

## Original Article

# Anti-inflammatory effects of *Boletus edulis* polysaccharide on asthma pathology

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**Abstract:** Asthma is a chronic airway disease common around the world. The burden of this disease could be reduced with new and effective treatments. Here, the efficacy of a polysaccharide extract from the *Boletus edulis* (BEP) mushroom, which has demonstrated anti-inflammatory properties, was tested in a mouse model of asthma. Five groups of BALB/C mice were developed; one group served as a control and did not have asthma induction. The other four groups of mice were sensitized by ovalbumin challenge. FinePointe™ RC animal airway resistance and pulmonary compliance was used to assess airway function in asthma models. Three of the 4 model groups received treatments: one received pravastatin, one received dexamethasone, and one received BEP. Histopathology of lung tissues was performed using H&E and AB-PAS staining. Levels of cytokines IL-4 and IFN- $\gamma$  were detected using ELISA, qRT-PCR, and Western blotting. Cyclophilin A was measured by Western blot, and flow cytometry was used to determine the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells. BEP treatment resulted in improvements in lung pathology, IL-4 level ( $P < 0.05$ ), and IFN- $\gamma$  level ( $P < 0.05$ ) similar to traditional dexamethasone treatment. Further, the proportion of anti-inflammatory CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells significantly increased ( $P < 0.05$ ) compared to untreated asthma models, and expression of cyclophilin A significantly decreased ( $P < 0.05$ ). Thus, *Boletus edulis* polysaccharide reduces pro-inflammatory responses and increases anti-inflammatory responses in mouse models of asthma, suggesting this may be a novel treatment method.

**Keywords:** *Boletus edulis* polysaccharide (BEP), asthma, mouse models, anti-inflammatory responses, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells

## Introduction

Asthma is one of the most common chronic respiratory diseases worldwide [1]. Its characteristic symptoms include wheezing, difficulty breathing, repetitive paroxysmal cough, and airway hyper-responsiveness [2]. Airway inflammation, which is both the central pathogenic feature and the principal clinical manifestation, induces airflow obstruction and bronchial hyper-responsiveness [3]. This process has a complex pathogenesis involving both genetic and environmental factors. One of the mechanisms underlying airway inflammation is an imbalance in T helper immune cells [4-10]. In asthma, T helper type 2 (Th2) cells are functionally upregulated, while Th1 cells are inhibited, which enables Th2 cytokines to promote inflammation. Interleukin-4 (IL-4), secreted by Th2 cells, induces airway inflammation by activating

eosinophils as well as promoting IgE secretion [11]. The level of IL-4 is increased in bronchoalveolar lavage fluid of patients with asthma [12]. Further, when Th1 cells are suppressed they cannot secrete IFN- $\gamma$  to inhibit IgE secretion; IgE is upregulated in allergy and asthma, a process that promotes inflammation [13].

However, a Th1/Th2 imbalance is not the sole mechanism underlying asthma pathogenesis; other immunological pathways regulate airway inflammation. In particular, regulatory T cells (Treg), especially those producing the proteins CD4, CD25, and FOXP3 (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>), contribute to this process [14-17]. Treg cells are essential for inducing and maintaining immunological tolerance to foreign and self-antigens (including allergens). CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells are a subset of CD4<sup>+</sup>T lymphocytes, which can inhibit immune response and induce

immune tolerance by secreting inhibitory cytokines (IL-10, TGF- $\beta$ , and IL-35) or mediating cell-cell contact [15, 16]. Given the increasing incidence of asthma around the world [1], finding new ways to ameliorate the immune response is critical to reducing the burden of this disease.

The edible fungus *Boletus edulis* produces a polysaccharide (BEP, molecular weight 113 kDa) that exhibits a variety of biological activities, including anti-tumor, immune stimulation, and anti-oxidation [18-22]. Given its demonstrated anti-inflammatory activities, we hypothesized that it may be useful in altering the immune response in asthma. Here, the effects of BEP were assessed in a mouse model of asthma by investigating the IL-4 and IFN- $\gamma$  content and immune cell counts (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells) after BEP administration and in comparison with traditional asthma therapies. The findings will provide new ideas for the treatment of asthma.

## Methods

### *Experimental animals*

Seventy-five female BALB/c mice ranging in weight from 8-20 g were purchased from Shanghai Lab Animal Research Center (Shanghai, China). The mice were divided into 5 groups: 15 mice in the control group, 15 in the untreated asthma group, 15 in the pravastatin group, 15 in the *Boletus edulis* polysaccharide group, and 15 in the dexamethasone group.

### *Mouse model of asthma*

To establish a mouse model of asthma, mice were sensitized using the ovalbumin (OVA) method presented by Szeffler et al. [23]. Briefly, after being divided into 5 groups and adapted to the environment for 7 days, each mouse in the 4 non-control groups was injected with 25  $\mu$ g OVA (albumin, from chicken egg white, SIGMA-ALDRICH, batch number: 080M7012V) and 1 mg aluminium hydroxide gel (Imject Alum, Thermo, batch number: NE169583) (dissolved in PBS, 200  $\mu$ L) on days 0 and 7. Mice in the control group were mock-sensitized by intraperitoneal injection of an equivalent volume of PBS, with the same injection site as the asthma groups. Next, mice in the 4 non-control groups were stimulated to induce asthma beginning on day 14 by being placed in a closed container

and inhaling 6% OVA solution by ultrasonic atomization (402A ultrasonic atomizer, Yuwell Medical Equipment, Jiangsu, China) for 30 minutes once daily for 7 consecutive days. Mice in the control group inhaled PBS, at the same frequency and for the same duration as the asthma group. On day 21, 5 mice were taken from each group to be assessed for airway response. On day 22, the remaining mice were euthanized to measure inflammatory markers.

### *Treatment modalities*

Mice in each group were administered a mock treatment or chemical treatment as follows: (1) Control group received atomized PBS only; (2) Untreated asthma group, atomized PBS; (3) Pravastatin group, pravastatin atomization inhalation at a dose of 3.33 mg/kg (pravastatin sodium tablets, Daiichi Sankyo Pharmaceutical, Shanghai, batch number: 110150), once daily beginning on day 7 of model construction; (4) *Boletus edulis* polysaccharide group: intragastric administration of BEP (Edible Fungus Institute of Lishui City, Zhejiang, China) at a dose of 300 mg/kg, once per day, beginning on day 7 of model construction; (5) Dexamethasone group, intraperitoneal injection of dexamethasone at 2 mg/kg (dexamethasone sodium phosphate, Cisen Pharmaceutical, batch number: 1211146411) on half an hour before each stimulation, until the end of the experiment.

### *Determination of indexes and methods*

Airway hyper-responsiveness was assessed on 5 mice per group using the FinePointe™ RC animal airway resistance and pulmonary compliance system (BUXCO, USA). Within 24 hours after the final stimulation procedure, each mouse was injected intraperitoneally with 60 mg/kg pentobarbital sodium. Then, a tracheal incision was introduced for intubation connection of the animal airway resistance and pulmonary compliance detection system. Respiratory frequency was set to 160 times/min. Mice were administered an atomized inhalation of 10  $\mu$ L acetylcholine (Shanghai Joe Feather Biological Science and Technology Co., Ltd, Shanghai, China), at ascending concentrations of 0 mg/mL, 1 mg/mL, 2 mg/mL, 4 mg/mL, 8 mg/mL, and 16 mg/mL. The maximum airway resistance within 3 minutes after atomizing inhalation was measured to indicate maximum resistance of the mouse at the corresponding

**Table 1.** Airway resistance in mice with asthma following treatment ( $\bar{x} \pm s$ , cmH<sub>2</sub>Osec/mL)

Treatment Group	1 mg/mL	2 mg/mL	4 mg/mL	8 mg/mL	16 mg/mL
Control	0.07±0.04	0.20±0.07	0.26±0.06	0.36±0.10	0.51±0.16
Untreated asthma	0.64±0.27*	0.82±0.29*	1.19±0.21*	1.47±0.27*	1.63±0.05*
Pravastatin	0.13±0.04 <sup>#</sup>	0.29±0.09 <sup>#</sup>	0.66±0.25 <sup>#</sup>	0.62±0.10 <sup>#</sup>	0.62±0.28 <sup>#</sup>
<i>Boletus edulis</i> polysaccharide	0.11±0.22	0.23±0.28	0.91±0.18	0.90±0.18 <sup>#</sup>	1.31±0.18 <sup>#</sup>
Dexamethasone	0.15±0.07 <sup>#</sup>	0.40±0.24	0.78±0.53	0.84±0.05 <sup>#</sup>	1.41±0.04 <sup>#</sup>

Note: \*P<0.01, compared with normal group; <sup>#</sup>P<0.05, compared with model group.

concentration of acetylcholine. At the end of the experiment, the mice were euthanized by an overdose of anesthetics.

The remaining 10 mice per group were subjected to other measures. Bronchoalveolar lavage fluid (BALF) was collected from mice that were first anesthetized then killed by intraperitoneal bleeding. The thorax was accessed to ligate the furcation root of the right lung, and the mouse received tracheal intubation. Next, 0.5 mL cold PBS were introduced and suction was performed 2-3 times, with a recovery rate of about 80%, to obtain BALF. BALF samples were mixed and centrifuged at 1500 rpm for 10 minutes, then the supernatant was stored at -80°C for detection of cytokines; the precipitate from the above centrifugation step and freeze at -80°C for PCR and immunoblotting. Finally, the lung lobe was collected, fixed with 4% formalin, and embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin or Alcian blue/periodic acid-schiff, and samples were examined under light microscope.

#### ELISA

Enzyme-linked immunosorbent assays (ELISA) were used to detect cytokines in BALF with IL-4 (Lot: L1304010390), IL-17 (Lot: L130426057), and IFN-gamma (Lot: L130426058) kits (USCN, Wuhan, China). 96-well- Plates with were prepared with blank wells and standards. Standard wells received 50 µL standard substances for IL-4, IL-17 or IFN-gamma at different concentrations. Sample wells received 50 µL of BALF. Standard wells and sample wells received 100 µL horseradish peroxidase (HRP)-labeled antibody. Plates were sealed and incubated at 37°C for 60 min. Wells were washed and patted dry 5 times. Next, 50 µL each of substrate A and B were added, and plates were incubated at 37°C for 15 min. All wells then received 50 µL stop buffer, and OD value was measured on

a microplate reader (SPECTRA max Plus 384, Molecular Devices) at 450 nm within 15 min.

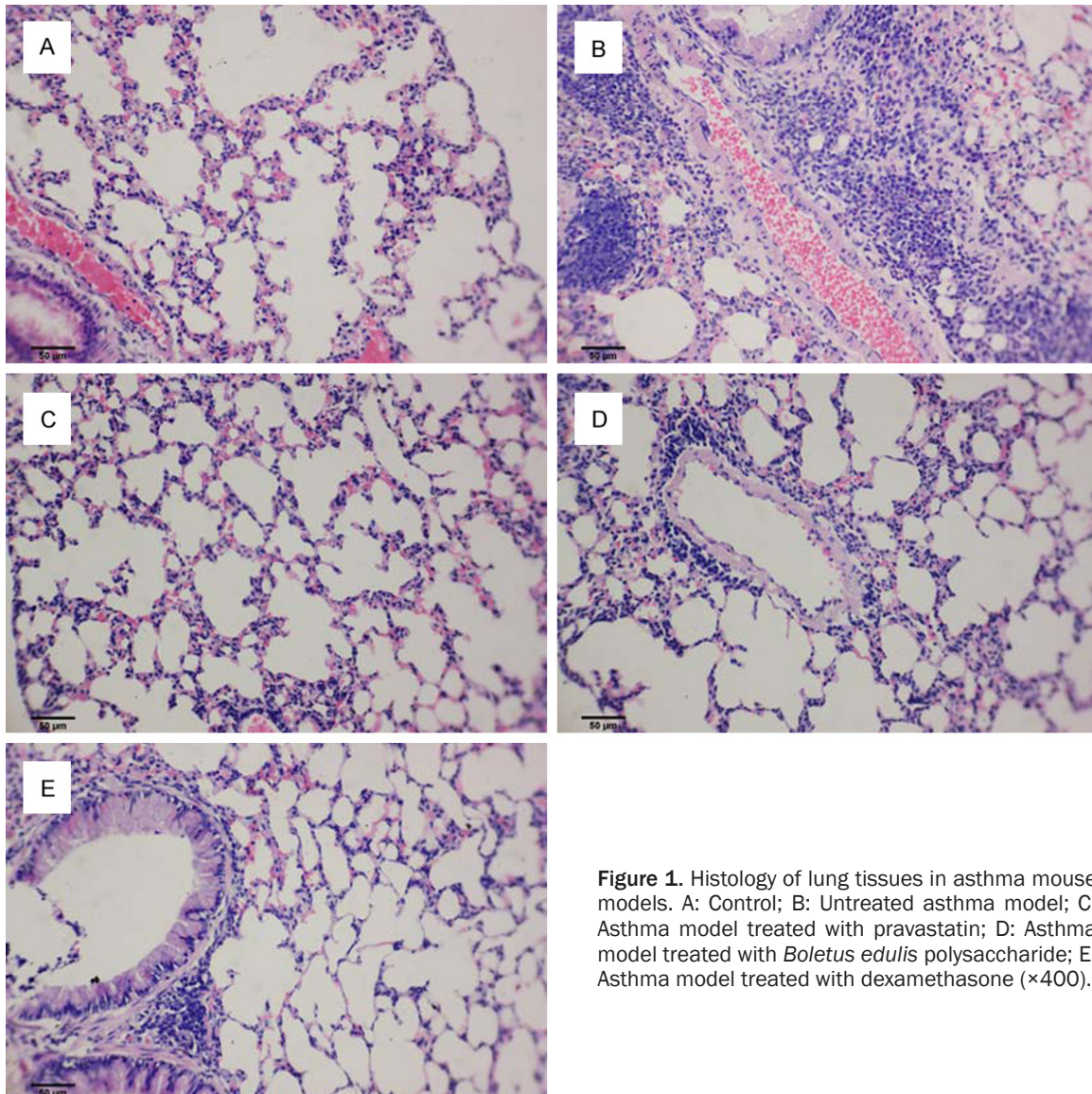
#### Protein extraction and western blotting

The BALF was centrifuged (600g×) at 4°C for 5 minutes to collect cells and extract total protein. BCA total protein quantification assay kit (Beyotime Biotechnology, P0012) was used to measure the protein concentration, and 30 µg total protein were separated by 10% SDS-PAGE. Samples were transferred to PVDF membrane (Millipore Corporation, IPVH00010), sealed with TBST containing 5% skim milk powder, and incubated for 1 hour at room temperature. Membrane was washed with PBST, then primary antibodies for proteins of interest or internal reference [anti-IFN-g (Santa Cruz Biotech, SC52557); anti-IL-4 (Abcam, AB11524); anti-b-actin (Santa Cruz Biotech, SC47778)] were added. Membrane was incubated with shaking at room temperature for 1 hour, washed with PBST, and incubated with secondary antibody [goat anti-rat IgG-HRP (Bioworld, SC2006); goat anti-mouse IgG-HRP (Bioworld, BS12478)] at room temperature with shaking for 1 hour. Staining was developed with an ECL Plus chemiluminescence kit (Millipore, Bedford, MA, USA). ChemiDoc XRS+ System (Bio-RAD) was used to visualize immunoblots, with gray value analysis used to determine relative expression of proteins.

#### Quantitative RT-PCR

The BALF was centrifuged to obtain cell precipitates for total RNA extraction using high-purity total RNA rapid extraction kits from Shanghai Generay Biotech Co. Ltd (GK3012). After detection of purity and solubility of RNA solution, Revert Aid First Strand cDNA synthesis kit (Fermentas Corporation, K1622) was used to reverse transcribe in a 20 µL reaction system comprising 4 µL 5× Reaction Buffer, 1 µL of 20





**Figure 1.** Histology of lung tissues in asthma mouse models. A: Control; B: Untreated asthma model; C: Asthma model treated with pravastatin; D: Asthma model treated with *Boletus edulis* polysaccharide; E: Asthma model treated with dexamethasone ( $\times 400$ ).

U/ $\mu$ L Ribolock TM RNase Inhibitor; 2  $\mu$ L 10 mM dNTP mix, and 1  $\mu$ L RevertAid TM M-MmLv Reverse Transcriptase. Reaction mix was heated at 25°C for 5 minutes, then 42°C for 60 minutes, and at 70°C for 5 minutes, then terminated. cDNA was diluted and stored at -70°C.

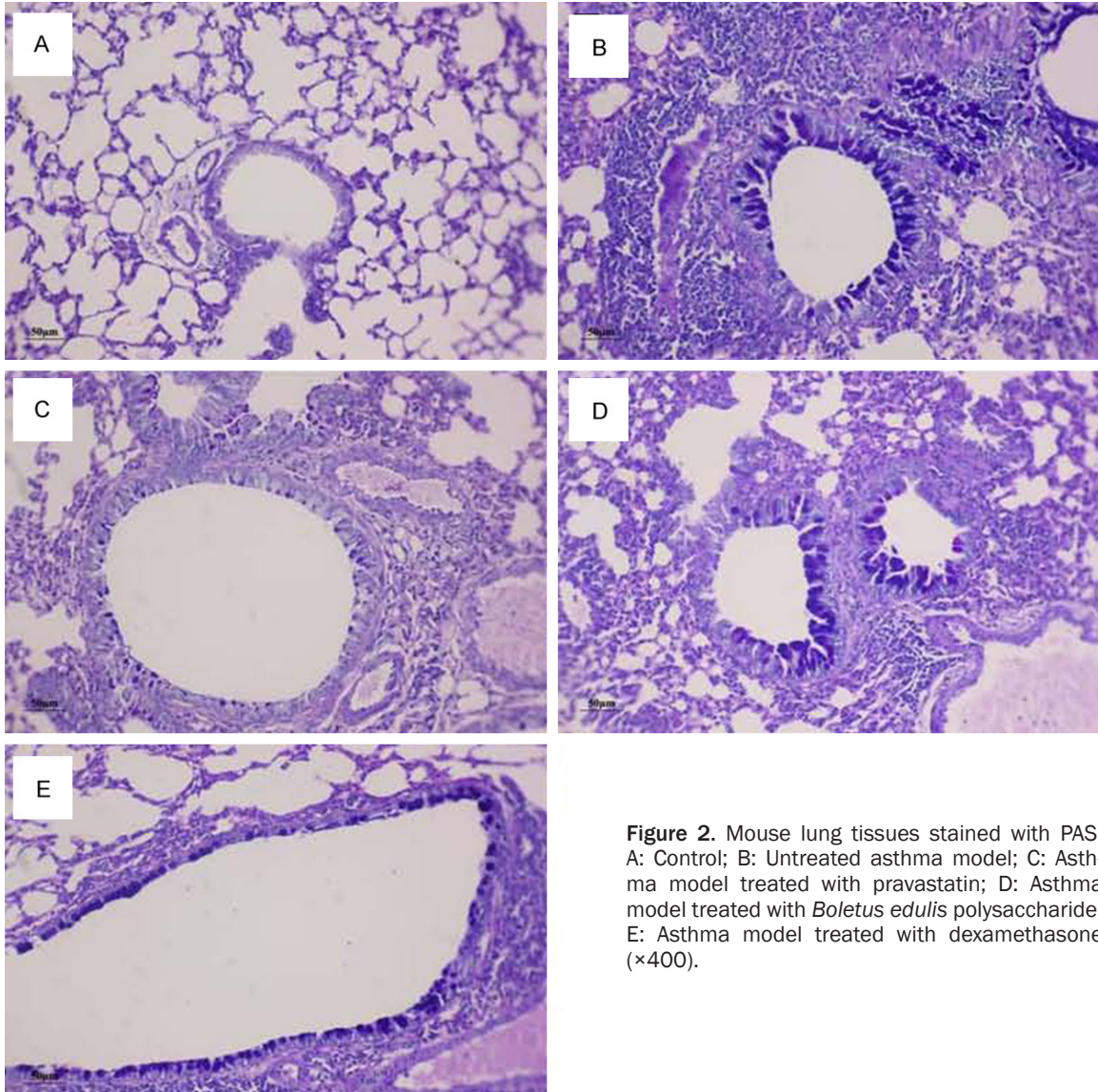
qRT-PCR amplification was performed using the following primer sequences. Mouse Actin forward, GAGACCTTCAACACCCAGC and mouse Actin reverse, ATGTCACGCACGATTCCC were based on the target Genbank ID 11461 to produce PCR fragments of 263 bp. The mouse IL17-4 forward, TCATCTGCTCTTCTTCTCG, and mouse IL17-4 reverse, TGGCGTCC CTTCTCCTGT, were based on targeting Genbank ID 16189 to produce PCR fragments of 114 bp. Mouse

IFN- $\gamma$  forward, TGCCTCAACTGCTCTCCAC, and mouse IFN- $\gamma$  reverse, GAGGCATCAACTGACAGGTCT, were based on Genbank ID 15977 to produce PCR fragments of 179 bp. IQ SYBR Green Supermix (12.5  $\mu$ L, Bio-Rad, 170-8882AP) was used in a total 25  $\mu$ L reaction including 1  $\mu$ L each of 10  $\mu$ M primers and 10.5  $\mu$ L cDNA. Reactions were run at 50°C for 3 minutes, 95°C for 3 minutes, and 40 cycles of 95°C for 10 seconds and 59°C for 20 seconds. The  $2^{-\Delta\Delta Ct}$  method to determine relative expression levels of mRNA.

#### Detection of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells

BALF was assessed under a microscope; approximately 106 cells were collected by centrifugation.





**Figure 2.** Mouse lung tissues stained with PAS. A: Control; B: Untreated asthma model; C: Asthma model treated with pravastatin; D: Asthma model treated with *Boletus edulis* polysaccharide; E: Asthma model treated with dexamethasone ( $\times 400$ ).

gation at 1500 rpm for 5 minutes. Each sample was probed with 0.125  $\mu\text{g}$  anti-mouse CD4 (Abcam, Shanghai, China) and 0.06  $\mu\text{g}$  anti-mouse CD25 antibody (Abcam, Shanghai, China) and incubated at 4°C away from light for 30 minutes. Samples were washed with flow cytometry staining buffer twice and 1 mL resuspended cells was added to Fixation/Permeabilization working solution and incubated at 4°C overnight. Samples were washed with 1 $\times$  permeabilization buffer twice, centrifuged, and added with flow cytometry staining buffer to a total of 100  $\mu\text{L}$  reaction volume. 0.5  $\mu\text{g}$  Fc blocking agent (CD16/32, Abcam) were added, and samples were incubated at 4°C away from light for 15 minutes. Next, 0.5  $\mu\text{g}$  anti-mouse/

rat FOXP3 antibody (Abcam) were added, and samples were incubated at 4°C away from light for 30 minutes. Samples were washed twice with 1 $\times$  permeabilization buffer, re-suspended with 500  $\mu\text{L}$  1 $\times$  permeabilization buffer, and detected by flow cytometer.

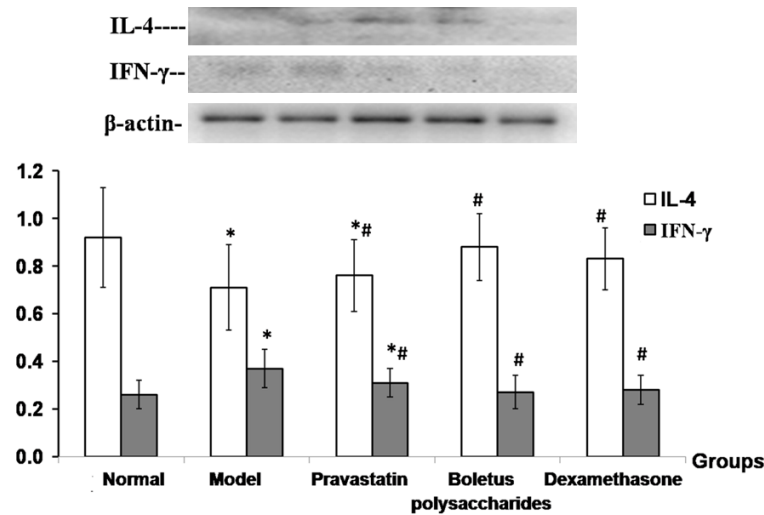
#### Statistical methods

All data were analyzed using SPSS17.0 software. The results are expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). The inter-group comparisons of means were performed using univariate analysis of variance and pairwise comparison between groups, with  $P < 0.05$  as significantly different.

**Table 2.** IL-4 and IFN- $\gamma$  levels in BALF and lung tissues (pg/mL)

Treatment Group	BALF		Lung tissues	
	IL-4	IFN- $\gamma$	IL-4	IFN- $\gamma$
Control	48.19 $\pm$ 5.33	273.89 $\pm$ 44.98	429.53 $\pm$ 34.49	915.97 $\pm$ 144.05
Untreated asthma	35.20 $\pm$ 5.55*	350.59 $\pm$ 72.80*	296.63 $\pm$ 45.51*	1322.18 $\pm$ 226.75*
Pravastatin	39.18 $\pm$ 4.19* <sup>#</sup>	303.99 $\pm$ 51.34 <sup>#</sup>	358.43 $\pm$ 68.78* <sup>#</sup>	1243.66 $\pm$ 101.58* <sup>#</sup>
<i>Boletus edulis</i> polysaccharide	40.88 $\pm$ 6.48* <sup>#</sup>	292.97 $\pm$ 49.12 <sup>#</sup>	349.00 $\pm$ 41.49* <sup>#</sup>	1123.72 $\pm$ 365.82 <sup>#</sup>
Dexamethasone	44.21 $\pm$ 8.55 <sup>#</sup>	288.61 $\pm$ 25.81 <sup>#</sup>	342.58 $\pm$ 47.06* <sup>#</sup>	1100.22 $\pm$ 139.77 <sup>#</sup>
F	6.355	3.275	9.604	5.086
P	0.001	0.019	0.001	0.002

Note: \*P<0.01, compared with normal group; <sup>#</sup>P<0.05, compared with model group.



**Figure 3.** Protein expression of IL-4 and IFN- $\gamma$  in lung tissues. Relative protein expression of cytokines in the lung of mouse models of asthma after indicated treatment as detected by Western blotting and normalized against  $\beta$ -actin. Notes: \*P<0.01, compared to control group; <sup>#</sup>P<0.05, compared to untreated asthma model group.

## Results

### Mouse model of asthma: airway resistance

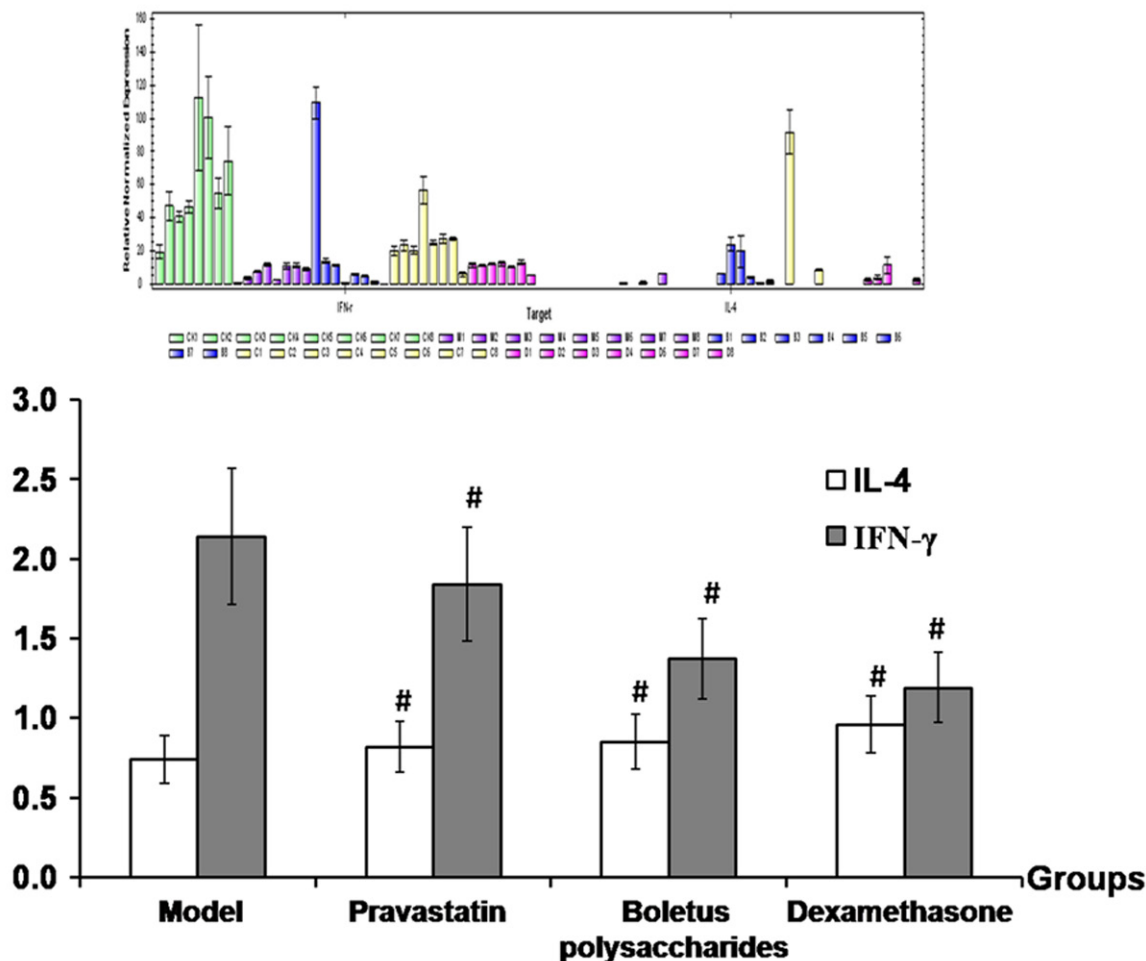
The mouse model of asthma was induced using standard atomized exposure to OVA [23]. We measured airway resistance to confirm induction of asthma. Mice exposed to OVA inhalation exhibited significant airway resistance at acetylcholine concentrations of 1, 2, 4, 8, and 16 mg/mL (P<0.05; **Table 1**). To determine whether drug treatment resulted in improved airway resistance, we compared airway resistance after acetylcholine challenge between untreated mice with asthma and various treatment groups (**Table 1**). Compared with the untreated asthma model group, the model mice treated

with pravastatin had significantly less airway resistance at an acetylcholine concentration of 16 mg/mL (P<0.05). Similarly, model mice treated with *Boletus edulis* polysaccharide (BEP) had significantly less airway resistance at acetylcholine concentrations of 1 and 16 mg/mL (P<0.05). The airway resistance of model mice treated with dexamethasone was significantly less at acetylcholine concentrations of 2, 4, 8, and 16 mg/mL (P<0.05).

### Lung tissue pathology

To determine whether treatment with asthma drugs (pravastatin and dexamethasone) or BEP resulted in improvements

in lung pathology following acetylcholine challenge, we compared H&E-stained tissue samples between untreated asthma model mice and treated asthma model mice. Control mice had lung tissues, including pulmonary alveoli, bronchi of the lung, and pulmonary interstitium, with clear structure and absence of congestion in the alveolar wall, inflammatory exudate in the alveolar and bronchial lumen, obvious lesion of epithelial cells in the lung bronchi, increase of mucous cells, or significant peribronchial inflammatory cell infiltration (**Figure 1A**). In untreated mice with asthma, significant lesions were observed in the lung tissues, as were inflammatory cell infiltration around the bronchus and in the alveolar sep-

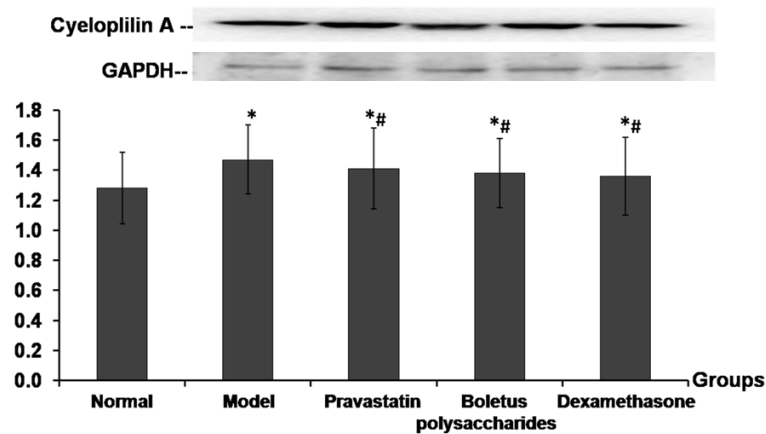


**Figure 4.** mRNA expression of IL-4 and IFN- $\gamma$  in lung tissues. Relative mRNA expression of cytokines in the lung of mouse models of asthma after indicated treatment as detected by qRT-PCR. Notes: #P<0.05, compared to untreated asthma model group.

tum, eosinophils and lymphocytes around the bronchus, thickening of the alveolar wall, and loss of the alveolar structure (**Figure 1B**). Further, one mouse exhibited severe bleeding in the lung. In mice treated with pravastatin, there was a reduced lesion degree compared to untreated mice; the degree of peribronchial inflammatory cell infiltration and proportion of eosinophils were also lower. Similarly, the alveolar wall was mildly thickening, and did not exhibit bleeding, congestion, or other symptoms (**Figure 1C**). Mice treated with BEP had a reduced lesion degree compared to untreated controls (**Figure 1D**). Three mice had a lesion degree similar to that of the dexamethasone group (**Figure 1E**); but the remaining 5 mice had more lesions compared with the dexamethasone group. The inflammatory cell infil-

tration mainly occurred around the bronchi and blood vessels, with lymphocytes as the main cell types; there were fewer eosinophils, no obvious bleeding, and no congestion in the alveolar lumen. Mice treated with dexamethasone exhibited a reduced degree of lesions compared with the untreated group, and were similar to mice treated with pravastatin (**Figure 1E**). The alveolar wall was thickened, but did not exhibit bleeding, congestion, or other symptoms.

Lung tissues were also visualized with AB-PAS staining to detect acidic mucosubstances (blue) and neutral mucosubstances (red), or both (purple, most common). No obvious mucosubstance was detected in the bronchus in the control group (**Figure 2A**), while purple muco-



**Figure 5.** Protein expression of cyclophilin A in lung tissues. Relative protein expression of cyclophilin A in the lung of mouse models of asthma after indicated treatment as detected by Western blotting and normalized against GAPDH. Notes: \* $P < 0.01$ , compared to control group; \*\* $P < 0.05$ , compared to untreated asthma model group.

substance was seen in bronchia of lung tissues in the untreated asthma model group (**Figure 2B**). In contrast, the mucosubstances detected in lung tissues in mice treated with pravastatin (**Figure 2C**), BEP (**Figure 2D**), and dexamethasone (**Figure 2E**) were reduced to different degrees compared with the untreated asthma model group. Mice treated with dexamethasone or pravastatin exhibited less mucosubstance staining than the BEP-treated mice.

#### *Levels of IL-4 and IFN- $\gamma$ in BALF and lung tissues*

Inflammatory markers IL-4 and IFN- $\gamma$  were assessed in both BALF and lung tissues. The levels of IL-4 in BALF (**Table 2**) and in lung tissues (**Figure 3**) were lower in the untreated asthma group compared with control group, but tended to increase in each treatment group compared to the untreated group ( $P < 0.05$ ). In contrast, the IFN- $\gamma$  levels in BALF (**Table 2**) and in lung tissues (**Figure 3**) were increased in the untreated asthma model group compared with the control group, but tended to decrease in each treatment group compared to the untreated group ( $P < 0.05$ ).

The protein levels were validated at the gene level by quantitative RT-PCR. In accordance with the observed protein levels, mRNA expression of IL-4 in lung tissues in the untreated model was lower than in the control group (**Figure 4**), but tended to increase in each treat-

ment group compared to the untreated mice ( $P < 0.05$ ). mRNA expression of IFN- $\gamma$  in lung tissues in the untreated group was higher compared to the control group, but tended to decrease in each treatment group compared to the untreated mice ( $P < 0.05$ ).

#### *Protein expression of cyclophilin A in lung tissues*

Cyclophilin A is another protein that reflects the level of inflammation in asthma; thus, cyclophilin A was assessed in the mouse models in this study. Western blot test results showed that the protein expression of cyclophilin

A in lung tissues was higher in the untreated asthma model group compared with the control group, but tended to decrease in each treatment group compared to the untreated mice ( $P < 0.05$ ), as shown in **Figure 5**.

#### *Proportion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells in BALF*

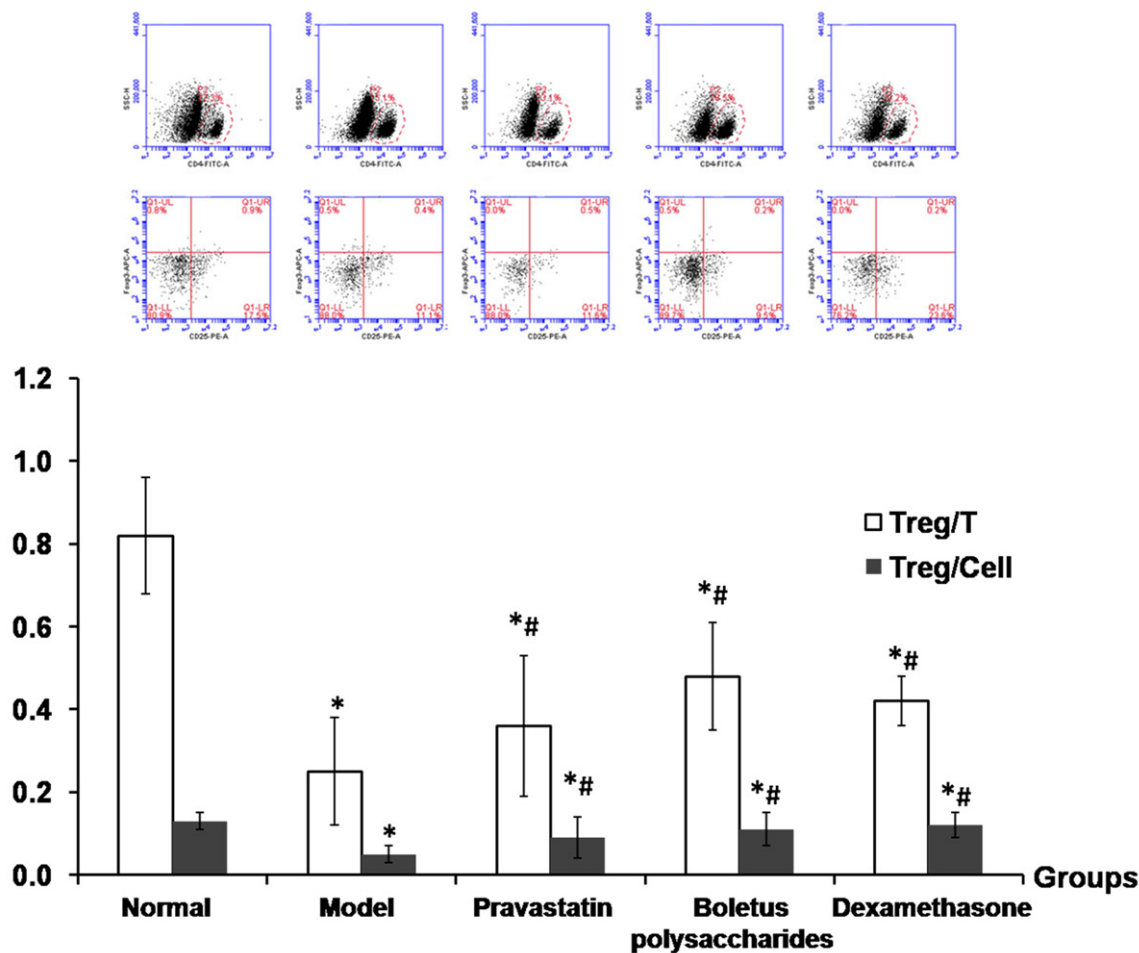
BALF cell contents were sorted by flow cytometry to determine the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells. The CD4<sup>+</sup>T cells (Treg/T) and cells in lymphocytes population (Treg/Cell) were lower in the untreated asthma model group compared to the control group, but tended to increase in each treatment group compared to the untreated model group ( $P < 0.05$ ), as shown in **Figure 6**.

#### **Discussion**

Asthma has become a serious public health concern, and thus research effort is often devoted to identifying new and effective methods for the treatment of asthma. In this study, we tested the potential for *Boletus edulis* polysaccharide to reduce asthma severity in a mouse model. The findings reveal that BEP may be a viable treatment alternative to current asthma therapies.

OVA stimulation is a useful method to induce asthma in mice [23]. Here, OVA stimulation resulted in symptoms associated with asthma,





**Figure 6.** Proportion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells in BALF. The proportions of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells were measured in bronchoalveolar lavage fluid from the lung of mouse models of asthma after indicated treatment. Notes: \*P<0.01, compared to control group; #P<0.05, compared to untreated asthma model group.

especially increased airway resistance and lung pathology, demonstrating successful modeling. The features of asthma observed in this model were ameliorated, at least in part, by treatment with pravastatin, dexamethasone, or BEP. In particular, BEP treatment resulted in the greatest improvement in airway resistance. BEP was also able to induce reductions in inflammatory cell infiltration that were comparable with those of dexamethasone treatment. Further, mucosubstance deposition in lung tissues was reduced in BEP-treated mice compared with untreated mice.

Inflammation is central to asthma pathology, as the main cause of airflow obstruction and airway hyper-responsiveness [24]. Imbalances of Th1/Th2 cells can promote inflammatory processes. Activated Th1 and Th2 cells secrete the

cytokines IFN- $\gamma$  and IL-4, respectively. IFN- $\gamma$  promotes resistance to infection, antitumor activity, and immune regulation by activating macrophages, improving expression of MHC class I and class II molecules, and promoting antigen presentation [25]. IFN- $\gamma$  is also an IL-4 antagonistic factor that can inhibit the IgE-mediated immune response that promotes bronchial asthma [26]. Further, IFN- $\gamma$  can promote Th1 cell differentiation, inhibit the reaction of Th2 cells, inhibit eosinophil gathering in the airway mucosa and IgE production in the blood, and inhibits human bronchial epithelial cell proliferation [27, 28].

IL-4 is the only cytokine for B cells producing IgE [29, 30]. In the alveolar lavage fluid of patients with moderate and severe asthma inflammation, the generation of IL-4 and IgE are

directly related [31]. In addition, secretion of IL-4 in asthma patients with acute episodes is significantly higher than in remission patients and normal controls [32, 33]. Similarly, IL-4 concentration increases in serum of children with asthma, while IFN- $\gamma$  concentration decreases [32, 33]. Accordingly, the BALF and lung tissues of mice treated with BEP exhibited higher IL-4 and lower IFN- $\gamma$  (protein and mRNA) compared with the untreated asthma model group. However, these two indicators were both slightly more normalized in the dexamethasone treatment group.

Importantly, many clinical studies have found that imbalances cannot completely explain asthma pathogenesis. The importance of the regulatory T cells, which express specific cell surface markers CD4 and CD25 [34, 35], has been recognized. Indeed, Treg cells can induce and maintain immune tolerance for foreign antigens and autoantigens (including allergen) through a variety of mechanisms, which are essential for controlling inflammation in asthma. In particular, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells play an important role in the pathogenesis of asthma [36]. Prior studies have reported that CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells are significantly fewer in the peripheral blood and bronchoalveolar lavage fluid of children with asthma compared with healthy controls [37]. In the peripheral blood of patients with moderate and severe asthma, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg numbers are significantly lowered compared with the mild asthma and healthy control groups [38-42]. In addition, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells can reduce airway mucus secretion, inhibiting airway smooth muscle proliferation and collagen synthesis [38-42]. Together these findings highlight the importance of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells in controlling asthma airway inflammation. In our study, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells in BALF with CD4<sup>+</sup>T cells (Treg/T) and cells in lymphocytes population (Treg/Cell) was significantly increased in mice treated with BEP compared with the untreated mice.

Cyclophilin protein (CyPA) belongs to the immunophilin protein family and is an intracellular receptor for cyclosporin A. It is also an extracellular chemokine stimulating factor, which has significant chemotactic enhancement effect for T cells and eosinophils [43]. The serum CyPA

level in acute asthma patients is significantly higher than that in stationary patients and healthy controls [44]. In this study, the protein expression of CyPA of BEP-treated mice was lower than in untreated asthma model mice ( $P < 0.05$ ) in lung tissues.

In summary, OVA induction of asthma in mice was verified by clinical symptoms, lung pathology, and immune markers. Treatment of induced asthma with *Boletus edulis* polysaccharide resulted in improved pathology, airway resistance, and immune markers similar to dexamethasone treatment.

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### Disclosure of conflict of interest

None.

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