



Shegan-Mahuang Decoction ameliorates asthmatic airway hyperresponsiveness by downregulating Th2/Th17 cells but upregulating CD4 + FoxP3 + Tregs



Cheng-Chuang Lin¹, Yuan-Yuan Wang¹, Si-Min Chen¹, Yun-Tao Liu, Ji-Qiang Li, Fang Li, Jie-Chen Dai, Tong Zhang, Feifei Qiu, Huazhen Liu, Zhenhua Dai*, Zhong-De Zhang**

Section of Immunology & Joint Immunology Program, The Second Clinical Medical College of Guangzhou University of Chinese Medicine, Guangdong Provincial Academy of Chinese Medical Sciences, Guangzhou, Guangdong, 510006, China

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ABSTRACT

Ethnopharmacological relevance: Shegan-Mahuang Decoction (SMD), also named Yakammaoto or Shegan-Mahuang Tang, is a classic formula of traditional Chinese medicine with nine herbs, including *Asarum sieboldii* Miq., *Aster tataricus* L.f., *Ephedra sinica* Stapf, *Belamcanda chinensis* (L.) Redouté, *Pinellia ternata* (Thunb.) Breit., *Schisandra chinensis* (Turcz.) Baill., *Tussilago farfara* L., *Zingiber officinale* Roscoe, and *Ziziphus jujuba* Mill. SMD was originally discovered by Zhang Zhongjing in Eastern Han dynasty. It has been widely used as traditional medicine to treat flu-like symptoms in China and Japan for around twenty centuries. It was also utilized for the treatment of the early stage of acute asthma. However, the immune mechanisms underlying its therapeutic effects remain unknown.

Aim of the study: This study was set to investigate the effects of SMD on asthmatic airway hyperresponsiveness and its impacts on adaptive immunity in a mouse model of asthma.

Materials and methods: The HPLC fingerprint profile of the water extract of SMD recorded 22 peaks, including those equivalent to guanosine, chlorogenic acid, tectoridin, 6-gingerol and wuweizisu B, as described previously (Yen et al., 2014). Airway hyperresponsiveness was assessed by measuring the airway resistance. Cellular infiltration was measured via H&E staining and immunohistochemistry while gene expression was analyzed using real-time RT-PCR. Treg frequency was determined through flow analysis whereas cytokine production in the supernatant was evaluated using ELISA. Finally, mTOR and NF-κB signalings were analyzed via Western blotting.

Results: We found that SMD largely corrected the imbalance of Th cell subsets in asthmatic mice with a significant inhibition of Th2 and Th17 cytokine production, thereby reducing asthmatic airway hyperresponsiveness. Moreover, lung function tests showed that SMD reduced airway hyperresponsiveness while immunohistochemical analyses demonstrated that SMD attenuated pulmonary infiltration of CD3⁺ and CD4⁺ T cells. Further, we observed a significant increase in the proportion of CD4 + Foxp3 + Tregs in SMD-treated asthmatic mice. We also found that SMD downregulated gene expression of GATA3 and ROR-γt in murine lung tissue. In addition, both mTOR- and NF-κB-related protein expressions were reduced in the lung tissue of SMD-treated mice. SMD inhibited Th2/Th17 cytokine production by CD4⁺ T cells and also their mTOR activity in vitro.

Conclusions: Our findings demonstrate that SMD attenuates asthmatic airway hyperresponsiveness by hindering Th2/Th17 differentiation, promoting CD4 + Foxp3 + Treg generation and suppressing mTOR and NF-κB activities.

Abbreviations: AHR, airway hyperresponsiveness; Dex, dexamethasone; mTOR, mechanistic target of rapamycin; SMD, Shegan-Mahuang decoction; SMD-H, high dose of SMD; SMD-L, low dose of SMD; and Th cell, T helper cell

* Corresponding author. Section of Immunology & Joint Immunology Program Guangdong Provincial Academy of Chinese Medical Sciences 55 Nei Huan Xi Lu, College Town Guangzhou, Guangdong, 510006, China.

** Corresponding author.

E-mail addresses: zdai2009@outlook.com (Z. Dai), doctorzdd99@163.com (Z.-D. Zhang).

¹ CCL, YYW and SMC contributed equally to this work and share the first author.

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1. Introduction

Asthma is a chronic inflammatory airway disease with reversible airflow obstruction, persistent airway hyperresponsiveness (AHR) and airway remodeling (Pawankar, 2014). Symptoms, including wheezing, shortness of breath, chest tightness and/or coughing, may fluctuate but worsen over time, leading to respiratory failure. As a public health problem, asthma affects over 300 million individuals globally and leads to heavy economic burden for the society. Although the pathogenesis of asthma is complex, researches have indicated that most phenotypes of asthma are associated with pathogenic Th2 and Th17 cells (Olin and Wechsler, 2014; Wenzel, 2012). Meanwhile, Treg cells maintain auto-immune tolerance by inhibiting the generation and proliferation of autoreactive Th cells. They regulate the differentiation of Th1, Th2 and Th17 in different microenvironments (Rothstein and Camirand, 2015). Several cellular signaling pathways, such as mTOR and NF- κ B pathways, are changed in asthma, resulting in continuous inflammation. The mTOR pathway plays an important role in the differentiation and proliferation of Th and Treg cells (Powell and Delgoffe, 2010) while NF- κ B is a potent nuclear transcription factor that promotes the expression of proinflammatory mediators and the progression of airway inflammation (Christman et al., 2000; Oh et al., 2011).

Although asthma is generally considered to be well managed by inhalation of corticosteroids, long-term use of corticosteroids can cause various side effects. In addition, about 40% of asthmatics fail to respond to corticosteroids (Martin et al., 2007). Thus, there is a need for alternative therapeutic options. Traditional Chinese Medicine (TCM) has been shown to have a therapeutic effect on asthma (Liu et al., 2016; Wang and Zhang, 2017). Shegan-Mahuang Decoction (SMD), also named Yakammaoto, is a classic TCM formula with nine herbs, including *Asarum sieboldii* Miq., *Aster tataricus* L.f., *Ephedra sinica* Stapf, *Belamcanda chinensis* (L.) Redouté, *Pinellia ternata* (Thunb.) Breit., *Schisandra chinensis* (Turcz.) Baill., *Tussilago farfara* L., *Zingiber officinale* Roscoe, and *Ziziphus jujuba* Mill. HPLC fingerprint profile of the water extract of SMD recorded 22 peaks, mainly including those equivalent to guanosine, chlorogenic acid, tectoridin, 6-gingerol and wuweizisu B, as described previously (Yen et al., 2014). SMD was originally discovered by Zhang Zhongjing in Eastern Han dynasty. It has been widely used as a traditional medicine to treat flu-like symptoms in China and Japan for around twenty centuries. As one of the common TCM formulas, SMD has been traditionally used to relieve asthmatic symptoms (Chen et al., 2012; Chen and Hu, 2010; Guo and Liu, 2016; Zhao and Pang, 2019). It has also been reported that SMD improves cough variant asthma, post-infection cough, bronchitis and other airway injuries (Chen et al., 2012; Wang and Zhang, 2017; Yen et al., 2014). Previous studies have further revealed that SMD can treat asthma by regulating the production of inflammatory mediators, such as IFN- γ , IL-4, IL-10, IL-13, and TNF- α (Chen and Hu, 2010; Zhou et al., 2010). However, the exact immune mechanisms underlying the attenuation of asthma by SMD remain unclear.

Here, we studied whether SMD would ameliorate asthmatic airway hyperresponsiveness by suppressing T cell responses. In these

experiments, we found that SMD significantly suppressed murine asthmatic airway hyperresponsiveness and airway inflammation induced by OVA. SMD decreased the infiltration of CD3⁺ and CD4⁺ T cells and the expression of transcription factors GATA-3 and ROR- γ t in lung tissue, while reducing production of IL-4, IL-17A and IL-23 in the serum. Moreover, SMD increased the frequency of CD4⁺ FoxP3⁺ Tregs in the lymph nodes and spleens from asthmatic mice. Further studies in vitro demonstrated that SMD reduced the production of Th2 and Th17 cytokines by CD4⁺ T cells. We also observed the suppression of mTOR and NF- κ B signaling by SMD in asthmatic lung tissue and inhibition of mTOR signaling in cultured T cells. Thus, SMD attenuates asthmatic airway hyperresponsiveness by downregulating Th2/Th17 responses while enhancing Treg generation.

2. Materials and methods

2.1. Animals

BALB/c mice (female, 6–8 weeks old, body weight 20 $\pm$ 2 g) were purchased from Experimental Animal Center of Southern Medical University (Guangzhou, China). All mice were housed in a pathogen-free room with controlled conditions. Our research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, as found in the US guidelines (NIH publication #85–23, revision in 1985). All experiments were approved by the Institutional Animal Care and Use Committee of Guangdong Provincial Academy of Chinese Medical Sciences and the Medical Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University.

2.2. Preparation of SMD

The SMD formula consists of nine herbs, including *Asarum sieboldii* Miq., *Aster tataricus* L.f., *Ephedra sinica* Stapf, *Belamcanda chinensis* (L.) Redouté, *Pinellia ternata* (Thunb.) Breit., *Schisandra chinensis* (Turcz.) Baill., *Tussilago farfara* L., *Zingiber officinale* Roscoe, and *Ziziphus jujuba* Mill. The HPLC fingerprint profile of the water extract of SMD or yakammaoto recorded 22 peaks, including those equivalent to guanosine, chlorogenic acid, tectoridin, 6-gingerol and wuweizisu B, as described previously (Yen et al., 2014). The composition of the herbal formula, weight ratio of the herbs and voucher/batch numbers are shown in Table 1. The medicinal herbs were manufactured by Kangmei Pharmaceuticals Inc. (Puning, Guangdong, China) and ordered through the Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China). A voucher specimen was deposited at the Herbarium of Guangzhou University of Chinese Medicine (Code: GUCM). All herbs are traditionally used to treat patients in China. According to the standard of traditional decoction method of the herbs, herbs were soaked in pure water for 30 min, then boiled over high heat for a while and simmered for 40 min. After that, the liquid was filtered and concentrated through a rotary flask, resulting in low concentration of SMD (0.63g crude herbs per milliliter water) or high

Table 1
Composition of SMD.

Chinese medicinal plant	Family	Voucher number	Batch number	Weight (g)
<i>Ephedra sinica</i> Stapf	Ephedraceae	180106671	0772	4.0
<i>Pinellia ternata</i> (Thunb.) Freit.	Araceae	180302	1801	4.0
<i>Zingiber officinale</i> Roscoe	Zingiberaceae	180100547	0642	4.0
<i>Tussilago farfara</i> L.	Asteraceae	180203821	7433	3.0
<i>Aster tataricus</i> L.f.	Asteraceae	180306011	5280	3.0
<i>Ziziphus jujuba</i> Mill.	Rhamnaceae	180106791	1690	2.0
<i>Belamcanda chinensis</i> (L.) Redouté	Iridaceae	1712001	1858	1.5
<i>Asarum sieboldii</i> Miq.	Aristolochiaceae	180100581	0463	1.5
<i>Schisandra chinensis</i> (Turcz.) Baill.	Schisandraceae	180302331	3101	1.0

concentration of SMD (2.52 g crude herbs per milliliter water). Each mouse received 0.15 ml, making a low dose of 4.7 and high dose of 18.9 g/kg. These doses were within the ranges published by others (Zhang et al., 2014; Zhao et al., 2010).

2.3. OVA-induced asthma mouse model and animal treatment

Mice were randomly divided into control group, model group, high dose of SMD group (SMD-H, 2.52 g/ml), low dose of SMD group (SMD-L, 0.63 g/ml) and Dexamethasone (Dex) group. For systemically administered agents, the conversion factor of SMD usage is based on clinical usage, resulting in the dose of SMD for mice is 10 ml/kg of body weight. Except for the control group, mice were sensitized with OVA (Thermo, USA) (50 µg, intraperitoneal injection) and 2 mg of adjuvant AL (OH)₃ (Thermo, USA) on days 0, 6, and 13. On the 21st day, mice were placed in a self-made nebulizer and exposed to aerosol OVA for 60 min. Atomized OVA challenge was repeated daily for two weeks. Half an hour before this challenge, mice from SMD-treated groups were administered orally with SMD, and mice of DEX group were intraperitoneally injected with DEX. The model group was intraperitoneally injected with an equal amount of saline.

2.4. Measurement of airway hyperresponsiveness

After the last OVA challenge, mice were anesthetized with 1% pentobarbital sodium, connected to an instrument (Buxco, USA) for measuring murine pulmonary function through percutaneous tracheotomy. The respiratory rate was set to 140 beats/min. Methacholine, with different concentrations (3.125 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, and 50 mg/ml) was used to induce airway responsiveness. The airway resistance R (cmH₂O/s/ml) was detected by the machine (DSI-BUXCO-RC, St. Paul, USA).

2.5. Measurement of cytokines

The levels of cytokines (IL-4, IL-17a and IL-23) in mouse serum were determined using the Th2/17 Magnetic Bead Panel 96-Well Plate Assay kit according to the corresponding protocol provided by the manufacturer (Millipore, Billerica, MA, USA). The level of IL-4 and IL-17a in cell culture supernatant was measured using ELISA kit (Boster Inc., Guangzhou, China) according to the manufacturer's instructions and the absorbance was read at 450 nm in a microplate spectrophotometer (Thermo Fisher Scientific, USA).

2.6. Histological analysis and immunohistochemistry

Mouse lung tissues embedded in paraffin were cut into 3 µm thick sections, and then placed on glass slides for the usage of H&E and immunohistochemical staining. For immunohistochemical staining, slides were incubated with primary anti-CD3 (1:100) or anti-CD4 (1:100) monoclonal antibody (Abcam, USA) at 4 °C overnight, followed by the corresponding secondary antibody. Slides were finally colored with 3'-diaminobiphenyl (DAB, Sigma-Aldrich). The positive expression area of CD3 and CD4 in the image was measured using Image J software.

2.7. Flow cytometric analysis

Draining lymph nodes and spleen cells were collected and surface markers were stained with mouse FITC-CD4 antibody (BioLegend, USA). After incubation, the cells were fixated and permeabilized using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent Kit (eBioscience, USA), and then incubated with mouse APC-Foxp3 antibody (eBioscience, USA). Finally, the frequency of CD4+ Foxp3+ Tregs was measured by FACSCalibur (BD Biosciences, USA).

2.8. Western blot analysis

Total protein samples from lung tissues or cultured CD4⁺ T cells were obtained with RIPA lysis buffer. The concentration of the protein was analyzed using a BCA kit (Thermo Fisher Scientific). 10% SDS-PAGE gel electrophoresis was performed to separate the proteins, which then were electro-transferred onto a PVDF membrane. TBST containing 5% BSA was used to block the non-specific binding to the membrane. The membrane was incubated with anti-P70S6k, anti-phospho-P70S6k, anti-P65, anti-phospho-P65, anti-RelB or anti-GAPDH antibody (Cell Signaling Technology, USA) at 4 °C overnight. Next, the membrane was washed and incubated with a HRP-conjugated secondary antibody. At last, signals were detected by the ECL method (Beyotime, China) and analyzed using Image J program software.

2.9. Quantitative real-time reverse transcription PCR (qRT-PCR)

Total mRNA was extracted from lung tissue using TRIzol reagent (Invitrogen, USA), and then cDNA was generated using a reverse transcription kit (TAKARA Bio Incorporation, Kusatsu, Japan). Quantitative PCR was then performed in an ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA) using the Quantitect SYBR Green PCR Kit (Takara, Beijing, China). All reactions were carried out according to the manufacturer's protocol. GAPDH gene was used as an internal standard gene, and the data were quantitatively analyzed by the 2^{-ΔΔCT} method. The value in the Model group was set to 1.0, and all data were shown as relative mRNA expression (fold change). The primer sequences are listed in Table 2 below:

2.10. Assays of SMD cytotoxicity in vitro

The cytotoxicity of SMD to CD4⁺ T cells was examined using CCK-8 assay. FACS-sorted CD4⁺ T cells were cultured in 96-well plates in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin. SMD was added to each well at different concentrations (250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.63 mg/ml and 7.81 mg/ml, respectively) in quadruplicate. 96 h later, 20 µL of CCK-8 was added to each well and incubated at 37 °C for 4 h. The absorbance was detected by a microplate spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 450 nm.

2.10.1. T cell stimulation in vitro

FACS-sorted CD4⁺ T cells (5 × 10⁵ cells/well) derived from BALB/c mice were stimulated with anti-CD3/anti-CD28 Abs in the absence or presence of SMD (31.25 or 15.63 mg/ml) or DEX (5 µg/ml). For Th17 differentiation, TGF-β1 (5 ng/ml, Peprotech) and IL-6 (10 ng/ml, Peprotech) were also added to the culture. Culture supernatants were finally harvested and evaluated for cytokines IL-4 and IL-17A via ELISA 96 h later.

2.11. Statistical analysis

Comparisons of means were conducted using one-way ANOVA and Student's *t*-test. Data were presented as the mean ± SEM. GraphPad

Table 2
Primer sequences of target genes.

Target gene	Primer sequence (5'→3')
GATA3 (forward)	ATTACCACCTATCCGCCCTAT
GATA3 (reverse)	CGGTTCTGCCATTCAATTTAT
RORγt (forward)	ACAAATTGAAGTGATCCCTTGC
RORγt (reverse)	GGAGTAGGCCACATTACACTG
GAPDH (forward)	GGAGAGTGTTCCTCGTCCC
GAPDH (reverse)	ACTGTGCCGTTGAATTTGCC

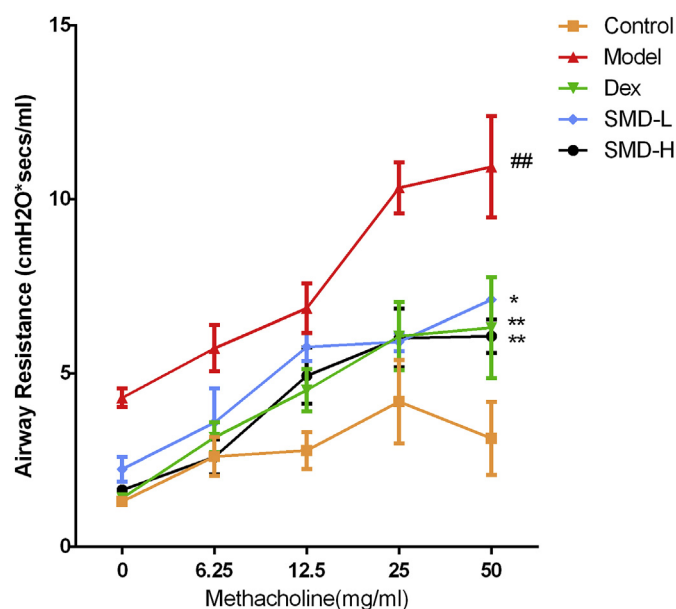


Fig. 1. SMD ameliorates airway hyperresponsiveness in asthmatic mice. Bronchial challenge test was performed to detect airway resistance after the last OVA inhalation. Methacholine was used to induce airway hyperresponsiveness while airway resistance of asthmatic and normal mice was measured. Data are presented as means $\pm$ SEM ($n = 4-5$ mice per group, $##p < 0.01$ vs. Control group; $**p < 0.01$ and $*p < 0.05$ vs. Model group). One of three separate experiments is shown.

Prism 8 (GraphPad Software, USA) was used for graphing and analyses. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. SMD attenuates airway hyperresponsiveness in asthmatic mice

A mouse model of asthma induced by nebulized OVA was used in our experiment. We observed that mice with high dose SMD or Dexamethasone treatment were more active than control group (data not shown). Besides, we investigated the protective effects of SMD on asthmatic AHR via bronchial provocation test. As illustrated in Fig. 1, the airway resistance of the experimental Model group was significantly higher than that of the Control group, indicating that we have successfully established an asthmatic mouse model. Both low and high dose of SMD obviously reduced the airway resistance of asthmatic mice compared to the model group. Interestingly, treatment with high doses of SMD was nearly as effective as Dexamethasone in decreasing the airway resistance.

3.2. SMD alleviates airway inflammation in asthmatic mice

To study the pulmonary inflammation of asthma mice, H&E staining on lung tissue was performed 14 days after SMD treatment, and we found inflammatory cell infiltration around the trachea and blood vessels in the lung tissue of asthmatic mice, together with thickened alveolar wall and fluid extravasation (Fig. 2). We also demonstrated that SMD treatment, at either low or high doses, significantly reduced inflammatory cell infiltration and destruction of alveolar walls compared to untreated asthmatic mice. Besides, there were less eosinophils and mucus plugs in the lungs of SMD treated group than in the model group. These results suggest that SMD may ameliorate asthmatic symptoms through lessening airway inflammation in the lung.

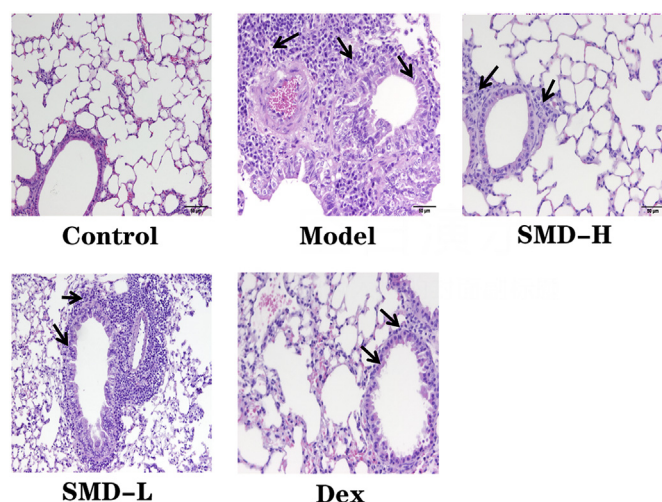


Fig. 2. SMD attenuates pulmonary inflammation in asthmatic mice. OVA-induced asthmatic mice were treated with high-doses of SMD (SMD-H), low-doses of SMD (SMD-L) or Dex for 14 days. Then, H&E staining on lung tissues of asthmatic mice were performed to assess inflammatory cell infiltration and airway remodeling. One of three sets of images is shown.

3.3. SMD treatment inhibits the infiltration of $CD3^+$ and $CD4^+$ T cells in the lung

To investigate whether SMD exerts an effect on T cells in asthmatic mice, OVA-induced asthmatic mice were treated with SMD or Dex for 14 days. The infiltration of T cells in mouse lung tissues was then detected by immunohistochemistry. As shown in Fig. 3A & 3B, both low and high doses of SMD obviously reduced $CD3^+$ T cell infiltration ($P < 0.01$) in lung tissues of asthmatic mice compared to asthmatic mice without treatment. On the other hand, there were much fewer $CD4^+$ T cells infiltrated in lung tissue of asthmatic mice treated with high doses, but not low doses, of SMD than those mice without SMD. These data suggest that only high doses of SMD can suppress $CD4^+$ T cell infiltration while both low and high doses of SMD can inhibit $CD3^+$ T cell infiltration in the lung.

3.4. SMD downregulates Th2/Th17 related transcription factor expression and cytokine production

It has been reported that asthma phenotypes are positively associated with Th2 and Th17 development. Naive T cell differentiation into Th2 cells is largely dependent on key transcription factor GATA-3 (Hirose et al., 2017). On the other hand, nuclear orphan receptor- γ t (ROR- γ t) is a specific transcription factor that induces differentiation of Th17 cells (Ivanov et al., 2006). Therefore, the expression of ROR- γ t and GATA-3 in lung tissue of asthmatic mice was measured via real-time RT-PCR 14 days after treatment with SMD or DEX. As shown in Fig. 4A, the model group, which was exposed to OVA, showed a significant increase in the mRNA expression of ROR- γ t and GATA3 compared with control group ($p < 0.01$). The mRNA expression of GATA-3 and ROR- γ t was significantly decreased in mice treated with high doses or low doses of SMD as well as DEX. There was no significant difference in protective effects of high doses versus low doses of SMD.

On the other hand, Th2/Th17 related cytokines in serum derived from control or SMD-treated mice were measured. We observed that OVA significantly increased IL-4, IL-17A and IL-23 production in the serum compared to control group (Fig. 4B). High doses of SMD (SMD-H) could reduce the production of IL-4 ($P < 0.01$), IL-17A ($P < 0.05$) and IL-23 ($P < 0.05$) in the serum. However, there is no significant difference between model group and low-doses group (SMD-L). These results indicate that SMD inhibits Th2/Th17 related transcription factor

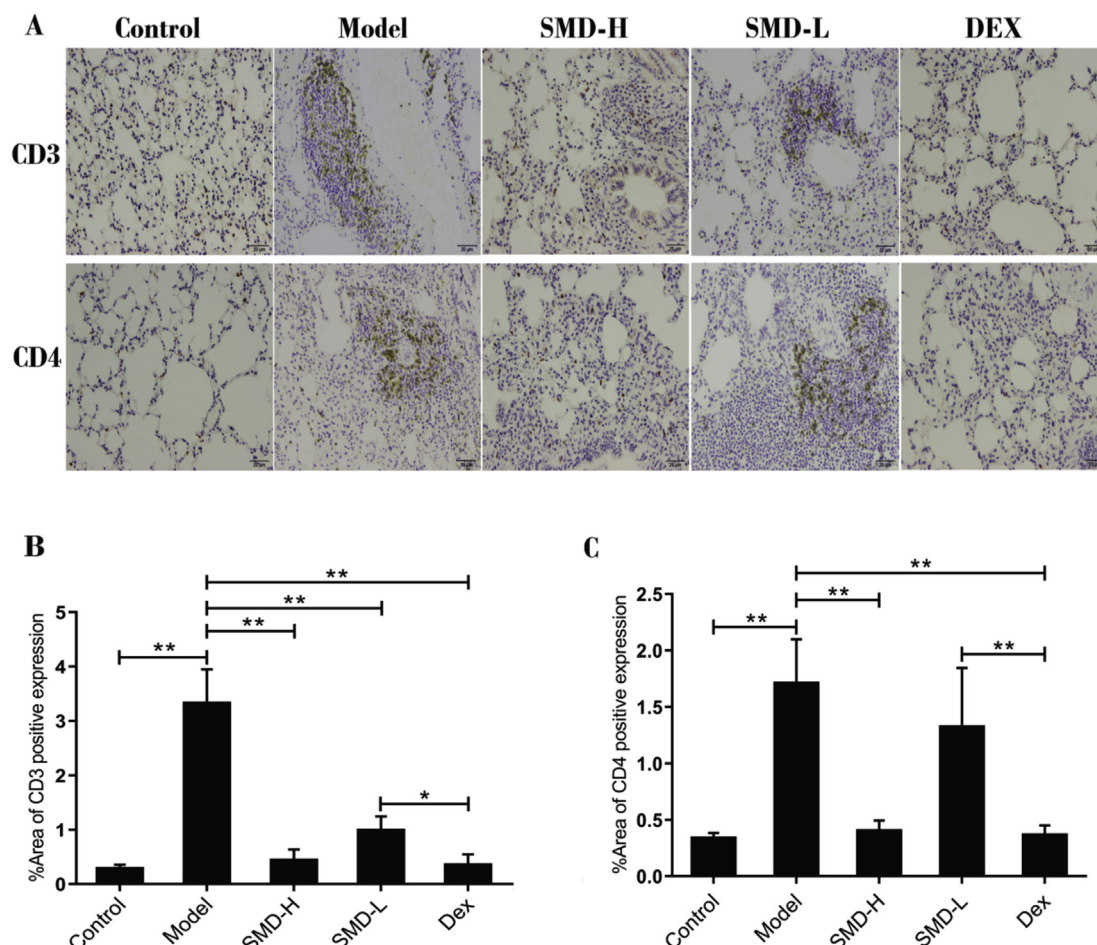


Fig. 3. SMD inhibits infiltration of CD3⁺ and CD4⁺ T cells in lung tissues of asthmatic mice.

(A) Immunohistochemical staining on lung tissues was performed to detect infiltrating CD3⁺ and CD4⁺ T cells in control or asthmatic mice 14 days after treatment with SMD (Magnification: 200X). The CD3-positive (B) or CD4-positive area (C) in lung tissue was calculated using Image J software ($n = 3-4$). The data are expressed as mean $\pm$ SD ($\#p < 0.01$ vs. Control group; $**p < 0.01$ and $*p < 0.05$ vs. Model group). A representative of three independent experiments is shown.

expression and cytokine production, thereby reducing airway inflammation in asthmatic mice.

3.5. SMD induces CD4⁺Foxp3⁺ Tregs in asthmatic mice

Treg cells can inhibit the proliferation of Th cells and play a role in controlling asthma. To investigate the effects of SMD on Tregs, cells from the spleen and draining lymph nodes of asthmatic mice were analyzed via flow cytometry 14 days after SMD treatment. The results showed that OVA obviously downregulated CD4⁺Foxp3⁺ Tregs ($p < 0.01$) in both spleen and lymph nodes from asthmatic mice (Fig. 5). High-doses, but not low-doses, of SMD significantly increased the proportion of CD4⁺Foxp3⁺ Treg cells ($p < 0.01$) in the spleen of asthmatic mice when compared with model group without SMD (Fig. 5A). In contrast, either low-doses or high-doses of SMD augmented the frequency of CD4⁺Foxp3⁺ Treg cells in lymph nodes of the asthmatic mice (Fig. 5B). As a positive control, DEX also did the same. These findings suggest that amelioration of asthmatic airway hyperresponsiveness by SMD is probably dependent on its induction of CD4⁺Foxp3⁺ Treg cells.

3.6. SMD inhibits mTOR and NF- κ B pathways in lung tissues of asthmatic mice

To determine whether SMD regulates mTOR and NF- κ B pathways in

lung tissues of asthmatic mice, we analyzed the changes of these two signaling pathways via western blotting two weeks after SMD treatment. As shown in Fig. 6A-B, both high doses and low doses of SMD significantly inhibited the expression of P70S6K and phosphorylated P70S6K (P-P70S6K) in lung tissues ($p < 0.01$), so did DEX. Furthermore, we examined the expression level of NF- κ B signaling P65 and RelB. As shown in Fig. 6C-E, the expression of P65, P-P65 or RelB was significantly higher in the OVA-induced group (Model) compared with control group. SMD treatment obviously decreased the expression of RelB and P-65 compared to OVA only group (Model). Meanwhile SMD also reduced the phosphorylation of P65 (P-P65). These results suggest that SMD can effectively inhibit both mTOR and NF- κ B pathways in lung tissues of asthmatic mice.

3.7. SMD inhibits IL-4 and IL-17A production by CD4⁺ T cells in vitro

To first determine potential cytotoxicity of SMD, FACS-sorted CD4⁺ T cells were cultured for 96 h to measure their viability using CCK-8 assays. As shown in Fig. 7A, up to 31.25 mg/ml of SMD did not alter cell viability at all. The cytotoxicity of SMD was significant at a concentration of 62.5 mg/ml. To ask if SMD has an impact on expression of Th2 and Th17 cytokines in vitro, FACS-sorted CD4⁺ T cells were cultured under normal or Th17 primed condition without or with SMD or Dex for 96 h. As shown in Fig. 7B, either 31.25 mg/ml or 15.63 mg/ml of SMD, which was non-cytotoxic, significantly reduced the protein

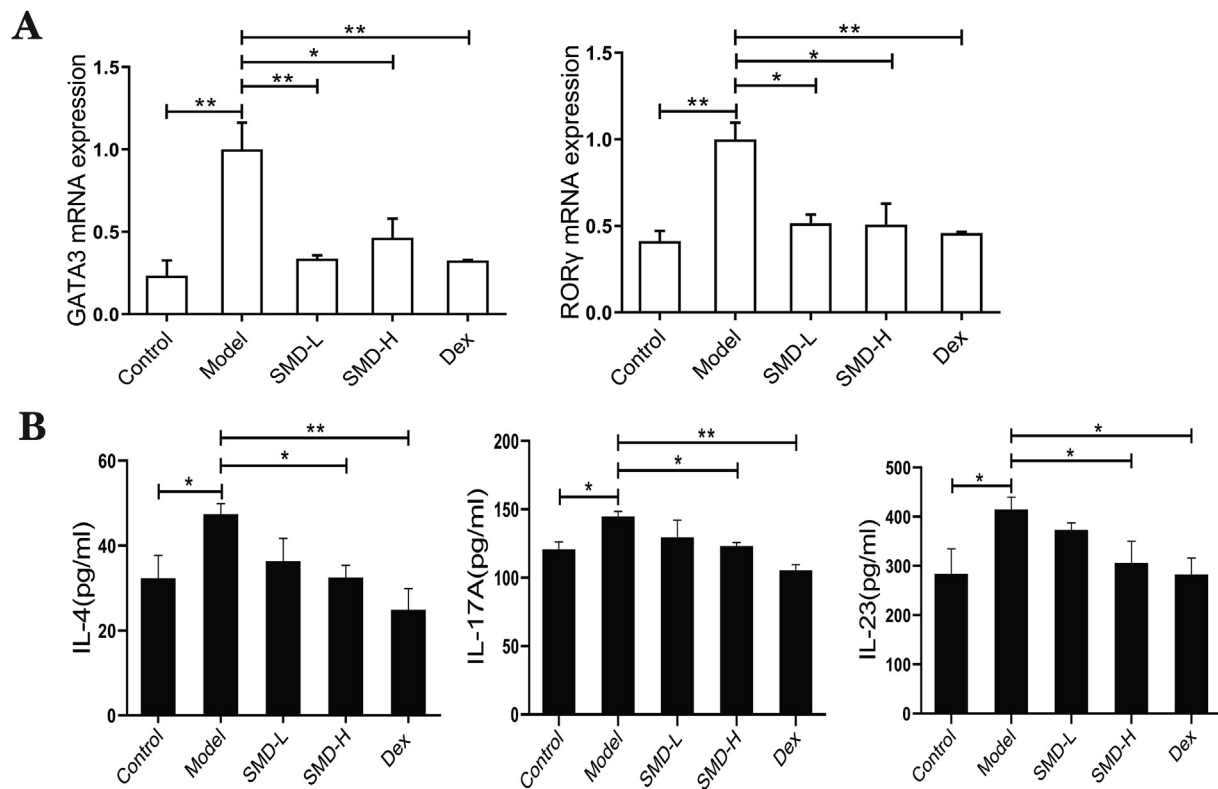


Fig. 4. Effects of SMD on mRNA expression of Th2/Th17 related transcription factors and protein levels in the serum of asthmatic mice.

(A) The mRNA expression of transcription factors GATA-3 and RORγt in lung tissue of OVA-induced mice was measured by RT-PCR 14 days after treatment with SMD or DEX. (B) The protein levels of cytokines IL-4, IL-17A, and IL-23 in mouse serum were measured after treatment with SMD or DEX. Data are expressed as mean $\pm$ SEM. (n = 4–5 mice per group, ##p < 0.01 vs. Control group; **P < 0.01 and *p < 0.05 vs. Model group). One representative of three independent experiments is shown.

production of IL-4 and IL-17A in the supernatant (Fig. 7B). Furthermore, SMD appeared to be more effective in suppression of production of these cytokines than Dex. These findings suggest that SMD inhibits Th2/Th17 differentiation in vitro.

3.8. SMD also inhibits the activation of mTOR pathway in CD4⁺ T cells in vitro

To investigate if SMD regulates CD4⁺ T cells via altering mTOR

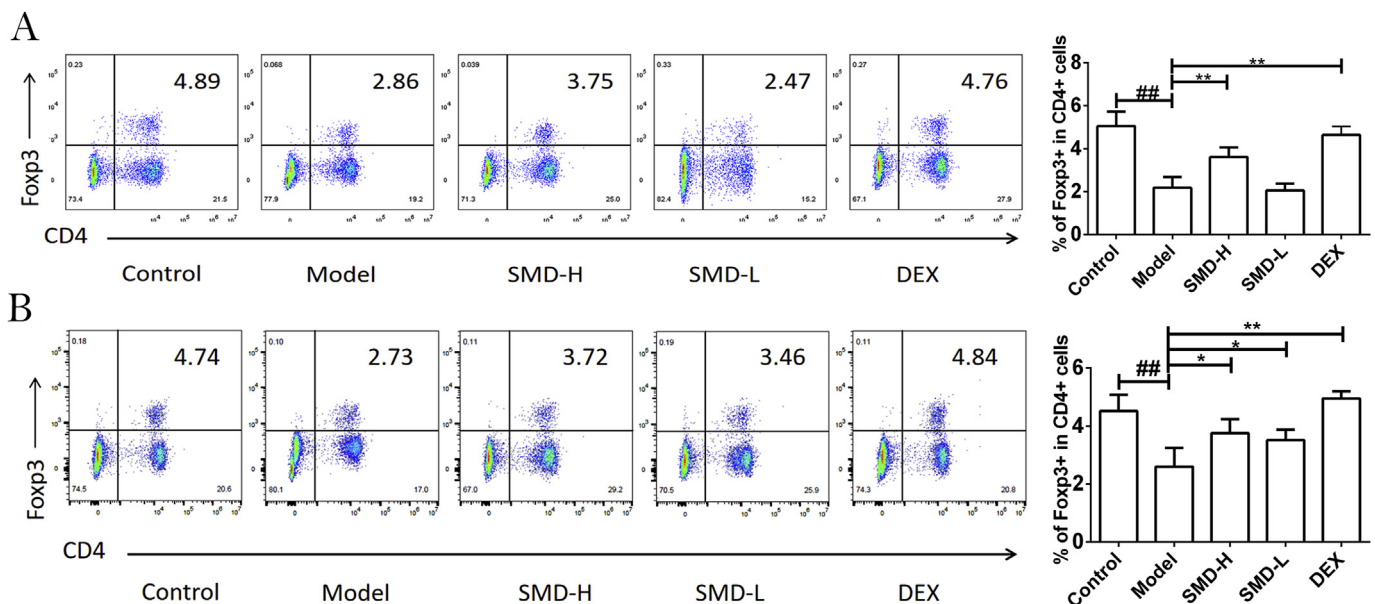


Fig. 5. SMD increases CD4 + Foxp3 + Treg frequency in asthmatic mice.

Spleen (A) or lymph node (B) cells were isolated from the OVA-induced asthmatic mice treated with SMD or DEX, and the percentages of CD4⁺Foxp3⁺ Treg cells were determined using a flow cytometer 14 days after SMD treatment. Data of column graph were presented as means $\pm$ SEM. (n = 4–5 mice per group, ##p < 0.01 vs. Control group; **P < 0.01 and *p < 0.05 vs. Model group). A representative of three separate experiments is shown.

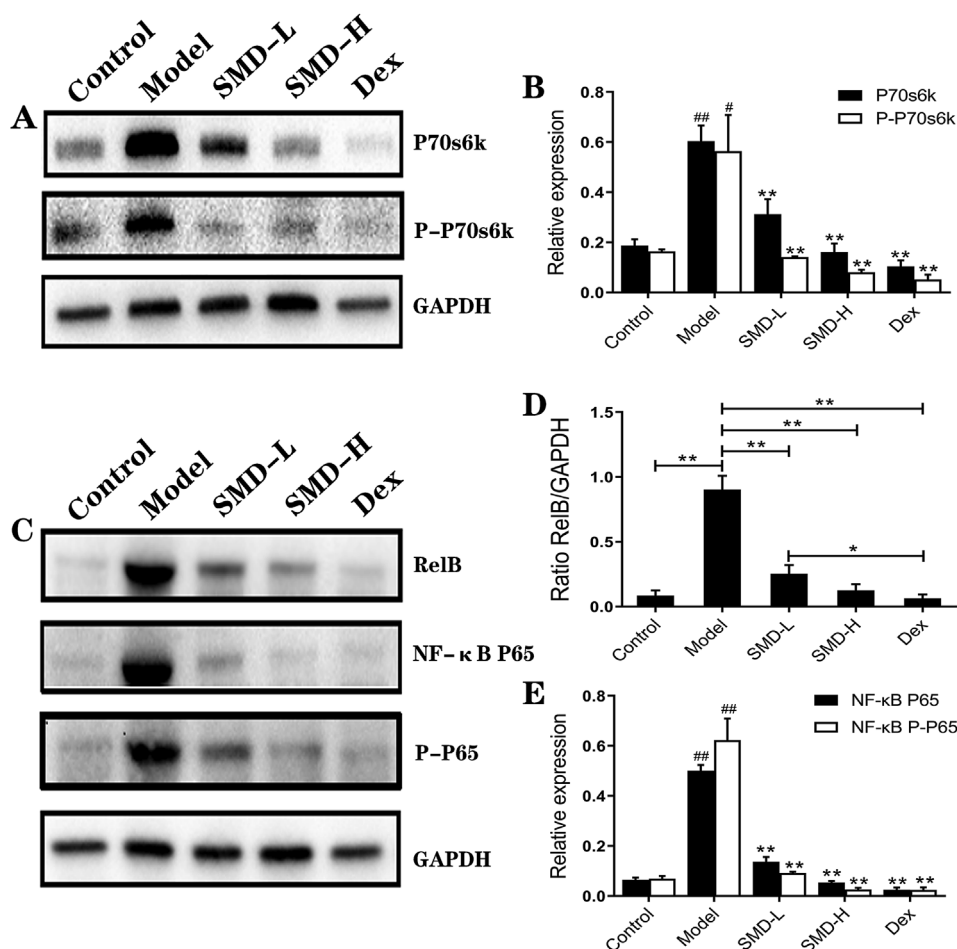


Fig. 6. SMD suppresses the activation of mTOR and NF-κB pathways in lung tissues of asthmatic mice.

(A-B) The total proteins of P70S6k and phosphorylated P70S6k (P-P70S6k) in lung tissue of asthmatic mice were determined via Western blotting two weeks after SMD treatment. (C-E) The proteins of RelB, P65 and P-P65 in the lung tissues were also measured via western blotting. Data of column graphs on right panels were presented as means $\pm$ SEM from three separate experiments ($n = 3$ mice per group, Model vs Control, $\#p < 0.01$ and $\#p < 0.05$ vs. Control group; $**p < 0.01$ and $*p < 0.05$ vs. Model group). GAPDH was used as a loading control.

pathway, CD4⁺ T cells were stimulated using anti-CD3 and anti-CD28 antibodies in the presence of SMD for 48 h and then harvested for analysis. Similar to the in vivo results, both high-concentration (SMD-H: 31.2 mg/ml) and low-concentration (SMD-L: 15.6 mg/ml) of SMD treatment dramatically inhibited the mTOR activity of CD4⁺ T cells by downregulating expression of both P70S6K and phosphorylated

P70S6K (P-P70S6K), and so did DEX (Fig. 8). These results indicate that suppressive effects of SMD on CD4⁺ T cells are likely dependent on its inhibition of mTOR signaling in CD4⁺ T cells.

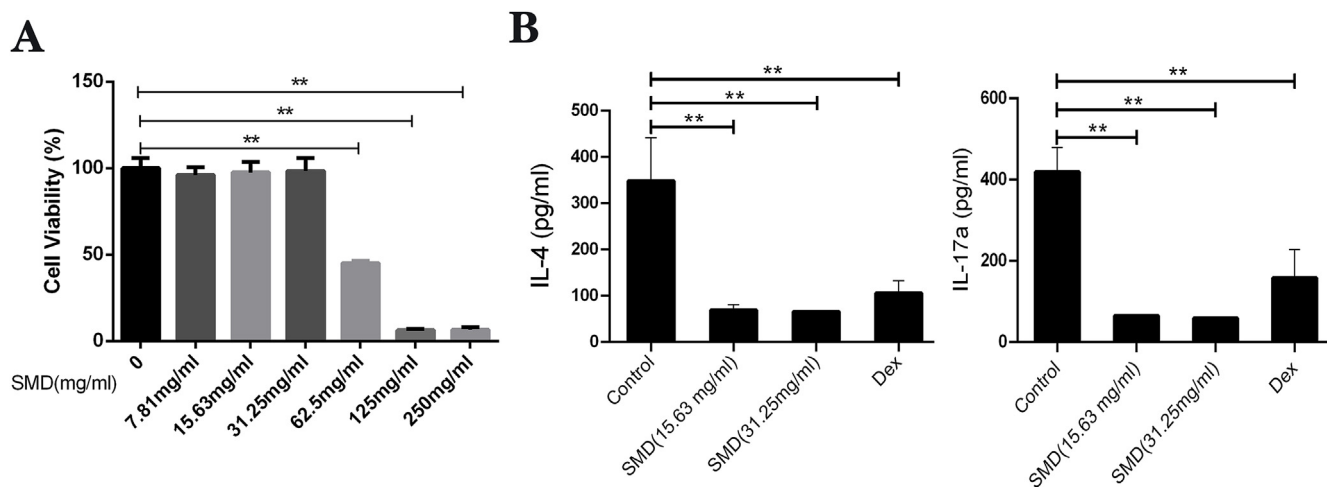


Fig. 7. SMD reduces production of IL-4 and IL-17A by CD4⁺ T cells in vitro.

(A) CCK-8 assay was used to analyze the potential cytotoxicity of SMD on CD4⁺ T cells 96 h

after cell culture with anti-CD3/anti-CD28 Abs. Data are presented as means $\pm$ SEM from three independent experiments ($**p < 0.01$, $n = 4$ wells). (B) FACS-sorted CD4⁺ T cells from BALB/c mice were cultured and stimulated with anti-CD3/anti-CD28 Abs in the presence or absence of SMD for 96 h. For Th17 development, TGF- β 1 and IL-6 were added to the culture. The supernatant was measured for relevant cytokine production via ELISA ($n = 4$ wells per group, $**p < 0.01$).

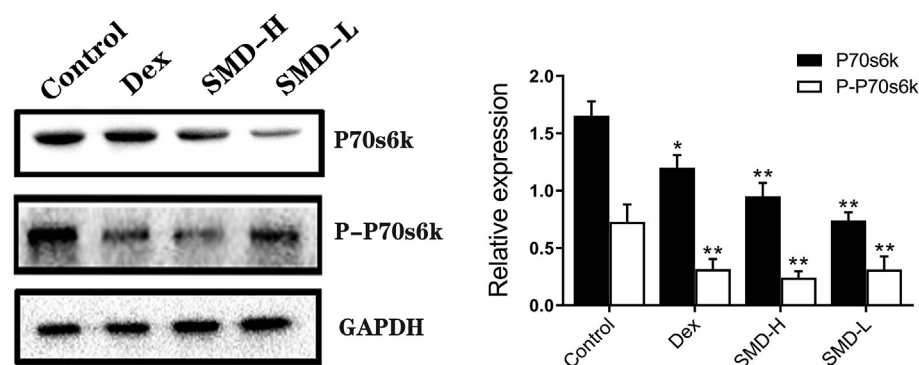


Fig. 8. SMD inhibits mTOR signaling in CD4⁺ T cells in vitro.

FACS-sorted CD4⁺ T cells were cultured and stimulated with anti-CD3/anti-CD28 Abs in the presence or absence of SMD-H (31.2 mg/ml) or SMD-L (15.6 mg/ml) for 48 h. Then the activation of mTOR pathway was analyzed by western blotting. The total protein (P70S6K) and phosphorylated P70S6K (P-P70S6K) in CD4⁺ T cells were measured using western blotting. GAPDH was used as a loading control. Data are expressed as mean $\pm$ SEM (n = 3 mice per group, **p < 0.01). A representative of three independent experiments is shown.

4. Discussion

As a chronically inflammatory respiratory disease, asthma is characterized by wheezing, coughing, bronchial contraction, chest tightness and shortness of breath (Reddy and Gupta, 2014). Mucus hypersecretion in the airway obstructs airflow into the respiratory tract, causing breathing difficulties, suffocation and even death (Thornton et al., 2008). Over 300 million people are suffering from this respiratory disease and asthma incidence is rapidly increasing in both developed and developing countries (Asher and Pearce, 2014; Braman, 2006). A wide range of therapies, such as β_2 -adrenergic drugs, conventionally inhaled corticosteroids, IgE blockers and methylxanthines, have been developed to control asthmatic attacks. Although conventional inhalation of corticosteroids among them is considered to be the most effective treatment (Busse et al., 2001; Dahl, 2006), it would usually cause severe systemic side effects (Cave et al., 1999). In addition, conventional doses of hormones could not effectively treat asthma in clinic over time. Thus, the challenge of asthma therapy now is to find new effective drugs without serious side effects (Ito et al., 2006).

In the present study, we focused on the effects of SMD, a traditional Chinese medicinal formula, on asthmatic airway inflammation and immune mechanisms underlying its effects on asthma using OVA-induced asthmatic mouse models. The inhalation of nebulized OVA led to morphological changes of murine lung tissues, inflammatory cell infiltration in the bronchiolar walls, airway goblet cell hyperplasia and mucus hypersecretion. In this study, we found that SMD treatment significantly alleviated airway inflammation and AHR, accompanying with decreased infiltration of CD3⁺ and CD4⁺ T cells in lung tissues of asthmatic mice, suggesting that SMD indeed provides an effective treatment option for asthma. However, it remains to be determined if SMD would cause any side effect.

The pathogenesis of asthma is complex and heterogeneous, involving various immune cells and proinflammatory cytokines (Holgate, 2012). As a prototypical Th2-cell-mediated disease, previous experiments have demonstrated the importance of Th2 cell cytokines in promoting allergic airway inflammation (Lambrecht and Hamad, 2014). Th17 cells, a recently discovered T lymphocyte subset different from Th1 and Th2 (Kimura and Kishimoto, 2011), can also cause high airway responses (Chen et al., 2017). A main Th17 cytokine IL-17A stimulates airway epithelial cells, endothelial cells and stromal cells to secrete proinflammatory factors that recruit and activate of neutrophils, resulting in asthmatic inflammatory responses (Movassagh et al., 2017). Previous studies have also shown that transcription factor GATA-3 promotes the production of Th2-related cytokines, including IL-4 (Yagi et al., 2011), while transcription factor ROR γ induces the differentiation of Th17 cells (Ivanov et al., 2006). Our analysis of murine lung tissue showed that SMD treatment dramatically decreased the mRNA expression of GATA3 and ROR γ in lung tissue of asthmatic mice. Besides, SMD could inhibit the production of not only IL-4 but also IL-17 and IL-23 in serum of asthmatic mice. Our study in vitro also demonstrated that SMD inhibited Th2 and Th17 cytokine production by CD4⁺

T cells in vitro. On the other hand, Th17/Treg balance plays an important role in the inflammatory process of asthma. We observed a significant increase in the proportion of CD4⁺ Foxp3⁺ Treg cells in the lymph nodes and spleen from mice treated with SMD compared to asthmatic model group without SMD. These findings revealed that SMD treatment effectively protected mice from OVA-induced allergic asthma by upregulating CD4⁺ Foxp3⁺ Treg cells while suppressing Th2/Th17-mediated immune responses. Understanding of immune mechanisms responsible for the anti-asthmatic effects of SMD would help improve its therapeutic effects in the future.

NF- κ B signaling pathway is generally associated with inflammatory responses as well as asthma, which involves the enhanced gene and protein expression of asthma-related proinflammatory cytokines, chemokines, growth factors and multiple enzymes (Edwards et al., 2009), while mTOR signaling pathway is found to mainly regulate the differentiation and activation of CD4⁺ T cells (Angela et al., 2016). We found that the phosphorylation of NF- κ B was downregulated in lung tissue of SMD-treated asthmatic mice compared with that of asthmatic model group, implying that SMD indeed is anti-inflammatory in asthma. On the other hand, we found that SMD inhibited mTOR signaling pathway in both lung tissue of asthmatic mice and CD4⁺ T cells in vitro, suggesting that SMD may exert its suppressive effects on T cells via inhibition of T-cell mTOR signaling.

In conclusion, SMD significantly alleviated methacholine-induced lung inflammation and AHR, and reduced T cell infiltration around the bronchia and mucus secretion. SMD alleviated asthma inflammation through increasing Treg cell numbers, suppressing Th2 and Th17 cell differentiation, and blocking mTOR and NF- κ B signaling pathways. Thus, our findings provided the first evidence that SMD attenuates asthmatic airway hyperresponsiveness by hindering Th2/Th17 differentiation while promoting CD4⁺ Foxp3⁺ Treg generation. Our results will likely help expand clinical practice using SMD as an effective drug formula, an alternative to a conventional immunosuppressant, to treat human asthma.

Author contributions

CCL, YYW and SMC performed experiments and wrote primary manuscript; YTL, JQL, FL, JCD and TZ analyzed the data; FQ and HL designed some experiments; ZD designed the experiments and edited the manuscript; ZDZ provided essential agents and oversaw this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2020.112656>.

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