Anti-inflammatory Mechanism of Astragaloside IV in the Recurrence of Asthma: Critical Role of Memory T Cells and OX40/OX40L Signaling Pathway

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Research Article

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Abstract

Background: Astragaloside IV (AST) has been confirmed to have antiasthmatic effects by our research. However, the underline mechanism is unclear. Memory CD4(+) T cells play an important role in the recurrence of asthma, the study try to explore the immunoregulatory effect from memory T cells.

Methods and Results: Flow cytometry was used to determine the counts of memory T cells in blood and spleen; Pulmonary tests, inflammatory cytokines in blood and inflammatory cells in bronchoalveolar lavage fluid were measured to evaluate the inflammatory response. The central memory CD4(+) T cells (T_{CM}) were isolated and adoptive transferred into nude mice via tail intravenous, after OVA challenge 3 days, the lung inflammation was measured. Results shown the T_{CM} phenotype was increased in spleen but the T_{EM} was decreased. On the contrary, the number of activated T_{EM} was increased, but the number of inactivated T_{CM} was decreased in asthmatic mice circulation; AST treatment significantly decreased T_{CM} in the spleen and T effector phenotypes in blood. OX40/OX40L pathway expression were significantly increased in asthma spleen, AST pretreatment inhibited the OX40 signal pathway expression. Additionally, the adoptive transferred T_{CM} s from AST treatment mice had relieved inflammation compared with asthma group.

Conclusions: This study indicates that AST can ameliorate asthma inflammation by decreaseing the ration of T_{EM}/T_{CM} in blood. The anti-inflammatory mechanism maybe involved in the OX40/OX40L pathway.

1. Introduction

Persistent allergic inflammation in the lower airway may require the presence of abundant effector T cells[1]. Superior functional attributes of secondary effectors arising from memory CD4 T cells, as compared to primary effectors arising from naïve precursors, play an important and underappreciated role in secondary immune responses. A large number of effector cells of asthma are generated from T_{CM} s in secondary responses, thereby providing a major source of effector cells that ultimately facilitate Ags clearance[2]. T_{CM} belong to memory CD4(+) T cells group, which include CC chemokine receptor (CCR) 7^{high} T_{CM} and $CCR7^{low}$ T_{EM} [3, 4]. Both T_{EM} and T_{CM} can be found in the circulation. In contrast to T_{EM} , T_{CM} cells can migrate to the secondary lymphoid organs and quickly proliferate in response to infiltrating antigen-presenting cells (APCs). T_{CM} cells express CCR7 and L-selectin (CD62L), which regulate the recirculation of T_{CM} entering into lymphoid organs when reencounter with specific Ags[4–6]. Following antigen capture in peripheral tissues, dendritic cells also upregulate CCR7 and downregulate CD62L[7, 8]. The recent research showed that patients with asthma had a profound increase of $CCR7^{high}$ memory CD4(+) T cells, and the frequency of $CCR7^{high}$ memory CD4(+) T cells was correlated with asthma severity[9]. CD44 is used for phenotyping murine memory T cell subsets, including effector memory T cells (T_{EM} , $CD62L^{low}$ $CD44^{high}$) and central memory T cells (T_{CM} , $CD62L^{high}$ $CD44^{high}$).

T_{CM} activation is mediated not only by antigen stimulation through T cell receptors but also by costimulatory signals through costimulatory molecules. Among several costimulatory molecules, the tumor necrosis factor (TNF) receptor family member OX40 has an essential role in the survival and homeostasis of memory T cells[10, 11]. OX40/OX40L is an important signal pathway that mediates the apoptosis and proliferation of antigen restimulated human memory T cells[12]. A previous study found that Th2 inflammations in the lung were inhibited if OX40/OX40L signaling pathway was blocked[13]. Different studies also reported that OX40 signals contribute to the generation of T_{EM}[14, 15]. Therefore, in this study, the expression of the OX40/OX40L costimulatory signal pathway was measured before and after the AST treatment to explore the regulated mechanism.

The roots of Astragali Radix are known as “Huangqi” and are widely used in traditional Chinese medicine for treatment of inflammation. Astragaloside IV is a main bioactive component from the aqueous extract of Astragalus membranaceus which has potent anti-inflammatory, antioxidative, antifibrotic, antihypertensive, and myocardial protective activities [16–18]. T_{CM}s display core molecular signatures and functional attribute characteristics of stem cells, the main sources of effector cells of T_{EM}[19], however, current common anti-asthmatic therapeutics, including corticosteroids, can not block activation of T_{CM}[20, 21]. In this study, we explored the treatment mechanism of AST in the regulation of proliferation of T_{CM}s.

2. Materials And Methods

2.1 Mice

All mice were bred and maintained at the Animal Facility of the Fudan University of Medicine. 6 weeks female BALB/c and 5 weeks nude mice were originally obtained from the Shanghai SLAC Co. (Shanghai, China). Mice were acclimated for 1 week to the housing condition before the experiments began, and then 2-3 mice per cage housed in a laminar air flow room with a relative humidity of 55.5%. Each room was maintained at 22°C with a light-dark cycle of 12h light and 12h dark throughout the experiment. Ethics statement Animal care and manipulation was in agreement with institutional guidelines, which are in accordance with the Guide for the Care and Use of Laboratory Animals. All animal experimental procedures used in this study were approved by the Fudan University of Animal Ethics Committee. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2 Induction of asthmatic mice model

6 weeks female BALB/c mice were developed by OVA sensitization and inhalation as described previously[22]. In brief, on days 0, 7, and 14, mice were sensitized with an intraperitoneal (i.p.) injection of 0.2 mL alum-precipitated antigen in phosphate-buffered saline (PBS) which contained 80 µg of OVA mixed with 2 mg aluminum hydroxide. After sensitization, the mice were exposed to aerosolized OVA for 30 min/day on days 20–24. The concentration of OVA was 1.5% to induce the asthmatic condition. The

AST treatment group mice were given 40, 20, or 10 mg/Kg, Astragaloside IV, respectively (Y0001171, C41H68O14, molecule weight: 784.97, Winherb Medical Science Co., Ltd Shanghai, CAS 84687-43-4) (dexam 1mg/kg or 0.9% NaCl) by intraperitoneal injection. The doses of AST used in the study refer to the relevant literature and data[23, 24]. The animal study protocols were approved by the Institutional Animal Care and Use Committee of Fudan University.

2.3 Bronchoalveolar lavage (BAL) and histological examination

The BALB/c mice or nude mice were euthanized with amobarbital sodium. The left lungs were lavaged four times with SA (0.5 ml each), and approximately 1.6 ml was consistently recovered with gentle handling. The cell suspension was centrifuged at 1,500 rpm for 10 minutes at 4°C. The cells were resuspended into 1 ml of saline with 1% BSA, and the total cell numbers were counted with a hemocytometer. Right lungs were perfused with PBS to remove blood and the left lung lobe was fixed in 10% methanol and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) as previously described[25]. The goblet cells in small bronchi were identified with AB-PAS staining.

2.4 The cell culture

2.4.1 Isolation of splenocytes

The BALB/c mice or nude mice were euthanized with amobarbital sodium. Their spleens were collected at necropsy. Each spleen was aseptically homogenized using a glass tissue grinder. The cell suspension was centrifuged at 300 x g for 5 minutes at 4°C. The pellet was treated with erythrocyte lysis buffer (0.19 M NH₄Cl) for 5 to 10 minutes at 4°C, centrifuged (as above), and washed twice with RPMI 1640 medium (Invitrogen/Gibco BRL, Grand Island, NY, USA). Splenocytes were then suspended in complete RPMI 1640, that is, medium supplemented with 10% fetal calf serum (Invitrogen/Gibco BRL, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin)(Dojindo Laboratories, Kamamashiki, Kumamoto, Japan). The viability using trypan blue (Invitrogen/Gibco BRL, Grand Island, NY, USA) exclusion was determined.

2.4.2 The splenocytes cells culture

The splenocytes cells were diluted in complete medium and dispensed into 96-well plates at 5×10^5 cells/well/100 µL. Cells were cultured in 96-well plates (Corning Inc., New York, NY, USA.), and viability was assessed using a commercial kit (Cell Counting Kit-8, Dojindo Laboratories, Japan) in accordance with the manufacturer's instructions. The details of the protocol have been described in our previous research. All samples were analyzed in triplicate.

2.5 Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) were used to isolate splenocytes. Splenocytes were collected, cells were isolated using magnetic microbeads and magnetic-activated cell sorting columns according to the manufacturer's protocol(all reagents were from Miltenyi Biotec, Bergisch Gladbach, Germany). T cells

were counted directly after MACS. T cell viability was determined by trypan blue exclusion. CD4(+) T cells and T_{CM} purity levels were always found to be 98% and 96%, respectively.

2.6 Adoptive transfer of T_{CM}s into nude mice

Six groups of nude mice(5 weeks) were transfused with CD44^{high} CD62L^{high} CD4(+)T cells isolated from spleen cells of OVA-stimulated asthma group, normal group, dexamethasone and AST(3 different doses) treated BALB/c mice via tail intravenous injection respectively. According to the research[26], 1.0×10^6 T_{CM}s in 150 μ l PBS were injected into nude mice i.v. through the tail vein. One day after the cell transfer, all mice were given daily challenge with OVA for 3 days, as indicated in the figure legends. 14 days after the challenge, mice were euthanized, and their spleens and lungs were collected at necropsy. Splenocytes were detected by flow cytometry and the lung tissues were stained with hematoxylin and eosin (H&E).

2.7 Determination of spleen weight and spleen index

After adoptive transfer of T_{CM}s and continuous OVA stimulation for 3 days, the nude mice were terminated at the 7th day. The spleen was immediately separated. The surrounding connective tissue and fat were excluded. The organs were dried of surface moisture using filter paper and weighed. Viscera indexes for the spleen were calculated by the following formula: organ index = organ weight (mg)/body weight (g).

2.8 Flow cytometry

At 50 days, the mice were killed, blood and spleen samples were collected. Multicolor flow cytometry were used to quantify the expression of CD62L $\square$ CCR7 $\square$ CD44 on CD4(+) T cells present in peripheral blood. The cells were lysed with Pharm Lyse buffer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cell suspensions were washed and incubated in PBS supplemented with 1% bovine serum albumin (BSA; Sigma-Aldrich), 1% mouse serum (Life Technologies). Cells were then stained with antibodies in 1% BSA/1% mouse serum/ PBS for 45 min on ice. Antibodies used were: anti-CD4-PerCP-Cy5.5, anti-CCR7-PE, anti-CD62L-APC, anti-CD44-FITC(all from BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo. MFI (mean fluorescence intensity) was determined by subtracting the MFI of the FMO (fluorescence minus one) control from the MFI of the stained sample.

2.9 Real-time PCR

Total RNA was extracted using TRIzol (Sigma Chemical Co. St. Louis, MO, USA) according to the manufacturer's instructions. Quantification was performed with a two-step reaction process: reverse transcription (RT) and PCR. Each RT reaction consisted of 0.5 mg RNA, 2 mL of PrimerScript Buffer, 0.5 mL of oligo dT, 0.5 ml of random 6 mers, and 0.5 mL of PrimerScript RT EnzymeMix I (TaKaRa Bio, Japan), in a total volume of 10 mL. Reactions were performed in a PCR system 9700(Applied Biosystems,USA) for 15 min at 37°C, followed by heat inactivation at RT for 5 s at 85°C. The 10 mL RT reaction mix was then diluted $\times 10$ in nuclease-free water and held at -20°C . Real-time PCR instrument with 10 mL PCR reaction mixture that included 1 mL of cDNA, 5 mL of $2 \times$ SYBR Green I MASTER (Roche,

Swiss), 0.2 mL of forward primer, 0.2 mL of reverse primer and 3.6 mL of nucleasefree water. Reactions were incubated in a 384-well optical plate (Roche, Swiss) at 95°C for 10min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s. Each sample was run in triplicate for analysis. At the end of the PCR cycle, melting curve analysis was performed to validate the specific generation of the expected PCR product. The primer sequences were designed in the laboratory and synthesized by Generay Biotech(Shanghai, China), based on the mRNA sequences obtained from the NCBI database. Primers used for PCR amplification are listed in Table 1. The expression levels of mRNAs were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primers used for PCR amplification

Gene	Forward primer	Reverse primer
OX40	TTCTTGCCTGTCCGCCTACT	GCTGCTGAACCCACACATACAT
OX40L	TGCTTCTGTGCTTCATCTATGT	TGGTAACTGCTCCTCTGAGTCT
IFN-r	CATAGATGTGGAAGAAAAGAG	AGAGTCTGAGGTAGAAAGAGATA
IL-4	TAGTTGTCATCCTGCTCTTCTT	CTCACTCTCTCTGGTGTTCTTC
IL-17A	GGCTGACCCCTAAGAAAC	ATTCCTTGCTGAAAATCAATAG
IL-13	CAGCCTCCCCGATACCAAAAT	CCCCAGCAAAGTCTGATGTGA
GADPH	CTTTGGCATTGTGGAAGGGC	CAGGGATGATGTTCTGGGCA

2.10 Western blot analysis

Spleen tissues were homogenized in buffer with a protease inhibitors and incubated for 30min at 4°C. Tissue or cell debris were removed by centrifugation (12 000 × g, 15 min), then boiled 5 min at 98°C with SDS-PAGE loading buffer, and stored at -80°C until used. The protein concentration was determined by BSA method. 30mg of protein were electroblotted onto a PVDF membrane, following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated 1h with 5% milk at room temperature, and then incubated overnight at 4°C with 1:1000 dilution of anti-OX40 antibody(Santa Cruz Biotech, CA, sc-21000) and 1:1000 dilution of β -actin antibody(Santa Cruz Biotechnology, sc-130656), respectively. Blots were washed three times with Tween-20/Tris-buffered saline (TTBS) and then incubated with a 1:10000 dilution of HRP-conjugated secondary antibody for 1h at room temperature. The optical density (OD) of the target proteins was shown as a proportion of b-actin OD.

2.11 Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS v.11, Chicago, IL, USA). The significance of the differences was determined by one-way ANOVA, comparisons of parameters between 2 groups were performed with LSD or Games-Howell test. Qualitative data between groups were compared by Pearson's χ^2 test or Fisher exact test, as appropriate. The data

analysis of qPCR gene expression levels of the control group were set at 1-fold, and gene expression changes of other groups were expressed as the normalized “fold” change in mRNA expression compared with the control group. The significance thresholds were 0.05 in all analysis.

3. Results

3.1 The anti-inflammatory effect of AST on asthmatic mice

We first measured the anti-inflammatory effects of AST in an OVA-induced asthmatic mouse model. Our results showed that AST significantly inhibited the lung inflammation of asthmatic mice at day 50, as shown in Fig. 1.

3.2 AST reduced the ratio of T_{EM}/T_{CM} in circulation blood

We found that the central memory T cells had a different distribution between blood and spleen in the asthma group after OVA challenge. The asthmatic mice had a greater percentage of T_{CM} cells in the spleen, while higher percentage T_{EM} in the blood. These results suggest that the asthmatic mice had a higher number of functional effector T cells in the blood (Fig. 2). As summarized in Fig. 3, in the circulation blood, the effector memory phenotype cells percentage was significantly reduced after AST treatment, while, T_{CM} subpopulations were increased.

3.3 The treatment mechanism of AS-IV involved in the OX40/OX40L signal pathway

The result shown a significantly increased expression of OX40/OX40L. The mRNA expression of OX40 and OX40L were significantly increased in asthma group (Fig. 4A and 4B); meanwhile the protein expression of OX40 was also increased in asthma group (Fig. 4C and 4D). AST pretreatment partially counteracted the effects on OX40 signal pathway. Therefore, AST treatment significantly blocked the OX40 signaling pathway, thus contributing to the anti-inflammatory effect in asthmatic mice.

3.4 The anti-inflammatory effect of AST was achieved by inhibiting activation of T_{CM}

In this study, we magnetically activated a cell sorting memory T cells from the spleen of BALB/c mice treated with or without AST. $T_{CM}(CD4^+ CD44^{high} CD62L^{high})$ were injected into the tail vein of nude mice. The results showed that $CD4(+)CD44^{high}$ cells were significantly increased in asthma group; AST treatment significantly reduced the cells numbers (Fig. 5A and 5B). The spleen index was significantly increased in nude mice which received T_{CM} cells from the asthmatic group (Fig. 5C). Spleen weight was significantly reduced in AST treated group. The lung inflammation was exacerbated in the

asthma transfer group; however, the counts of T_{EM} was reduced and inflammation was alleviated in AST treated group (Fig. 5D, 5E and 5F)).

4. Discussion

T_{CM} can more rapidly and vigorously respond to repeated exposure to antigens, thus providing the immunological memory that mediates rapid recall responses in secondary lymphoid organs and lung tissues. Central memory T cells enter into secondary lymphoid organs, proliferate, and produce a higher percentage of effector memory phenotype cells, which are related to the acute inflammation in lung tissues[27]. A recent report also revealed that increased $CCR7^{high}$ central memory T cells were associated with asthma severity[28]. An absolute requirement of these control mechanisms is the ability to inhibit the differentiation of T_{CM} . Yet, a recent report showed that in severe asthma patients, the percentage of $CCR7^{high}$ memory T cells in the blood was significantly lower than in the control group; thus, the distribution between lung, blood and peripheral lymphatic organs may be disordered in severe asthma [29]. Previous research has described that AST had an anti-inflammatory effect and could prevent the recurrence of asthma[30]. In this study, we confirmed that $CD4(+) T$ cell was a key factor manipulated by AST to avoid an asthma attack. We found that AST treatment significantly reduced expression of markers, CCR7 and CD62L, in spleen lymphocyte. After AST treatment, the T_{CM} numbers are reduced in the spleen. On the contrary, the the activated T_{EM} numbers are increased in asthma group. After AST treatment, the T_{EM} numbers were significantly reduced. In *vivo*, the nude mouse was used to further assess the treatment effect of AST. We found that nude mice that received $CD44^{high} CD62L^{high} CD4(+) T$ splenocytes adoptive transfer from AST treated asthmatic mice suffered from minor inflammation in lung. These results indicate that regulation of the memory T cells distribution by AST are important way in the anti-inflammatory response. Next, we hypothesized that OX40/OX40L signal pathway might have an important role in the activation process of T_{CM} . Indeed, previous research found that $CD4(+) T_{EM}$ of asthmatic mice significantly expressed OX40 and OX40L, where OX40 signals were indispensable for the generation of T_{EM} precursor cells in the later stage of inflammation[14]. In this study, AST blocked OX40/OX40L signal pathway which is an important stimulated pathway of T_{EM} generation. Increased effector phenotype in circulation was significantly reduced after AST treatment, which has an important role in blocking asthma attacks.

The main limitation of the current study is that the anti-inflammatory mechanism of AST in the differentiation of the effector cells remained in the superficial phase; thus, future follow-up studies are needed to provide further verification for the reported findings.

5. Conclusion

This study indicates that AST can ameliorate asthma inflammation by decreasing the ration of T_{EM}/T_{CM} in blood. The anti-inflammatory mechanism maybe involved in the OX40/OX40L pathway. This novel

finding provides support for alternative therapies for the treatment of asthma recurrence based on targeting the regulation of subpopulations of memory CD4(+) T cells.

Abbreviations

Astragaloside IV, AST; CC chemokine receptor, CCR7; Central memory CD4(+) T cells, T_{CM}; Antigen, Ags; Effector memory T cells, T_{EM}; Antigen-presenting cells, APCs; L-selectin, CD62L; Tumor necrosis factor, TNF; Interleukin-7, IL-7; Ovalbumin, OVA; Intraperitoneal, i.p; Magnetic-activated cell sorting, MACS; Hematoxylin and eosin, H&E.

Declarations

6. Authorship confirmation statement

We declare that this manuscript entitled “Anti-inflammatory mechanism of Astragaloside IV in the recurrence of asthma: Critical role of memory T cells and OX40/OX40L signaling pathway” is original, has not been published before and is not currently being considered for publication elsewhere.

We would like to draw the attention of the Editor to the following publications of one or more of us that refer to aspects of the manuscript presently being submitted. Where relevant copies of such publications are attached.

7. Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

8. Funding

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9. Contributions of authors

Conceptualization: Lingwen Kong and Jingcheng Dong; Methodology: Lingwen Kong, Qingqing Yang and Hongying Zhang; Formal analysis and investigation: Lingwen Kong; Writing-original draft preparation: Lingwen Kong; Writing-review and editing: Lingwen Kong and Jingcheng Dong; Funding acquisition: Lingwen Kong and Jing Sun; Resources: Lingwen Kong; Supervision: Lingwen Kong and Jingcheng Dong.

10. Consent for publication

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

11. Compliance with Ethical Standards

Animal care and manipulation was in agreement with institutional guidelines, which are in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of Fudan University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

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Figures

Figure 1

The anti-inflammatory effects of AST on OVA-induced asthmatic mice. (A) Experimental procedure for creating our OVA-induced allergic inflammation model; **(B)** Airway hyperactivity response as measured to methacholine (Mch). **(C)** and **(D)** H&E and PAS staining of the lung sections. **(E)** The number of various immune cell populations found in the BALF after OVA challenge. * $p < 0.05$, ** $p < 0.01$ vs asthma group; # $p < 0.05$; ## $p < 0.01$ vs normal group.

Figure 2

A different subsets of CD4⁺ memory T cells in blood and spleen. Blood and spleen T cells were collected and measured on day 50 by flow cytometry. (A) Experimental design; (B), (C) and (D) The different counts of frequency (in percent) of the T_{CM} biomarker in blood and spleen; (E). The counts of the frequency (in percent) of the T_{CM} cells in blood and spleen; * $p < 0.05$ vs normal group; ** $p < 0.01$ vs normal group.

Figure 3

AST treatment reduce T_{CM} numbers in spleen and T_{EM} numbers in blood. (A) and (B) The counts of the frequency (in percent) of T_{EM} in spleen ; **(C)** The percentage of T_{EM} in blood; **(D) and (E)** The T_{CM} numbers in blood. * $p < 0.05$, ** $p < 0.01$ vs asthma group; # $p < 0.05$, ## $p < 0.01$ vs normal group.

Figure 4

The treatment mechanism of AST involved in the OX40/OX40L signal pathway. (A) and (B) The mRNA expression of OX40 and OX40L (day 20); **(C) and (D)** The protein expression of OX40 (day 20). # $p < 0.05$, ## $p < 0.01$ vs normal group; * $p < 0.05$ vs asthma group.

Figure 5

The anti-inflammatory effect of AST was performed by regulating T_{CM}. 1.0×10^6 CD44^{high} CD62L^{high} CD4⁺ T_{CM} cells were transferred into nude mice, the mice were then stimulated with OVA on day 1-3 post adoptive transfer, the inflammatory levels were measured and the distribution of memory CD4⁺ T cells was analyzed in the indicated tissues. **(A)** and **(B)** The counts of the frequency (in percent) of CD4⁺ and CD4⁺CD44^{high} cells in blood; **(C)** Spleen index after AST or dexamethasone treatment; **(D)** and **(E)** The expression of IL-4 and IL-13 in blood; **(F)** H&E staining of the nude mice lung sections. # $p < 0.05$, ## $p < 0.01$ vs normal group; * $p < 0.05$ vs asthma group; ** $p < 0.01$ vs asthma group.