group. In the AST treatment group, they were cultured with AST (100 µg/ml). The mixtures were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Cell pellets harvested after culture for 24 h were analysed for the subsequent isolation of mRNA and proteins of T-bet and GATA-3.

Determination of T-bet and GATA-3 Expressions

Total RNA was isolated from each lung tissue sample and the CD4⁺ T cells using TRIzol[®] Reagent (Invitrogen, USA), and reverse transcriptase polymerase chain reaction (RT-PCR) was performed for the quantification of mRNA in lung tissue. First-strain cDNA was synthesized from an equal amount of total RNA with M-MLV reverse transcriptase. Genes of T-bet and GATA-3 were amplified by PCR using sense and antisense primers of T-bet and GATA-3 as previously described [13], with some modifications. T-bet primers were designed corresponding to the murine T-bet gene sequence outside of the T-box domain to maintain its specificity for T cells. GATA-3 primers were designed according to the corresponding structures of murine genes. The primers were as follows: T-bet: sense 5'-AACCAGTATCCTGTTCCAGC-3', antisense 5'-TGTCGCCACTGGAAGGATAG-3'; GATA-3: sense 5'-CTTCCCAGAGCGAACCTGTG-3', antisense 5'-GCAGAACCCGTGTTTGACG-3', and β-actin: sense 5'-TCCTGTGCGATCCATGAACT-3', antisense 5'-GAAGCATTTGCGGTGCACGAT-3'. The PCR annealing temperatures were 58°C for T-bet and 55°C for GATA-3 and β-actin. Semiquantitative RT-PCR was performed using β-actin as an internal reference to normalize gene expression for the PCR templates. The PCR product sizes were as follows: 436 bp for T-bet, 269 bp for GATA-3, and 300 bp for β-actin. The PCR products were studied on 1.5% agarose gels, and the amplified bands were visualized after staining with ethidium bromide. The size of the amplified fragments was determined by comparison with a standard DNA marker.

Real-time RT-PCR was performed on CD4⁺ T cells. Reverse transcription was performed using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Quantitative real-time PCR was carried out with a LightCycler (Roche Diagnostics, Germany) using Premix Ex Taq (TaKaRa, Japan) to detect the products. The reactions were cycled 40 times under the appropriate parameters for the primers, and the fluorescence was measured every 15 s at the end of each cycle to construct the amplification curve. GAPDH was loaded as the control, with the primers forward 5'-CAATGTGTCGTCGTGGATCT-3', reverse 5'-GTCCTCAGTGTAGCCCAAGATG-3', and probe 5'(FAM)-CGTGCCGCCTGGAGAAACCTGCC(Eclipse)-3'. Specific primers and probes, respectively, for murine T-bet and GATA-3 were used: T-bet: forward 5'-CCTCTTCTATCCAACCAGTATCCTG-3', reverse 5'-TTCATAACTGTGTTCCCGAGGTG-3', and probe 5'-(FAM)TCCCAGCCGTTTC TACCCCGACCT(Eclipse)-3', and GATA-3: forward 5'-GCTACGGTGAGAGGTATCC-3', reverse 5'-TCCAGCCAGGGCAGAGATC-3', and probe 5'-(FAM)AGCCAGGTATGCCGCCCGCC(Eclipse)-3'. All determinations were repeated at least twice.

Determination of T-bet and GATA-3 Protein by Western Blot

Fifty micrograms of total proteins isolated from the whole lung and CD4⁺ T cells were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane for 2 h. The membrane was then incubated in blocking solution (5% dry milk in PBS/0.05% Tween 20) at room temperature for 1 h and subsequently exposed to

0.8 µg/ml of the monoclonal antibodies anti-GATA-3 (Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA), anti-T-bet (Santa Cruz Biotechnology Inc.), or anti-β-actin (Sigma Chemical Company, USA) overnight at 4°C. On the following day, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (1:1,000) at room temperature for 2 h, and enhanced chemiluminescence was used to detect and quantify the proteins on membranes following the manufacturer's instructions.

Ethical Consideration

The guidelines for the care and use of laboratory animals of the Nanjing Medical University were followed, and the protocols were approved by the Animal Ethics Committee of the Nanjing Medical University, Wuxi People's Hospital, and Jiangsu Provincial Department of Health.

Statistical Analysis

Data are described as means ± standard deviations (SD). Statistical significance was determined using ANOVA to compare independent mean values, followed by multiple comparisons by Dunnett's test, which was done in version 11.0 of the SPSS 11.5 software package (SPSS Inc., Chicago, Ill., USA). A p value of <0.05 was considered indicative of a statistically significant difference.

Results

AST Decreased Airway Responsiveness in OVA-Induced Asthma

To elucidate the effects of AST on airway hyperreactivity, we measured the airway resistance in anaesthetized mice using invasive whole-body plethysmography. As shown in figure 1, there were no significant differences in baseline airway resistance between the four groups. The airway resistance in the OVA group was obviously increased by acetylcholine chloride administration in a dose-dependent manner and slightly increased in the control group. AST at doses of 50 or 150 mg/kg sharply decreased the airway resistance compared with the OVA group (all p values <0.01; fig. 1).

AST Attenuated the Infiltration of Eosinophils into BALF, Inhibited the Infiltration of Eosinophils into the Lung, and Reduced the Thickness of the Bronchial Wall and Area of ASM

We examined changes of total cell levels in the BALF to determine the effects of AST on asthma. As shown in figure 2, the eosinophil levels markedly increased to levels greater than those of total leukocytes in the BALF of OVA-challenged mice. OVA-sensitized and -challenged mice treated with AST (50 and 150 mg/kg) displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis upon asthma induction. We found

Fig. 1. AST decreases airway responsiveness in OVA-induced asthma. AST at a dose of 50 or 150 mg/kg showed reduced airway responsiveness in comparison with that seen in the OVA group. Values are expressed as means $\pm$ SD. * $p < 0.01$, groups with AST at a dose of 50 or 150 mg/kg vs. OVA group.

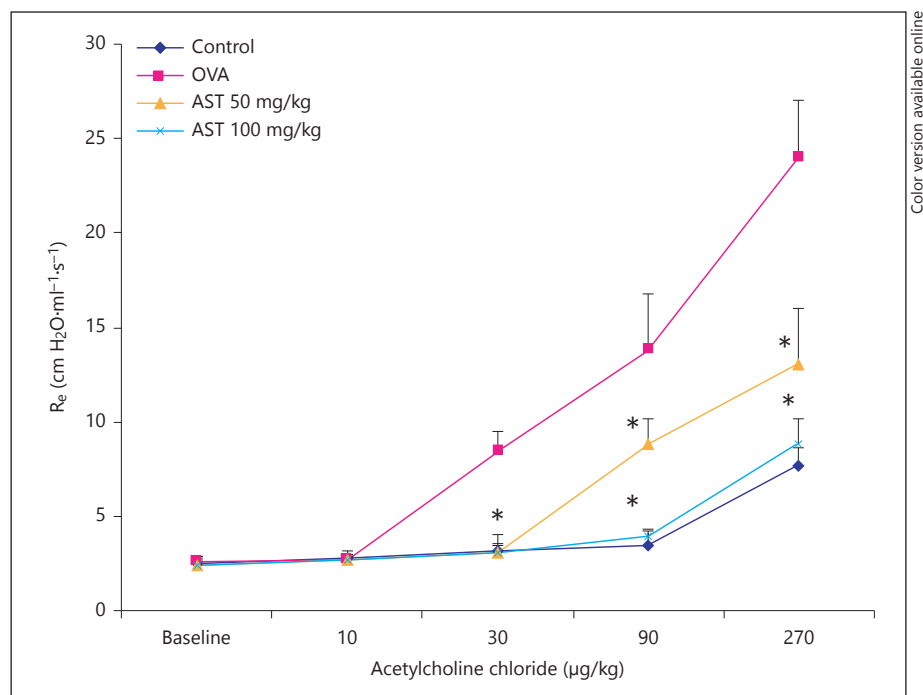
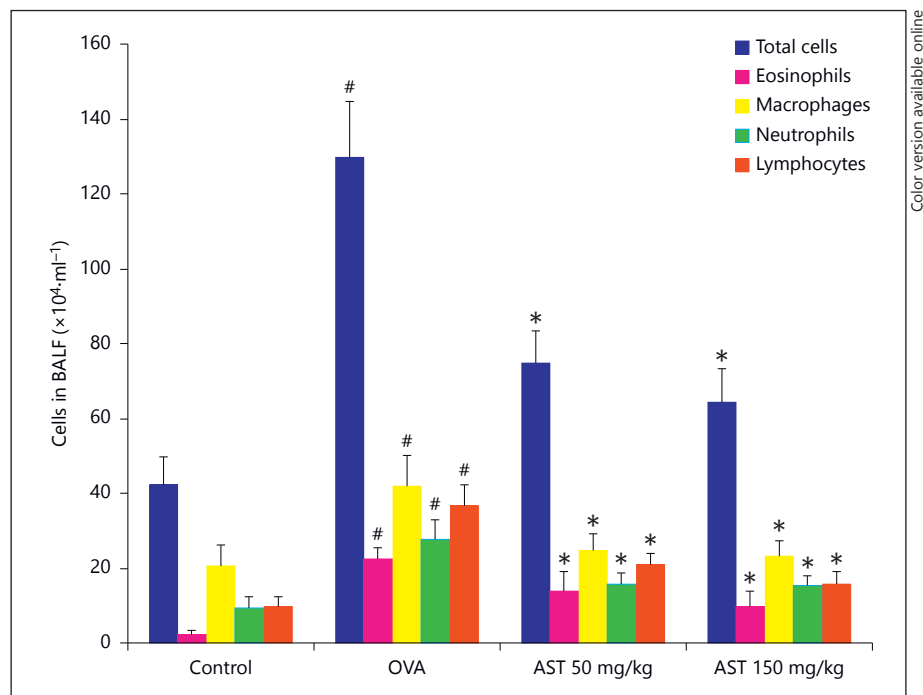


Fig. 2. Effect of AST on the recruitment of inflammatory cells in BALF. Cell amounts were assessed by direct counting under a light microscope in at least 5 squares of a haemocytometer after exclusion of dead cells stained with trypan blue. Values are expressed as means $\pm$ SD. # $p < 0.01$ vs. control group; * $p < 0.01$ vs. OVA group.



a 42.4–50.4% decrease in total cell amounts ($p < 0.01$; fig. 2) and a 39.2–44% suppression of eosinophil levels ($p < 0.01$; fig. 2) compared with the values in the OVA group.

In the further studies investigating the potential AST-induced suppression of lung inflammation, we as-

sessed the infiltration of eosinophils, thickness of the bronchial wall and area of the ASM in the lung. By morphometric analysis, the influx of eosinophils into the lung interstitium was significantly reduced after administration of AST compared with the asthmatic mice but

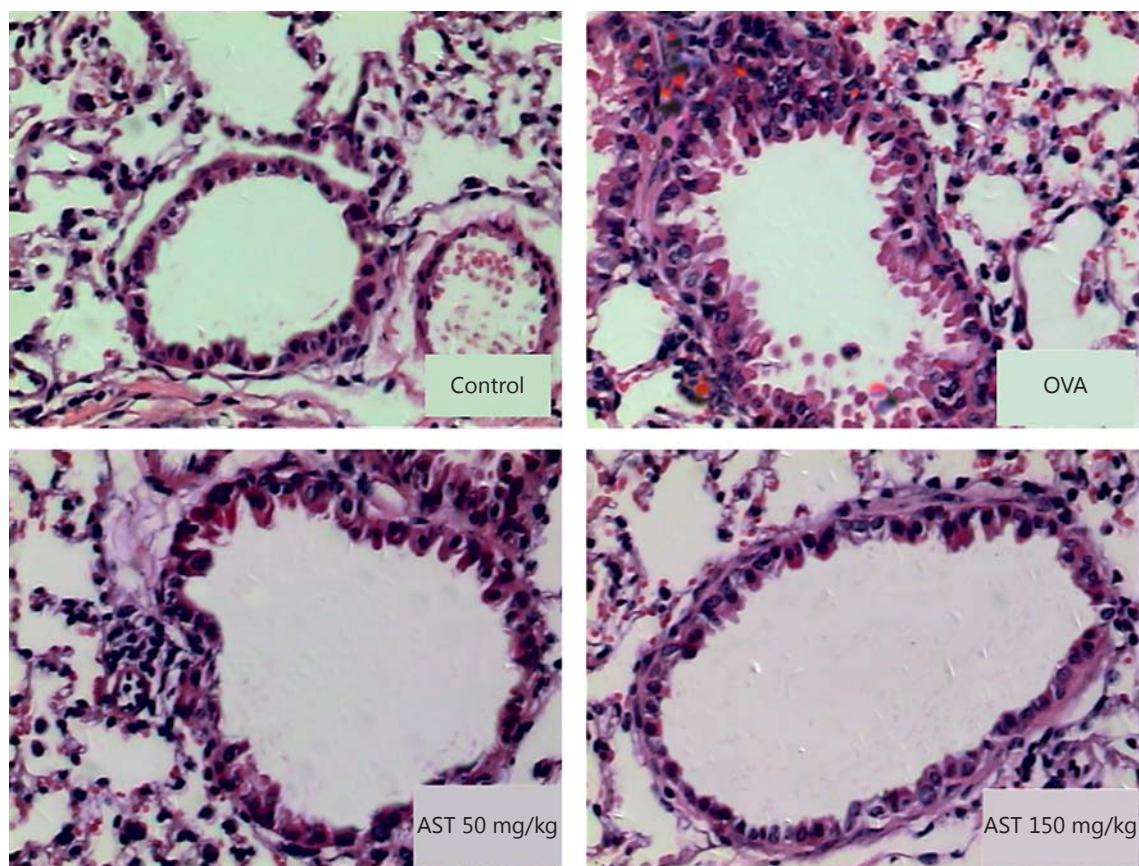


Fig. 3. Effect of AST on inflammatory cell recruitment into lung tissue. The histological examination of lung tissue was performed 24 h after the final OVA challenge. The lung tissues were fixed, cut into 5- μ m sections, and stained with H&E solution. $\times 200$.

increased compared with the control mice ($p < 0.01$; fig. 3, 4a). The thickness of the bronchial wall and area of the ASM were significantly reduced after administration of AST compared with the asthmatic mice. However, the AST-treated mice still had increases in these measures compared with the control group ($p < 0.01$; fig. 3, 4b, c).

Effects of AST on Th1 and Th2 Expressions in BALF

To determine whether AST influenced the secretion of cytokines into BALF, the levels of IL-4, IL-5, and IFN- γ in BALF were measured by ELISA 24 h after the final challenge. The OVA challenge triggered significant increases in IL-4 and IL-5 levels in BALF compared with the control fluid (fig. 5a, b). In OVA-sensitized and -challenged mice treated with AST, the rises in IL-4 and IL-5 levels were significantly suppressed, and the IFN- γ levels significantly increased, in BALF in contrast to the concentrations seen in the OVA group (fig. 5).

AST Decreased GATA-3 and Increased T-bet mRNA Expressions in vivo and in vitro

In order to assess the underlying mechanisms of AST, the mRNA expressions of the whole lung and CD4 $^{+}$ T cells were determined using RT-PCR and real-time PCR techniques. The mRNA expressions of GATA-3 were greatly elevated in the OVA group in comparison with the control group, and AST treatment significantly reduced the mRNA levels of GATA-3 after the OVA challenge (fig. 6a, b). In addition, OVA-treated mice showed a decrease in the mRNA levels of T-bet, and AST treatment markedly increased the mRNA expressions of T-bet after the OVA challenge (fig. 6a, c).

AST Decreased GATA-3 and Increased T-bet Protein Expressions in vivo and in vitro

We performed a Western blot analysis of total proteins derived from the lungs (fig. 7a) and CD4 $^{+}$ T cells (fig. 7b) to assess GATA-3 and T-bet protein expressions. As

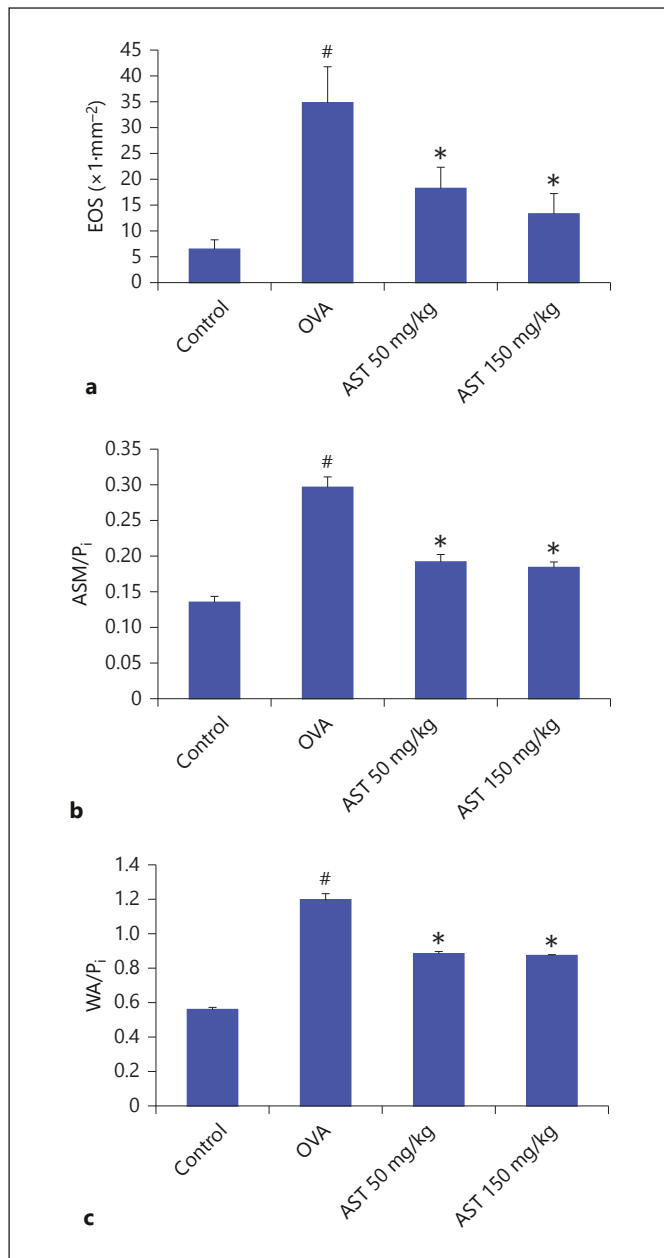


Fig. 4. Effect of AST on the infiltration of eosinophils (EOS; **a**), the area of ASM/ P_i (**b**) and the airway wall area (WA)/ P_i (**c**) in the lung. Values are expressed as means $\pm$ SD. [#] $p < 0.01$ vs. control group; ^{*} $p < 0.01$ vs. OVA group.

shown in figure 7, this analysis confirmed the downregulation of GATA-3 and upregulation of T-bet protein expressions in AST-treated lungs (fig. 7a) and CD4⁺ T cells (fig. 7b). Taken together, our findings indicate that AST treatment leads to a specific downregulation of GATA-3 and a significant upregulation of T-bet expressions in asthma in vivo and in vitro.

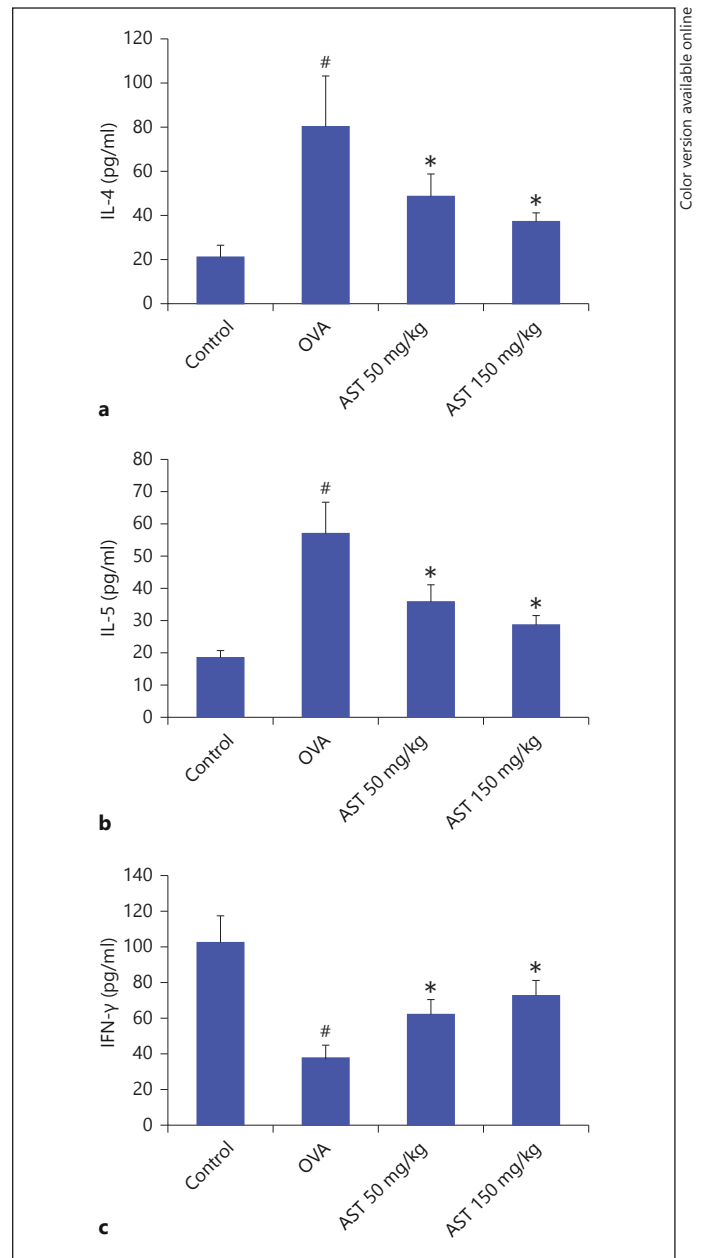


Fig. 5. Effects of AST on IL-4 (**a**), IL-5 (**b**), and IFN- γ levels (**c**) in BALF. Values are expressed as means $\pm$ SD. [#] $p < 0.01$ vs. control group; ^{*} $p < 0.01$ vs. OVA group.

Discussion

Asthma is a serious, sometimes life-threatening, respiratory disease that affects an estimated 300 million individuals worldwide [14]. Over the past decade, several studies have shown that both augmented Th2 responses and impaired Th1 responses, together with Th2-type cy-

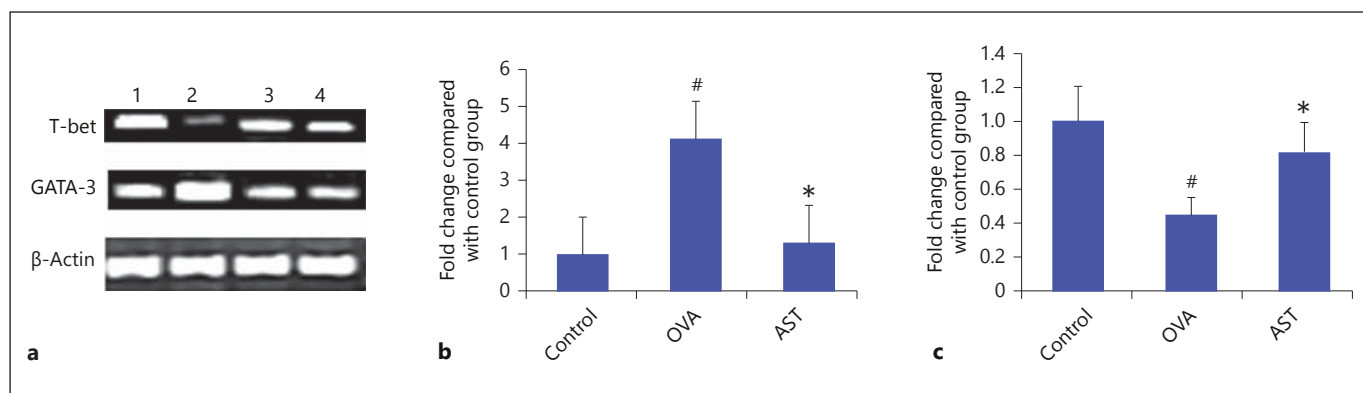


Fig. 6. Effects of AST on GATA-3 (**a, b**) and T-bet (**a, c**) mRNA expression levels in lung tissue (**a**) and CD4⁺ T cells (**b, c**). Values are expressed as means $\pm$ SD. 1 = Control group; 2 = OVA-treated group; 3 = AST-treated group (50 mg/kg); 4 = AST-treated group (150 mg/kg). [#] $p < 0.01$ vs. control group; ^{*} $p < 0.01$ vs. OVA group.

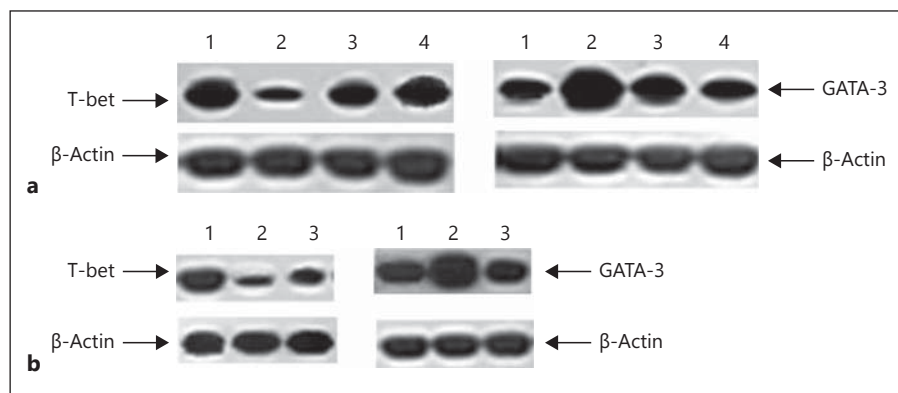


Fig. 7. Western blot analysis of T-bet, GATA-3, and β -actin expressions in the lungs of mice from the four groups (**a**) and in CD4⁺ T cells (**b**). 1 = Control group; 2 = OVA-treated group; 3 = AST-treated group (50 mg/kg); 4 = AST-treated group (150 mg/kg).

tokines, play a critical role in inflammation seen in asthma [15, 16]. The present study investigated the role of AST in Th1- or Th2-mediated airway inflammatory responses in OVA-induced chronic experimental asthma. Administration of AST markedly reduced IL-4 and IL-5 levels and increased IFN- γ levels in BALF. The reduction in IL-4 and IL-5 production caused by AST treatment is consistent with the results of the previous studies pertaining to the suppressive effect of AST on Th2 cytokine production. In addition, we also observed that AST increased the IFN- γ production in BALF. Our findings suggest that AST downmodulates the Th2 cytokine response, augments the Th1 cytokine response, and may enhance a shift in the Th1/Th2 balance towards the Th1 phenotype.

It has been proved that IFN- γ , IL-4, and IL-5 exert their effects on Th1 and Th2 differentiation by controlling the expression of the transcription factors T-bet [17] and GATA-3 [18]. GATA-3 belongs to the GATA family of transcription factors, which bind to the WGATAR

(W = A/T; R = A/G) DNA sequence through a highly conserved C4 zinc finger domain. Naive CD4⁺ T cells express low levels of GATA-3 mRNA [19, 20]. The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage and is downregulated in cells differentiating along the Th1 pathway [19, 20]. In keeping with the crucial role of Th2 cells in asthma, a significant increase in GATA-3 expression was demonstrated in asthmatic airways compared with that in control subjects [21]. Recent studies in transgenic mice have revealed that expression of a dominant negative form of GATA-3 can prevent allergic airway inflammation [22], indicating that GATA-3 plays a crucial role in the development of Th2-mediated lung inflammation [22].

In contrast, T-bet, a member of the T-box family of transcription factors, is a master regulator of Th1 lineage commitment [17, 23]. T-bet is induced by STAT1-mediated signals and strongly promotes IFN- γ and IL-12R β_2 expression during Th1 cell differentiation while repress-

ing Th2 differentiation [17, 24, 25]. Finotto et al. [26] observed a reduced expression of T-bet in human asthmatic airways. In the same study, they demonstrated that T-bet-deficient mice exhibit a phenotype reminiscent of human asthma even in the absence of allergen exposure. These findings suggest that T-bet might protect from asthma by predisposing the cells to differentiate to the Th1 rather than the Th2 direction and by further establishing and maintaining the Th1 phenotype. In addition, in one of the several genome-wide screens by Dizier et al. [27], linkage for asthma and asthma-related phenotypes was investigated. In this screen, they found linkage to the HOX2B gene, which is located close to T-bet in 17q21.32. Taken together, these findings suggest that T-bet might be a candidate gene for asthma and a regulator of Th1/Th2 differentiation.

However, it remains unclear whether AST plays a role in inhibiting the expression and/or function of GATA-3 and in increasing the expression and function of T-bet. Such a study would be of great significance for the therapy of allergic airway inflammation and hyperresponsiveness. Moreover, the potential mechanisms of action of such a therapy remain to be elucidated. The present study demonstrated a key role of the transcription factors GATA-3 and T-bet in the effector phase of a murine asthma model induced by OVA. We found that blockade of GATA-3 expression and elevation of T-bet expression in the lung caused by AST treatment lead to suppression of airway inflammation. In addition, using pulmonary function to assess airway hyperresponsiveness to acetylcholine chloride, we observed that AST significantly reduced airway hyperresponsiveness in a model of allergic asthma, suggesting a novel function of GATA-3 and T-bet as a key regulator of airway hyperresponsiveness in allergic asthma. Our findings suggest the potential therapeutic

utility of AST as a novel molecular tool for the treatment of allergic asthma. This approach has the potential advantage of blocking the production of several Th2 cytokines and simultaneously increasing Th1 cytokines.

The suppression of GATA-3 expression and elevation of T-bet expression caused by AST was accompanied by a suppression of Th2 cytokine synthesis and an increase in Th1 cytokine synthesis in vivo and suppressed the accumulation of eosinophils in the airways. This is important novel information that highlights GATA-3 and T-bet as key regulators of both T cell effector function and airway hyperresponsiveness in allergic airway inflammation. It should be noted that the aforementioned findings on the AST-induced suppression of airway inflammation were further validated by modulating the transcription of T-bet and GATA-3. All data presented a significant downregulation of OVA-induced airway hyperresponsiveness upon administration of AST, definitively demonstrating for the first time a critical regulatory role of GATA-3 and T-bet in airway hyperresponsiveness in allergic asthma.

Taken together, it is suggested that AST treatment is a novel selective approach to simultaneously suppress GATA-3 and increase T-bet expressions in asthmatic reactions in vivo and in vitro. In addition, we have demonstrated here that AST treatment is effective against asthma, suggesting that AST may be a novel molecular tool for the treatment of inflammatory disorders such as asthma in humans.

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