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CD4⁺CD25⁺FOXP3⁺ T cells, Foxp3 gene and protein expression contribute to antiasthmatic effects of San'ao decoction in mice model of asthma

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ABSTRACT

Objective: San'ao decoction (SAD) is a commonly used traditional combinatorial formula composed of Herba Ephedrae, Radix Glycyrrhizae and Amygdalus Communis Vas. Early studies showed that in the OVA sensitization asthmatic mice model its compatibility could lower airway reactivity and airway inflammatory cell infiltration. Based on the above results, this study mainly discussed San'ao decoction's immunomodulatory effects on Tregs.

Methods: UPLC–PDA–TOF–MS was applied to analyze chemicals of SAD, and under the optimized chromatographic and MS condition, the major components in SAD were well separated and detected within 22 min. An asthma model was established in BALB/c mice that were sensitized and challenged with ovalbumin. After 2 weeks' treatment, peripheral blood and bronchoalveolar lavage fluid (BALF) were assessed for inflammatory cell counts; histological change of lung tissue were detected; flow cytometry detection of splenic CD4⁺CD25⁺Foxp3⁺ Treg cells of the mice were counted; Foxp3 expression in lung tissues were examined as well.

Results: 22 Peaks signal chemical components in SAD were identified by UPLC–QTO–MS method. In terms of the percentage of eosinophile in peripheral blood and bronchoalveolar lavage fluid (BALF), SAD groups were significantly lower ($p < 0.01$) than model group. Compared with model group, lung histological changes of SAD groups were reduced; the proportion of CD4⁺CD25⁺Foxp3⁺ Treg cells in CD4⁺ cells of asthmatic mice also decreased; SAD significantly increased the proportion of CD4⁺CD25⁺Foxp3⁺ Treg cells and promoted Foxp3 expression in a mouse model of asthma.

Conclusion: These results suggest that the antiasthmatic effects of SAD are at least partially associated with CD4⁺CD25⁺Foxp3⁺ Treg cells.

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Introduction

Bronchial asthma is a chronic airway disease that is typically characterized by chronic airway inflammation, bronchial hyper-responsiveness and airway remodeling (Holgate et al., 2009). TH2 cell-derived cytokines have been implicated in eosinophil accumulation, mucus hypersecretion, AHR development, and lung remodeling (Munitz et al., 2008). 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 (Workman et al., 2009). 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 (Campbell and Ziegler, 2007). Several lines of evidence suggest that Tregs are intimately involved in asthma pathogenesis. Therefore, Tregs may be an attractive therapeutic target for the treatment of asthma (Boulet et al., 2012; Stock et al., 2006; Fontenot et al., 2003).

San'ao decoction (SAD) is a well-known Chinese prescription and has been used clinically to treat asthma and cough for thousands of years. SAD consists of three traditional Chinese medicines: Herba Ephedrae (*Ephedra sinica* Stapf), Semen

Abbreviations: SAD, San'ao decoction; Treg, regulatory T cells; Foxp3, forkhead box P3; OVA, ovum albumin; BALF, bronchoalveolar lavage fluid; CD4⁺CD25⁺FOXP3⁺ Tregs, CD4⁺CD25⁺FOXP3⁺ regulatory T cells.

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Table 1
Main components in SAD.

Peak no.	tR (min)	Assigned identity	Molecular formula
1	4.62	Catechin	C ₁₅ H ₁₄ O ₆
22			
2	4.90	Amygdalin	C ₂₀ H ₂₇ NO ₁₁
3	5.97	Epicatechin	C ₁₅ H ₁₄ O ₆
4	6.16	Vicenin	C ₂₇ H ₃₀ O ₁₅
5	7.18	Schaftoside/isoschaftoside	C ₂₆ H ₂₈ O ₁₄
6	8.05	Liquiritin apioside	C ₂₆ H ₃₀ O ₁₃
7	8.28	Isoliquiritin apioside	C ₂₆ H ₃₀ O ₁₃
8	10.94	Licuraside	C ₂₆ H ₃₀ O ₁₃
9	11.33	Formononetin	C ₁₆ H ₁₂ O ₄
10	13.29	Licorice saponin	C ₄₂ H ₆₃ O ₁₆
11	15.60	Licorice saponin G2	C ₄₈ H ₆₂ O ₁₇
12	19.35	Licorice saponin H2	C ₄₂ H ₆₂ O ₁₆
13	19.67	Glycyrrhizin acid/Licorice saponin K2	C ₄₂ H ₆₂ O ₁₆
14	2.05	[Norpseudoephedrine-CH ₃]-edrine	C ₉ H ₁₂ N
15	2.17	[norephedrine-CH ₃]-	C ₉ H ₁₂ N
16	2.44	Norpseudoephedrine	C ₉ H ₁₃ NO
14			
17	2.59	Norephedrine	C ₉ H ₁₃ NO
18	2.98	Methylephedrine/methylpseudoephedrine	C ₁₁ H ₁₇ NO
19	8.09	Isoliquiritigenin	C ₁₅ H ₁₂ O ₄
20	8.29	Liquiritigenin	C ₁₅ H ₁₂ O ₄
21	11.34	Ononin	C ₂₂ H ₂₂ O ₉
22	14.63	Licorice saponin A3	C ₄₈ H ₇₂ O ₂₁

Armeniaca amarum (*Prunus armwniaca* L. var. ansu Maxim) and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch), which are all recorded in Pharmacopoeia of the People's Republic of China (2010 Edition). It is a basic prescription for treating respiratory disease in Chinese medical science. In clinical practice, different herbal medicines added to SAD can obviously relieve different respiratory symptom, such as wind-cold type of common cold, bronchitis, Thresher's disease and so on (Li et al., 2010). However, it remains unknown whether Tregs are involved in the protective effect of SAD on asthma.

Materials and methods

Drugs and reagents

The ratio of Heaba Ephedrae, Semen Armeniaca Amarum, and Radix et Rhizoma Glycyrrhizae in SAD was 2:1:3. All crude herbs were purchased from Yongkang Pieces Co., Ltd., Bozhou, Anhui, China (20100109). The botanical identities of the herbs were well-defined by professor Wu Dekang of Medicinal Plant Department, Nanjing University of Chinese Medicine. The herbs were decocted and concentrated, with each milliliter containing 0.88 g of crude drugs.

OVA was purchased from Shanghai Bio Life Science and Technology Co., Ltd. (batch no. 100715); Dexamethasone Acetate tablets (batch no. 1 081211, 0.75 mg per tablet) provided by Zhejiang Xianju Pharmaceutical Co., Ltd., China were added into phosphate buffer solution (PBS) to be 0.24 mg/ml solution. Regulatory T cell Stai Mouse ning Kit #3 (eBioscience, E09667-1636).

Components in SAD were identified by UPLC–QTO–MS method and are mentioned in Table 1. Taking retention time (tR), mass fragment ions (m/z), UV absorption characteristics (lambda max), and relevant references into consideration, 22 peaks signal chemical components were identified. Total ion current is shown in Fig. 1 (Ma et al., 2012).

UPLC–MS finger print

UPLC conditions

UPLC was performed on a Waters Acquity UPLC system (Milford MA), consisting of a binary solvent delivery system, an autosampler and PDA detector system. An ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm i.d., 1.7 × 10^{−6} m; Waters) was used for all analyses. The mobile phase was composed of acetonitrile (A) and 0.1% formic acid (v/v) (B), with the following gradient elution: 0–8 min, 95–80% A; 8–12 min, 80–70% A; 12–15 min, 70–65% A; 15–18 min, 65% A; 18–21 min, 65–20% A; 21–23 min, 20–5% A; 23–24 min, 5% A; 25–30 min, 95% A. The flow rate of the mobile phase was 0.4 ml/min and the temperatures of maintained at 30 and 10 °C, respectively.

Mass spectrometric conditions

The MS analysis was performed on a Waters ACQUITY Synapt Q–TOF mass spectrometer connected to the Waters ACQUITY UPLC system via an electrospray ionization (ESI) interface. High purity nitrogen was used as the nebulizer and auxiliary gas and argon was the collision gas. The Q–TOS MS was operated in negative ion mode with a capillary voltage of 3 kV, a sampling cone voltage of 30 V, a cone gas flow of 50 l/h, a desolvation gas flow of 600 l/h, a collision energy of 6 V and full scan spectra from 100 to 1000 Da.

Mass accuracy was maintained by using LockSpray. The [M–H][−] and [M+H]⁺ ions of leucine-enkephalin at m/z 554.2615 and 556.2771 were used as the lock mass in negative and positive ESI modes, respectively. The concentration of leucine-enkephalin was 50 pg/ml and the infusion flow rate was 10 ml/min. Centroided data were acquired for each sample from 100 to 1200 Da, and dynamic range enhancement was applied throughout the MS experiment to ensure accurate mass over a wide dynamic range.

Sample preparation

For the traditional decoction, samples of Herba Ephedrae (6 g), Radix Glycyrrhizae (3 g) and Amygdalus Communis Vas (9 g) were accurately weighed and mixed, the mixed herbal powders were put into a flask containing 180 ml of water and boiled for 120 min. After this, the extracted solutions were centrifuged at 3000 rpm for 5 min and the supernatant was regarded as the sample of a traditional decoction.

Animals

A total of 60 Bal b/C mice (half male half female, 18–20 g, SPF grade, 6–8 weeks old) were used in this study, and were all provided by the Aiermei Technology Co., Ltd., Suzhou, China, with the certificate 2013839) 2007-0041. They were kept under the same laboratory conditions of 12-h alternate light–darkness cycle under 23–25 °C with free access to rodent laboratory chow and tap water.

Apparatus

The S-888E ultrasonic atomizer was purchased from Nanjing Dolphin Electronics Co. Ltd., China. The flow cytometry with CellQuest software was supplied by Beckmen Coulter. Western electrophoresis was purchased from Bio-Rad, America.

HPLC–MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany) and MS grade formic acid was purchased from Sigma–Aldrich (St. Louis, MO). Other chemicals and solvents were of analytical grade. Purified water was prepared in-house with a Millipore system (Bedford, MA).

Mice sensitization and drug treatment

60 BALB/c mice were randomly divided into normal group, model group, high dose of SAD group (SAD-H) (3.6 g kg^{−1}), low

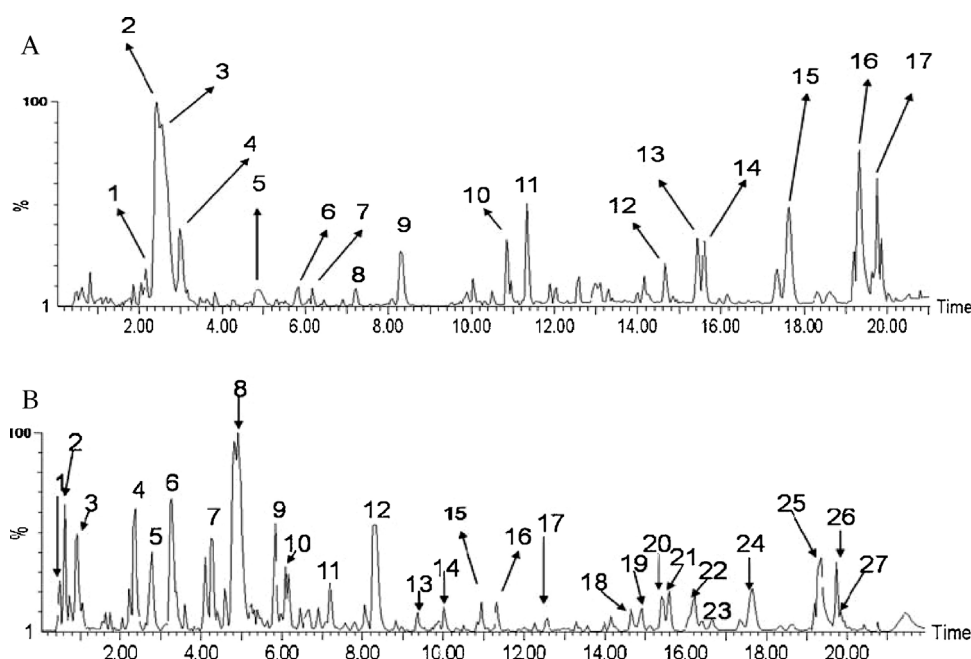


Fig. 1. Elementary particle flow graph (BPI) chromatogram monitored in positive ion mode for traditional decoction (A); BPI chromatogram monitored in negative ion mode for traditional decoction (B).

dose of SAD group (SAD-L) (1.8 g kg^{-1}), and dexamethasone (DM) group ($0.0075 \text{ mg kg}^{-1}$). Mice in the groups other than normal group were given 0.1 ml sensitized fluid (0.2 ml sensitized fluid containing 0.1 mg ovalbumin and 0.02 mg $\text{Al}(\text{OH})_3$) subcutaneously and intraperitoneally on the 1st and 8th day. From Day 15 to Day 28, they were given 5% OVA atomization for 20 min, 30 min before which the mice were administrated with their corresponding decoctions, while mice of normal group received the distilled water of equal volume and distilled water atomization.

Blood cytology

After the last challenge, 24 h, about 1 ml blood were taken from the eyes of mice, $20 \mu\text{l}$ of blood mixing in 0.38 ml EOS counting liquid, eosinophil counts in an optical microscope. Another blood smears fixed amount, Rui Ji's dye staining read 200 white blood cells under the microscope, statistics the number of various types of white blood cells, calculate the percentage of various types of white blood cells.

Bronchoalveolar lavage fluid (BALF)

Samples were obtained 24 h after the last challenge. Briefly, the mice were anesthetized and their tracheas were isolated by blunt dissection. A small caliber tube was inserted and secured in the airway. Two successive pooled volumes of 0.5 ml PBS were instilled and gently aspirated. Each BALF sample was centrifuged and the supernatants were stored at -70°C for further cytokine analyses. The total number of inflammatory cells was counted in $30 \mu\text{l}$ suspension after cell pellets were diluted with 0.5 ml PBS. Types of inflammatory cells of BALF pellets after H&E stain in cytospin preparation were determined by counting 200 cells, which were classified as macrophages, lymphocytes, eosinophils, and neutrophils.

Lung histology

The lungs of the mice sacrificed one day after the airway hyper-responsiveness assay were fixed in 4% formaldehyde. Each tissue

was sliced into $6 \mu\text{m}$ sections and stained with H&E stain (Mayer's hematoxylin & eosin). Finally, general tissue lesions and inflammatory cell infiltration were observed.

Flow cytometric analysis

Cells from the spleen 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 $0.5 \mu\text{g}$ 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 (Beckmen Coulter, USA).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from the cells as Yamamoto et al. (1997) previously described. First-strand cDNA synthesis was then performed from $1 \mu\text{g}$ of total RNA using oligo (dT) as a primer and reverse transcriptase (Fermentas, Lithuania). A sample of the reaction mixture ($20 \mu\text{l}$), corresponding to $1 \mu\text{g}$ of total RNA, was combined with $20 \mu\text{l}$ of the PCR reaction medium, including 0.5 U Taq DNA polymerase (Fermentas, Lithuania). Commercially obtained primers were used (Nanjing GenScript Technology Co., Ltd.). The foxp3 were: forward: 5-AGAAGCTGGGAGCTATGCAG-3; reverse: 5-GCTACGATGCAGCAAGAGC-3; GAPDH: forward: 5-AAA GGG TGT AAC GCA ACT AA-3; reverse: 5-GTG GAC ATC CGC AAA GAC-3 (100 bp).

Reverse transcription was performed by incubation at 42°C for 60 min, followed by heating at 95°C for 5 min, then incubated at $0-5^\circ\text{C}$ for 5 min. Polymerase chain reaction was performed by denaturation at 95°C for 5 min, followed by denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 40 s, followed by a final extension at 95°C for 10 min.

Table 2
The differential count of cells in blood of each group ($\bar{X} \pm S$, $n = 10$).

Group	Dose (g kg ⁻¹)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Normal	–	62.50 ± 8.22	37.19 ± 8.38	0.31 ± 0.46
Model	–	50.75 ± 8.24*	28.00 ± 8.74*	21.25 ± 5.30**
DM	0.0024	58.8 ± 8.25 ^{ΔΔ}	35.81 ± 7.11	4.06 ± 2.78 ^{ΔΔ}
SAD-H	3.6	58.29 ± 5.67	35.75 ± 5.43	5.90 ± 2.59 ^{ΔΔ}
SAD-L	1.8	56.06 ± 5.79	30.63 ± 7.21	13.31 ± 4.56 ^{ΔΔ}

Note: * $P < 0.05$, ** $P < 0.01$ vs Normal Group; ^Δ $P < 0.05$, ^{ΔΔ} $P < 0.01$ vs Model Group

Western blot analysis

The Total Protein was drawn from the lung tissue, got protein quantification using Bradford method, and diluted by denaturing Lysis Buffer. The equivalent samples were put into tubes under 95–100 °C for 5 min, and contain 50 μg proteins each. Then, the protein samples were subjected to SDS-PAGE (Nanjing Keygen Biotech Co., Ltd.). After electrophoresis, proteins were transferred, blocked in 5% skim milk powder bloke buffer with the proportion of 0.1 ml/cm² respectively (overnight at 4 °C). Appropriate amount of rat anti-Foxp3 mAb were added at the same time. Shakers were used to incubate the samples (overnight at 4 °C). The membranes were washed with PBST 4 times. The Secondary antibodies (combinations of the membrane and HRP) were diluted by the dilution (1:5000), incubated by the shakers for 2 h under room temperature. The membranes were washed with PBST another 4 times. 0.1 ml/cm² developing solution was used to calculate the amount. NC membranes with developing solution were laid aside under room temperature for 1 min. The membrane protein was posted on the X-ray film for exposure, and then developed in the developing machine. The time of exposure was adjusted until the best stripes were appeared. The signal intensity was quantified using Gray-scale analysis software (Image Tool 3.00).

Statistical analyses

The data were presented as the mean ± standard deviation. The statistics and data evaluation were respectively performed by SPSS 17 and one-way analysis of variance (ANOVA) followed by LSD or Dunnett's T3 posthoc analysis. $p < 0.05$ was considered to be significant.

Result

Identified chemical compounds of SAD

SAD, composed of *Herba Ephedrae*, *Radix Glycyrrhizae* and *Amygdalus Communis Vas*, forms a very complex matrix. The major components that can be detected in decoctions of SAD are alkaloids, flavonoids and saponin. UPLC–PDA–TOF–MS was applied to analyze chemicals of traditional and dispensing granule decoctions of SAD, and under the optimized chromatographic and MS condition, the major components in SAD were well separated and detected within

Table 3
The differential count of cells in BLAF of each group ($\bar{X} \pm S$, $n = 10$)10⁶/l.

Group	Dose (g kg ⁻¹)	Leukocyte	Lymphocytes	Neutrophils	Monocytes	Eosinophils
Normal	–	19.63 ± 5.58	2.47 ± 1.60	2.59 ± 1.51	14.53 ± 4.18	0.04 ± 0.07
Model	–	29.88 ± 3.68 [*]	7.8 ± 4.49 [*]	4.81 ± 1.63 [*]	13.88 ± 3.87	3.98 ± 1.97 [*]
DM	0.0024	10.50 ± 5.90 ^Δ	2.29 ± 2.08 ^Δ	2.09 ± 1.59 ^Δ	5.49 ± 2.21 ^Δ	0.63 ± 0.62 ^Δ
SAD-H	3.6	19.5 ± 8.62 ^Δ	4.09 ± 2.14 ^Δ	3.28 ± 2.51 ^Δ	10.95 ± 6.46 ^Δ	0.60 ± 0.60 ^Δ
SAD-L	1.8	24.13 ± 5.38 ^Δ	4.53 ± 2.53 ^Δ	4.14 ± 2.53	13.77 ± 3.35	1.69 ± 1.05 ^Δ

Note: * $P < 0.05$, ** $P < 0.01$ vs Normal Group; ^Δ $P < 0.05$, ^{ΔΔ} $P < 0.01$ vs Model Group

22 min. The representative chromatograms, monitored by PDA and MS, are shown in Fig. 1.

Comparison of airway inflammation in blood among groups

In terms of the percentage of lymphocyte in blood, the model group was significantly lower ($p < 0.01$) than the control group, while DM group was a bit higher ($p < 0.05$) than the control group; as for the percentage of neutrophil, there was no significant difference between each administered group and the model group; in terms of the percentage of eosinophile, model group was significantly ($p < 0.01$) higher than the control group, while DM group and SAD-H group were significantly lower ($p < 0.01$) than model group, and SAD-L was a bit ($p < 0.05$) lower than model group (Table 2).

Comparison of airway inflammation in BLAF among groups

The levels of total leukocytes, lymphocytes and eosinophils in the model group were significantly higher than those in the normal group ($p < 0.05$). Compared with the model group, the levels of total leukocytes, lymphocytes, neutrophils, monocytes and eosinophils in DM group were dramatically lower ($p < 0.01$). Meanwhile, the level of leukocytes, lymphocytes and eosinophils in SAD-H or SAD-L group was also lower than the model group ($p < 0.05$). As for the level of neutrophils and monocytes, there was no significant difference between the administered group and the model group ($p > 0.05$) (Table 3).

Histopathologic examination

In the normal group, the structure of pulmonary lobule was normal; no obvious denaturation, necrosis or exfoliation could be detected in alveolar epithelial cell; there was no hyperemia or edema on the alveolar wall; nothing exudated alveolar space and bronchial lumen; no inflammatory cell soaked the interstitial substance; the bronchial wall was intact; the thickness of smooth muscle was normal; its cells stayed in normal order; no alveolar epithelial cell could be found in the bronchial lumen. In the model group, the smooth muscle became much thicker; a large number of inflammatory cells, including eosinophiles, lymphocytes, macrophages and a small number of plasmocytes, soaked the lower layer and muscle layer of bronchial mucosa; a portion of the epithelia of the bronchial cilia exfoliated; adhering cilia either fell over, denatured, or necrotized. In comparison with the model group, each administered group witnessed some improvement, of

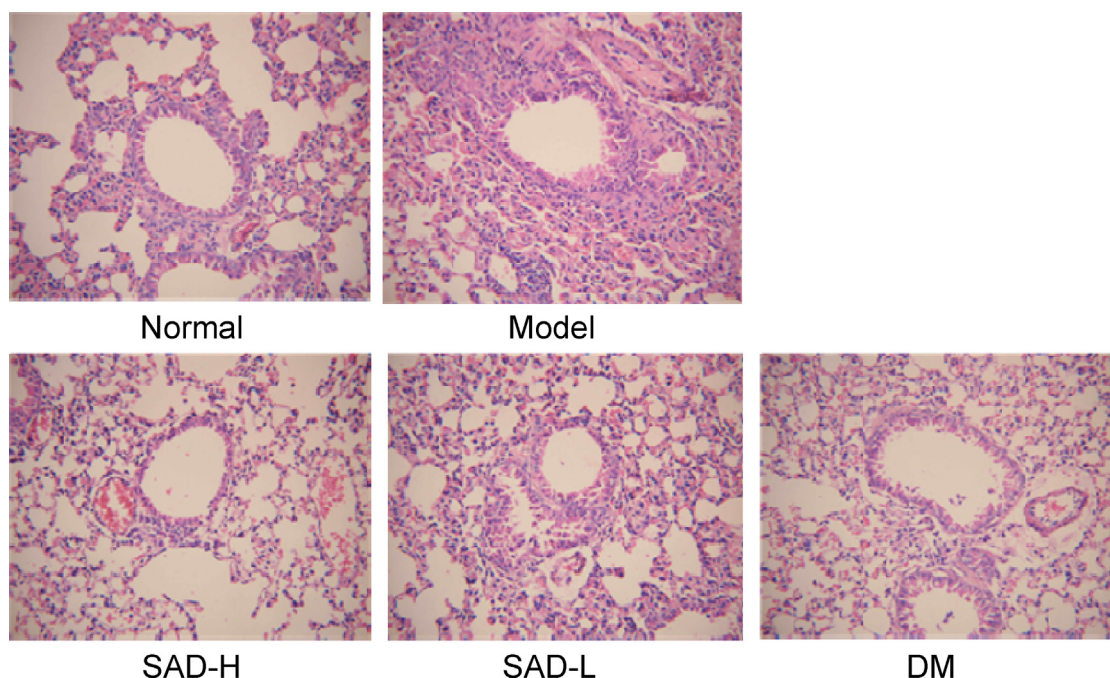


Fig. 2. HE trichrome staining 200 $\times$.

which DM group, SAD-H and -L groups improved more than the other groups (Fig. 2).

The effect of SAD on the CD4⁺CD25⁺Foxp3⁺ Treg population in spleen cells

CD4⁺CD25⁺Foxp3⁺ Treg cells play a significant role in the regulation of asthma, and the induction of allergen-specific Tregs has become an appealing strategy for asthma therapy. Thus, the relative proportion of Treg cells was determined in the spleen cells after oral treatment with SAD. The study showed that SAD-H group significantly increased the number of CD4⁺CD25⁺Foxp3⁺ Treg cells (Fig. 3). Notably, the oral administration of 3.6 g kg⁻¹ SAD significantly enhanced the frequency of Treg cells by up to 2.01%, compared to the model group (Table 4).

Result of the gene expression of Foxp3mRNA in the lung tissue

According to RT-PCR, Foxp3mRNA was expressed in the lung tissues of the normal group, and was poorly expressed in that of the asthma infected mouse group, resulting in a significant difference ($p < 0.05$) between the two groups. After the administration of dexamethasone, the expression of Foxp3 mRNA notably rose, but its content was lower than that of the normal group ($p < 0.05$). After the administration of high- and low-dosage SAD, the number of foxp3 expression significantly increased ($p < 0.05$), in comparison with the model group (Fig. 4 and Table 5).

Table 4
The percentage of CD4⁺CD25⁺Foxp3⁺ Treg in spleen cells.

Group	Dose (g kg ⁻¹)	Treg (%)
Normal	–	3.33 $\pm$ 0.12
Model	–	1.33 $\pm$ 0.40**
DM	0.0024	2.53 $\pm$ 0.73 $^{\Delta}$
SAD-H	3.6	2.01 $\pm$ 0.89 $^{\Delta}$
SAD-L	1.8	2.00 $\pm$ 0.30 $^{\Delta}$

Note: * $P < 0.05$, ** $P < 0.01$ vs Normal Group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs Model Group

Table 5
The gene expression of Foxp3mRNA.

Group	Dose (g kg ⁻¹)	Mean
Normal	–	1.01
Model	–	0.16*
DM	0.0024	0.84 $^{\Delta}$
SAD-H	3.6	0.54 $^{\Delta}$
SAD-L	1.8	0.52 $^{\Delta}$

Note: * $P < 0.05$, ** $P < 0.01$ vs Normal Group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs Model Group

Protein content of Foxp3 in the lung tissue

According to the result of Western blot analysis, the transcription factor foxp3 protein was expressed in the lung tissues of the normal control mouse group, and was poorly expressed in that of the asthma infected mice group, resulting in a significant difference ($p < 0.05$) between the two groups. After the administration of dexamethasone, the content of Foxp3 protein significantly increased; its content level was higher than that of the asthma infected mice group but lower than that of the normal control mice group ($p < 0.05$). After the administration of medium- and low-dosage SA, the number of foxp3 expression significantly increased ($p < 0.05$), in comparison with the model group (Fig. 5 and Table 6).

Discussion

Traditional Chinese medicine has a wealth of experience in treating asthma. Pathological changes in stages of asthma attack are

Table 6
Protein content of Foxp3 among groups.

Group	Dose (g kg ⁻¹)	Mean
Normal	–	1.05
Model	–	0.53*
DM	0.0024	0.74 $^{\Delta}$
SAD-H	3.6	0.72 $^{\Delta}$
SAD-L	1.8	0.60 $^{\Delta}$

Note: * $P < 0.05$, ** $P < 0.01$ vs Normal Group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs Model Group

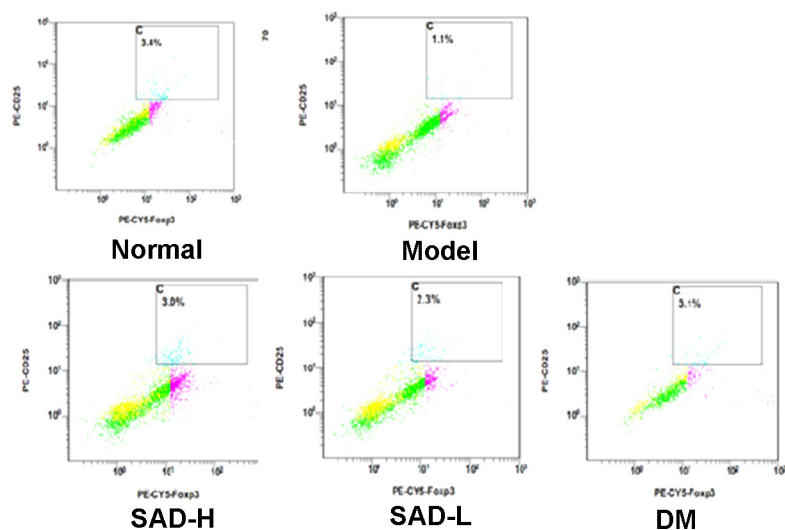


Fig. 3. The percentage of CD4⁺CD25⁺Foxp3⁺ Treg in spleen cells.

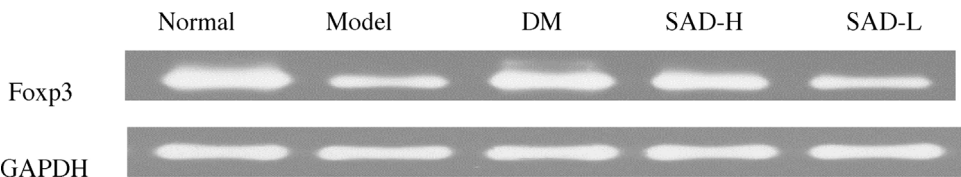


Fig. 4. The gene expression of Foxp3mRNA.

mainly characterized by onset of insidious phlegm by exogenous pathogenic factors and lung qi's failure to disperse and descend (Jin et al., 2012). Dispersing the lung and relieving asthma is one of the main treating methods. San'ao decoction, the classic formula of traditional Chinese medicine treating asthma, is composed of Herba Ephedrae, Semen Armeniacae amarum, Radix Glycyrrhizae. SAD mainly treats cough due to exogenous wind-cold syndrome marked by cold by wind pathogenic factor, stuffy nose, heavy voice, cough with profuse, headache and dizziness. By Song dynasty, its name first appears in prescriptions of peaceful benevolent dispensary. At that time, its set prescription consists of Herba Ephedrae, Semen Armeniacae amarum, and Radix Glycyrrhizae, disobeying the routine process method. That is why the formula is called San'ao decoction. Practitioners of later generations often use its modification to treat upper respiratory disease like cold and bronchitis, and have achieved good therapeutic effects in the clinic (Fan, 2006).

Early Orthogonal experiments showed that in the OVA sensitization asthmatic mice model its compatibility could lower airway reactivity and airway inflammatory cell infiltration. Based on the above results, this study mainly discussed San'ao decoction's immunomodulatory effects on Treg. The results suggested that EOS count increased obviously in peripheral blood of asthmatic mice by OVA sensitization in comparison with blank control group. There was inflammatory cell infiltration with a large number of neutrophil and lymphocyte in the mouse lung, edema in airway mucos, and a large number of inflammatory cells in

alveolar space, which coincided with pathological changes of asthma and thus suggested the successful setup of asthma model Compared with asthma model, EOS count in BALF in treatment group reduced obviously. High and medium dosage of San'ao decoction could improve the pathological changes of the lung and trachea, and raised CD4⁺CD25⁺Foxp3⁺ T cell population level in the OVA sensitization asthmatic mice. Medium and low dosage can make foxp3 gene and protein expression quantity in the OVA sensitization asthma rats up-regulating markedly (Barbas et al., 2008; Xu et al., 2010; Feng et al., 2009).

It's has been verified that asthma can cause serious imbalance of T lymphocyte subsets and various cytokine abnormalities. Existing researches contend that Th1/Th2 Imbalance Theory plays a significant role in asthma pathogenesis studies and thus it is widely accepted by scholars. However, after SAK- AGUCHI proposed in 1995 that T-cell (Treg) is able to regulate CD4⁺ T cell new subsets, recent discoveries have indicated that: Treg is a kind of T-cell that's capable of immunological regulation and suppression; through cell contact-dependent mechanism (including CTLA, GITR and Foxp3) or inhibitory cytokines (such as IL-10 and TGF-B) mechanism, Treg can restrain immune T-cells activation, maintain immune tolerance, and prevent immune diseases (Wang et al., 2011; Cavassani et al., 2006).

The results of flow cytometry tests show that the cell count of CD4⁺ of CD4⁺CD25⁺Foxp3⁺ in the spleen lymphocytes of asthmatic mice model is lower than the normal control group, which matches

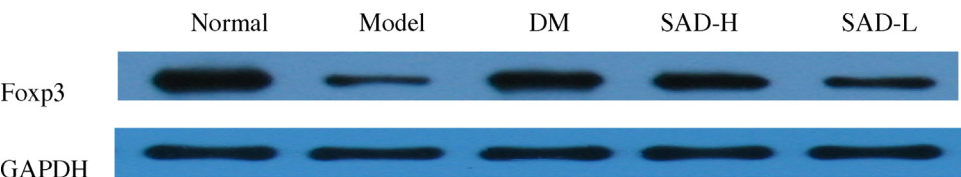


Fig. 5. Protein content of Foxp3.

former reports. Through exploration, the author found that the cell count and function of Treg in asthma patients decreased. However, if the Treg cell were input into the asthmatic animals' body, their airway hyperactivity, eosinophilic infiltration and the development of Th2 cytokine would be relieved (Barbas et al., 2008). Inadequate secretion of TGF- β , lower gene expression of Foxp3, the reduction of the cell count and the differentiation maldevelopment of regulatory T cells in CD4⁺CD25⁺ are the main reasons for children contracting asthma (Shi et al., 2010). Low, medium and high dosages of San'ao decoction can significantly improve the Treg level in the body of acute asthmatic mice, suggesting that San'ao decoction can cure asthma through adjusting the Treg level inside body.

The differentiation, maturation, and functioning of Treg are connected with the transcription factor Foxp3 to a significant extent (Luo et al., 2006). To reduce or remove Foxp3 can lead to a weakening functioning of Treg, while to add Foxp3 to CD25⁺effector T-lymphocyte enables the latter to restrain immunity, indicating the vital importance of Foxp3 to the functioning of Treg. It should be noted that the Foxp3 protein formula of asthma infected lung tissue is different from that of extrapulmonary tissue. The reason for this differentiation is yet to be found. Some studies hold that the expression of Foxp3 mRNA in asthma infected lung tissue rises strikingly, and in Treg drops sharply (Hori et al., 2003). This research found that the expression of Foxp3 mRNA in the lung tissues of asthma infected mice decreases significantly. Medium and low SAD dose groups demonstrated that the expression of Foxp3 mRNA in acute asthmatic mice rises remarkably, which suggests SAD may first increase Treg cells in the lung, and then increase the total Foxp3 protein content in Treg cells.

In summary, the study demonstrates that SAD can effectively attenuate airway inflammation and improve lung function and airway remodeling in a mouse model of asthma. More importantly, the number of CD4⁺CD25⁺Foxp3⁺ Treg cells and Foxp3 expression are significantly increased by SAD administration. These findings verify the possibility of SAD being a potent agent for asthma prevention and treatment, and demonstrate that CD4⁺CD25⁺Foxp3⁺ Tregs may contribute to the antiasthmatic effect of SAD.

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