Comparison of Two Erythronium sibiricum Bulb Polysaccharides in Anti-asthmatic Mechanisms Based on Th1/Th2 and Treg/Th17 Balances

Shanshan Gao
Xinjiang Medical University
Mei Wang
Xinjiang Medical University
Yue Zhou
Xinjiang Medical University
Rongchang Liu
Xinjiang Medical University
Xiangyun Xie
Xinjiang Medical University
Xue Li
Quality and Safety of Agri-products of Xinjiang Uygur Autonomous Region
Chunli Chen (z2212008@xjmu.edu.cn)
Xinjiang Medical University

Article

Keywords: Polysaccharide, Erythronium sibiricum bulb, Asthma, Th1/Th2, Th17/Treg

Posted Date: June 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1638594/v1

Abstract

This study investigated and compared the effects and mechanisms of two kinds of *Erythronium sibiricum* (Fisch. & C. A. Mey.) Krylov (*E. sibiricum*) bulb polysaccharides (E1P and ESBP) on asthma, which was based on helper T (Th) 1/Th2 and Th17/regulatory T cell (Treg) balances. E1P and ESBP improved pulmonary pathological states, reduced the recruitments of lung inflammatory cells and serum IgE in asthmatic mice. E1P and ESBP also suppressed Th2 and Th17 cytokine hypersecretion while enhancing Th1 and Treg cytokine secretion. The changes in related gene and protein expressions of Th1/Th2 and Th17/Treg balances induced by ovalbumin were reversed after the intervention of E1P and ESBP. Additionally, E1P lacked significant effects on IFNγ and IL-10 cytokines, RORγt mRNA and T bet protein compared with ESBP. *E. sibiricum* bulb polysaccharides ameliorated asthma by modulatingTh1/Th2 and Th17/Treg balances, and ESBP might exhibit a more comprehensive therapeutic action on asthma than E1P.

Introduction

As a major noncommunicable disease, asthma has affected about 262 million people and led to 461,000 deaths in 2019 around the world according to the World Health Organisation [1]. Asthma only could be controlled by medicines but cannot be cured due to its complex pathogenesis. The balances of helper T (Th) 1/Th2 and Th17/regulatory T (Tregs) cells play essential roles in asthma. As inflammatory mediators, Th2 and Th17 cells could drive the exacerbation of asthma by secreting cytokines and chemokines [2]. Th1 and Treg T cells have antagonistic effects on Th2 and Th17 cells, respectively, and potentially suppressive effects on asthma [3]. With the primary function of secreting cytokines, Th cells secrete cytokines to impact asthma. The overproduction of Th2 cytokines (such as interleukin(IL)-4, IL-5 or IL-13) and Th17 cytokine (IL-17) induced asthma, while increased Th1 cytokines (like IL-2 or Interferon (IFN)γ) and Treg T cytokines (such as IL-10 or transforming growth factor (TGF)β) alleviated airway damage in asthma [4–5]. Upstream regulators decide on the differentiation of Th cells. T-box expressed in T cells (T bet) and Gata-binding protein 3 (Gata3) are the typical molecular markers of Th1 and Th2, respectively. Fork-head box p3 (Foxp3), an indispensable pivotal regulator of Treg T cells, can suppress retinoic acid-related orphan receptor γt (RORγt) to block the increase in the number of Th17 cells [6]. In addition, Th17 and Treg T cells need a mutual TGFβ signal for differentiation [7], and TGFβ is mostly produced by bronchial epithelial cells or eosinophils [8]. Therefore, T bet, Gata3, TGFβ, RORγt and Foxp3 can balance Th1/Th2 and Th17/ Treg states.

Over the last decades, multiple pharmacological actions, such as immunomodulation, anti-inflammatory, antioxidant activity and anti-tumour activity of polysaccharides have been widely studied [9–10]. Considering that immunologic dissonance and inflammatory reaction are critical mechanisms of the asthma, many scholars have reported that polysaccharides could treat asthma through different mechanisms [11–14]. *Erythronium sibiricum* (Fisch. & C. A. Mey.) Krylov (*E. sibiricum*) bulb is a typical functional food that has been used as a folk medicine for centuries in Xin Jiang, China [15]. Following the

Kazakh ethnic medical text [16], *E. sibiricum* bulb is utilised to enhance energy and treat waist and knee soreness and lung-related ailments of patients. Our previous research proved that *E. sibiricu*m bulb polysaccharides exhibited bioactivities, such as anti-inflammatory [17] and immunomodulatory [18], which may partly explain the basis of using it as functional food or folk medicine. What is more, Ji-hye Seo et al. studied the asthma-inhibiting effect of *Erythronium japonicum* [19].

*E. sibiricu*m bulb crude polysaccharides can be extracted by two methods. The polysaccharide extracted by hot water decoction is named ESBP, while the other extracted by enzyme-assisted method is named E1P. The primary physiochemical identification indicated that the contents of uronic acid and protein in E1P were 5.32% ±0.16% and 0.18% ± 0.04%, and that of ESBP were 5.75% ± 0.39% and 0.33% ± 0.01%, respectively. Both E1P and ESBP were mainly composed of glucose and contained a small quantity of galactose and arabinose. However, the purity of E1P (89.53%) was higher than ESBP (58.5%), which may result from the less starch content of E1P than ESBP. *In vitro* studies indicated that E1P and ESBP could activate macrophages, and E1P exhibited better immunocompetence. However, the anti-asthmatic activities of E1P and ESBP have not been explored. In the present study, E1P and ESBP were used to explore their effects and mechanisms on asthma. We discussed and compared the anti-asthmatic effects of E1P and ESBP based on the balances of Th1/Th2 and Th17/Treg. This work provides insights into the development and utilisation of *E. sibiricum* bulb polysaccharides as functional food.

Methods And Materials

Reagents and kits

After getting the picking permission from Regional Natural Resources Bureau in Altay, dried *E. sibiricum* bulbs were purchased from Altay Regional Hospital of Traditional Chinese Medicine and identified by Prof. Palida Abulizi (College of Pharmacy, Xinjiang Medical University, Xinjiang, China). The Voucher specimens and licence (No.202100026) of *E. sibiricum* could obtain in the College of Pharmacy, Xinjiang Medical University. Experimental research and feld studies on the plants, including the collection of plant material complied with relevant institutional, national, and international guidelines and legislation. Ovalbumin (OVA) (SLCB8249) and aluminium hydroxide powder (WXBB1151V) were obtained from Sigma–Aldrich (MO, USA). Rabbit antibodies of Gata3 (AF6233), tbx21 (DF7759) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AF7021) were acquired from Affinity Biosciences (Changzhou, Jiangsu, China). Rabbit anti-RORγt (bs-23110R), rabbit anti-TGF β(bs-0086R) and rabbit anti-Foxp3 (bs-10211R) were supplied by Beijing Biosynthesis Biotechnology Co., Ltd. (Tongzhou, Beijing, China). Radio Immunoprecipitation Assay (RIPA) buffer was provided by Solarbio Life Sciences (Beijing, China). Enzyme linked immunosorbent assay (ELISA) kits (immunoglobulin E (IgE), IFNγ, IL-4, IL-13, IL-10 and IL-17) were bought from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). Kits of Prime Script® RT reagent and SYBR® Premix Ex Tap™ II were purchased from Takara Bio, Inc. (Otsu, Japan). Kits of ECL chemiluminescence and bicinchoninic acid (BCA) protein concentration assay were obtained from Lanjieke Technology Co., Ltd. (Beijing, China).

Preparations of E1P and ESBP

According to our previous experiments [17–20], hot water extraction and enzyme-assisted extraction were performed in scale-up methods to obtain ESBP and E1P, respectively. The yields of ESBP and E1P were determined by the phenol sulphuric acid method [21] and the water solubility were measured through the method used by Guo Lei [22].

Animals and study design

All animal investigational processes were performed under the guidelines provided by the Chinese Council on Animal Care, including any relevant details, and all experiments were permitted by the Animal Studies Committee of Xinjiang Medical University (registered number SYXK 2018-0003). The ethics approval number was IACUC20191101-07. This study was carried out in accordance with the ARRIVE guidelines[23]. BALB/c mice (female, 6–8 weeks old, 18-22g) were obtained from the Animal Centre of Xinjiang Medical University. Mice were maintained under constant temperature and humidity in pathogen free conditions at a 12:12 h (L:D) photoperiod with unlimited access to food and water. According to the dosage (30g/day) of *Erythronium sibiricum* bulb [16], the yield of polysaccharide and the body surface area conversion method [24], the dose of ESBP and E1P was designed. Mice were randomly and equally divided into nine groups (n = 12 each) as follows: control group, OVA-challenged group (untreated asthma group), dexamethasone (0.005 g/kg) + OVA group, ESBP (at doses of 0.9, 1.8 and 3.6 g/kg, respectively) + OVA groups and E1P (at doses of 1.5, 3 and 6 g/kg, respectively) + OVA groups. Murine chronic asthma model was induced and established by OVA [25–26]. Sensitisation liquid was prepared using 100 μg of OVA and 1 mg of aluminium hydroxide powder in 200 μL of saline. Mice in the non-control group were sensitised on days 1, 7 and 14 with 200 μL of the sensitisation liquid by intraperitoneal injection. The control group underwent the same way of injection with saline. From day 18, an ultrasonic nebuliser (402A, Jiangsu Yuyue Medical Equipment & Supply Co., Ltd.) was used to challenge mice with 5% OVA for 30 min three times each week for 8 weeks. Mice in the control group were challenged with saline. All the drugs were given by lavage administration each day during the 8 weeks.

Pulmonary sampling

After sacrificing all the mice, six mice in each group were used to collect bronchoalveolar lavage fluid (BALF) with the following method: 0.8 mL of sterilised saline water was introduced into the lung, withdrawn twice and collected, and this process was repeated three times. BALF was centrifuged to separate the supernatant, and cell pellets were then resuspended in 200 μL of saline water to complete the differential count of inflammatory cells. The six remaining mice were adopted for the collection of lung tissue. The left lung tissue of mice was used for pulmonary histopathological analysis, and the right lung tissue was stored at -80 ℃ to obtain protein and RNA.

Pulmonary histopathological analysis

The left lung was made into paraffin blocks with 4-micron thickness and used to carry out haematoxylin-eosin (HE) staining or Periodate acid-Schiff (PAS) analysis. An inverted light microscope (Nikon, Tokyo, Japan) was used to acquire images. Histopathological scores were classified and determined by four grades [14] as follows: grade 0, no inflammation; grade 1, very few inflammatory cells; grade 2, bronchi or vessels were surrounded by a thin 1-2-cell layers; grade 3, bronchi or vessels were surrounded by a 3-5-cell moderate layers; grade 4, bronchi or vessels were surrounded by a thick over 5-cell layers of inflammatory cells. The average optical density (AOD) represents the average response intensity of all selected objects (such as positive staining results) in the field of vision. PAS-positive cells were analysed as follows [27]: PAS images (200×) from each mouse were measured by Image Pro-plus6.0 for three times, and the mean AOD of each image was calculated and used to compare the statistics.

Analysis of BALF and serum

An automated whole blood analyser is recommended because of its advantage of higher accuracy than using a microscope or a counting chamber [28]. In the present study, an automatic blood analyser (Mingray, Shenzhen, China) was used for counting inflammatory cells in BALF. The BALF supernatant was analysed for IL-4, IL-10, IL-13, IL-17 and IFN-γ contents, while serum was used to analyse IgE levels by ELISA under the manufacturer's guidelines.

Preparation of RNA and real-time PCR (RT-PCR)

RNA was obtained in the portion of the right lung lobe by Trizol reagent (Waltham, MA, USA). Prime Script® RT reagent kit was used to synthesise cDNA samples by strictly following the manufacturer's instructions. RT-PCR was performed on the cDNA samples by SYBR® Premix Ex Tap™ II. GAPDH served as endogenous control, and the sequences of primer pairs are shown in Table 1. The reaction conditions of RT-PCR followed the manufacturer's instructions: 95°C for 30 s of pre-denaturation, followed by 40 cycles of denaturation at 95°C for 5 s and extension at 56°C for 34 s. Data were analysed in the Quant Studio 6 Flex detection system of Applied Biosystems Co. (Foster City, CA, USA). The abundance of target mRNA was indicated by the value of $2^{-\Delta\Delta CT}$.

Table 1
The sequence of primers

Gene	Forward Prime (5'-3')	Reverse Prime (5'-3')	Product (bp)
GAPDH	CCAATGTGTCCGTCGTGGATC	GTTGAAGTCGCAGGAGACAA	149
Gata3	CCCCATTACCACCTATCCGC	CCTCGACTTACATCCGAACCC	106
T bet	GTTGGTCTGACACCTGTGTT	TTCAAACCCTTCCTCTGCAC	154
Foxp3	TCTTGCCCATCTCTGTCTCA	TACGGGAATAGGAGGAGCAG	104
Ror(c)	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT	137
Tgfb1	CTTCAATACGTCAGACATTCGGG	GTAACGCCAGGAATTGTTGCTA	142

Western blot analysis

Lung tissues were lysed by RIPA for 45 min on ice and centrifuged at 14,000 rpm and 4 ℃ for 5 min to separate soluble protein. BCA kit was employed to measure the concentration of proteins. Proteins were separated by SDS-PAGE [29]. After the protein was transferred to a membrane, the membranes were treated with primary antibodies including GAPDH, Gata3, T bet, RORγt, TGFβ and Foxp3 at 4°C overnight. After incubation with a secondary antibody for 2 h, ECL kit was utilised to visualise the immunoreactive bands. Finally, the protein expression levels (band intensities) were quantified by Image J software.

Statistical analysis

Data are presented as mean ± standard error of the mean. One-way ANOVA was used in statistical analysis, and $p < 0.05$ suggested statistical significance. Data were analysed by SPSS 23.0, and statistical charts were drawn using Origin 2018.

Results

Comparison of yield and solubility between E1P and ESBP

The granules and gelatinisation of starch in crude polysaccharides contribute to lower yield and purity [30]. Amylase was used in the present study to eliminate starch and obtain higher yield. As predicted, the yield of E1P (52.5%) was remarkably higher than that of ESBP (33.7%), while the water solubility levels of E1P and ESBP were 22.93 ± 0.26 and 14.04 ± 0.05 mg/mL, respectively. These results suggested that amylase-assisted extraction improved the yield and water solubility of *E. sibiricum* bulb polysaccharides, consistent with previous reports [31–32].

E1P and ESBP improved the pulmonary pathological states

Tissue staining using H&E and PAS was performed to determine the effects of E1P and ESBP on the pulmonary pathological states of asthmatic mice (Fig. 1). As shown in Fig. 1A, the lung tissues of mice in the control group exhibited a healthy state with typical airway and alveoli. Compared with the control group, the lung of asthmatic mice in the OVA-challenged group suffered characteristic pathologic features of asthma-like disorganised alveoli and increasing proportions of inflammatory cells infiltrating around the bronchioles and vessels. Narrower tracheal lumen and thicker smooth muscle were observed, which confirmed the occurrence of airway remodelling in the OVA-challenged group. The improvements of pulmonary pathological states were detected in all medical intervention groups, and the curative effects of the two polysaccharides were found to be dose dependent (Fig. 1C). Figure 1B shows the representative PAS images, which were used to reflect the extent of mucus secretion and the state of goblet cells in the airway of mice in all groups [33]. Compared with mice in the control group, mice in the OVA-challenged group accumulated many mucus secretions in the airways, which became constricted. Goblet cell hyperplasia also developed in the bronchi. ESBP and E1P significantly reduced the mucus production and the number of goblet cells in the airway epithelium. In addition, the AOD of PAS-positive

cells was calculated for accurate evaluation (Fig. 1D). A significant increase in the AOD of PAS was observed after being challenged by OVA ($p < 0.001$), while the AOD was significantly decreased by the polysaccharides ($p < 0.001$) in a dose-dependent manner. E1P and ESBP could improve the pulmonary pathological states by reducing inflammatory infiltration, mucus secretions and hyperplasia of goblet cells in asthmatic mice.

E1P and ESBP alleviated the OVA-induced recruitment of inflammatory cells in the lung and reduced the serum IgE

A whole blood analyser was used to calculate the cells of BALF to classify and quantify the infiltrated inflammatory cells (Fig. 2). Compared with those in the control group, the number of total inflammatory cells and the recruitment of eosinophils and monocytes were significantly increased in the asthmatic model group ($p < 0.001$, $p < 0.001$ and $p < 0.05$, respectively). The total number of inflammatory cells was significantly decreased by treatment with E1P and ESBP in high-dose groups ($p < 0.01$ and $p < 0.05$, respectively). The recruitment of eosinophils also decreased in the high-dose group of E1P as well as mid- and high-dose groups of ESBP ($p < 0.01$). These results confirmed that *E. sibiricum* bulb polysaccharides could treat asthma by alleviating inflammatory infiltration of the lung. The indicator for allergy, IgE, in serum was detected to verify the capacity of the polysaccharides to treat asthma. Figure 3A suggests that IgE in the serum was elevated in the asthmatic model group ($p < 0.001$). Compared with the OVA-challenge group, all treated groups of the polysaccharides significantly reversed the levels of serum IgE ($p < 0.001$). The results suggested the potential of E1P and ESBP to treat asthma by reducing the serum IgE level.

E1P and ESBP balanced Th1/Th2 and Th17/Treg by effecting the cytokine levels

Inflammatory cytokine levels including typical Th1 cytokine IFNγ and Th2 cytokines IL-4 and IL-13 in BALF were detected by ELISA. As shown in Fig. 3B, the level of IFNγ was down-regulated by the OVA challenge ($p < 0.01$) compared with the control group. Based on the IFNγ level in the OVA-challenged group, the groups of polysaccharides showed up-regulated levels in a dose-dependent manner. Only those treated with high-dose ESBP showed increased IFNγ level with a statistical significance ($P < 0.01$). Changes in IL-4 and IL-13 cytokines levels are shown in Fig. 3C and D, respectively. Th2 cytokines including IL-4 and IL-13 were significantly increased after the OVA challenge ($p < 0.05$ and $p < 0.001$, respectively) and then significantly decreased by the intervention of ESBP and E1P in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively). Hence, ESBP could inhibit the development of asthma by increasing the cytokine level of Th1 and decreasing the cytokine level of Th2, while E1P only reduced the cytokine level of Th2.

Typical cytokines of Th17/Treg balance, including IL-17 and IL-10, were measured. As shown in Fig. 3E, the level of IL-10 was significantly decreased by the OVA challenge ($P < 0.001$). ESBP boosted the level of IL-10 after the OVA challenge in a dose-dependent manner and with a statistical significance in the high-

dose group ($P < 0.001$). The level of cytokine IL-17 was elevated after the OVA challenge ($P < 0.05$) and then decreased by E1P and ESBP in the mid-dose groups ($P < 0.05$ and $P < 0.05$, respectively) and high-dose groups ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 3F). Therefore, the data related to Th17/Treg balance in cytokine levels indicated that E1P and ESBP could suppress Th17 cytokine, while only ESBP could promote the status of Treg cytokine.

E1P and ESBP effected the expression levels of T bet and Gata3

As T bet and Gata3 have been considered as vital transcription factors of Th1 and Th2 cells in the upstream signal pathway, the pulmonary mRNA levels of T bet and Gata3 were analysed. As predicted, the level of crucial Th2 transcription factors Gata3 increased ($p < 0.05$), and that of Th1 transcription factors T bet decreased ($p < 0.01$) after the OVA challenge compared with those in the control group (Fig. 4A and B). The administration of E1P and ESBP significantly suppressed the Gata3 level ($p < 0.05$). The decrease in the T-bet of the OVA-challenged group was also attenuated by the polysaccharide treatments, but only the effects in the high-dose group reached statistical significance ($p < 0.01$). These results confirmed that *E. sibiricum* bulb polysaccharides can balance the Th1/Th2 by the reversing levels of transcription factors of T bet and Gata3 in asthma. To determine whether the same result can be observed in protein expression, the present work applied Western blot to quantify T bet and Gata3 protein in lung tissue (Fig. 5). The Western blot results proved that ESBP significantly reversed the protein expressions of T bet and Gata3 ($p < 0.05$ and $p < 0.05$, respectively, Fig. 5B and C), that induced by the OVA challenge. E1P reduced the protein expression of Gata3 with a statistical significance ($p < 0.01$) and slightly increased the protein expression of T bet without statistical significance. All these data suggested that E1P and ESBP could treat asthma by balancing the Th1/Th2 in asthma, and E1P lacked the promoting effects on the protein expression of T bet compared with ESBP.

E1P and ESBP influenced the expression levels of TGFβ, RORγt and Foxp3

RT-PCR was conducted to determine the expression levels of TGFβ, RORγt and Foxp3 in lung tissue, which are the key transcription factors of Th17 and Treg upstream. Figure 4C, D and E show the levels of the transcription factors TGFβ, RORγt and Foxp3, respectively. In accordance with other papers [34–35], the mRNA abundance of TGFβ and RORγt were up-regulated by the OVA challenge ($p < 0.001$ and $p < 0.05$, respectively). ESBP and E1P in the high-dose group decreased the mRNA level of TGFβ ($p < 0.001$ and $p < 0.001$, respectively), and only ESBP reduced the mRNA level of RORγt with a statistical significance ($p < 0.01$) compared with the OVA-challenged group. Foxp3, as an important transcription factor of Treg, was reduced after the OVA challenge ($p < 0.05$), and the effect was reversed by ESBP and E1P in the mid-dose and high-dose groups ($p < 0.05$ and $p < 0.001$, respectively). Meanwhile, the expression trends of TGFβ, RORγt and Foxp3 in the Western blot analysis were the same as those in RT-PCR (Fig. 6). The quantised data of RORγt protein level indicated that E1P also significantly decreased the expression of RORγt in the high-dose group ($p < 0.01$). Therefore, E1P and ESBP regulated the OVA-induced Th17/Treg imbalance by

reducing the overexpression of TGFβ and RORγt while promoting the expression of Foxp3. Compared with ESBP, E1P had a weaker inhibitory effect on RORγt mRNA.

Discussion

In recent years, increasing attention is being paid to asthma treatments due to the increasing incidence of asthma. Various polysaccharides are now considered as functional food or potential medicines to affect the development of asthma [36–37]. Our experiment firstly found that *E. sibiricum* bulb polysaccharides (E1P and ESBP) could improved the pulmonary pathological states, decreased the infiltration of inflammatory of lung cells and prevented airway remodelling in asthmatic mice. The action mechanisms of E1P and ESBP were also explored based on the Th1/Th2 and Th17/Treg balances.

Based on evidence, asthma is a kind of chronic disease with typical symptoms of airway inflammation, hyper-responsiveness and airway remodelling [38]. Airway inflammation involves multitudinous activated cells, such as eosinophils, neutrophils, monocytes and basophils, and eosinophils have been implicated in eosinophilic asthma with the exacerbation of illness [39]. For this reason, many therapeutics that aim to limit the numbers of eosinophils or deplete them are currently widely used to treat asthma. Pathologic mucus is challenging to clear due to its high viscosity and elasticity, thereby efficiently contributing to airway blockage [40–41]. As a biomarker of Th2-induced inflammation, elevated serum IgE has been considered to induce the exacerbation of asthma [42]. In the present study, the results of counting inflammatory cells and HE staining suggested that E1P and ESBP could reduce pulmonary inflammation in asthma. The data of PAS and HE indicated that the accumulation of mucus and airway remodelling in asthmatic mice were prevented by the two polysaccharides. The decreased serum IgE level of asthmatic mice reconfirmed the anti-asthmatic effect of *E. sibiricum* bulb polysaccharides.

The Th1/Th2 balance is critical for developing asthma. Asthma presents with an increase in Th2 cytokines and a decrease in Th1 cytokines. As the essential Th2 cytokines, IL-4 and IL-13 participate in the pathogenesis of asthma. An increase in IL-4 level can facilitate allergic sensitisation, switching to IgE and eosinophilia [43]. IL-13 also influences IgE production and eosinophil chemotaxis, and participates in smooth muscle contractility and goblet cell hyperplasia in different ways from IL-4 [44]. The Th1 cytokine IFNγ has the effects of suppressing fibroblast activity and antagonising the functions of Th2 cytokines [45]. Given that the differentiation of Th1 and Th2 cells was achieved by specific transcription factors, the abundance of related transcription factors could affect the balance between Th1 and Th2 cells. Gata3 is crucial to the differentiation of Th cells into Th2, and T bet is a master determinant of Th1 cells because the deficiency of T bet leads to the lack of Th1 immune responses [46]. Mutual restrain also exists between T bet and Gata3. Therefore, the imbalance between T bet and Gata3 changes the Th1/Th2 balance [47]. We also examined the mRNA abundance and the levels of protein expression of T bet and Gata3 in the lung. Our experimental data indicated that, in cytokines level, the imbalance of Th1/Th2 caused by asthma was reversed by ESBP and E1P. The difference was that ESBP increased the Th1 cytokine IFNγ and simultaneously decreased the Th2 cytokines IL-4 and IL-13, but E1P only reduced Th2 cytokines in

asthma. Meanwhile, ESBP and E1P could reduce the elevated quantity of Gata3 mRNA in the OVA-challenged group, and raised the transcription level of T bet that reduced by the OVA challenge. The experimental data of protein expression levels were consistent with the RT-PCR findings, confirming that ESBP and E1P can regulate the Th1/Th2 balance by simultaneously reducing Gata3 and increasing T bet expression in asthmatic reactions. Therefore, *E. sibiricum* bulb polysaccharides treated asthma by balancing Th1/Th2.

Along with the development of mechanism research of asthma, the Th17/Treg balance gradually came into prominence. In the cytokine level, the decreased cytokine production of Treg cells on asthma and the overproduction of Th17 cytokine exacerbate asthma [48]. As a typical cytokine of Th17 cells, IL-17 could promote neutrophilia in the lungs and mucus accumulation, thereby contributing to goblet cell hyperplasia and airway remodelling [49]. IL-10, a representative cytokine of Treg cells, affects asthma by diverse mechanisms in different situations because IL-10 could suppress Th2 and Th17 immune responses [50]. At the protein transcriptional level, TGFβ not only affects the differentiation of Th17 and Treg cells but also airway epithelia into myofibroblasts [7], thereby confirming that TGFβ is pivotal to airway remodelling and inflammation of asthma. Th17 cells are believed to help induce the inflammation of asthma, and their crucial molecule RORγt implicates the differentiation and maturation of Th17 cells [51]. Conversely, the functions of Foxp3 include inhibition of Th17 differentiation and driving Treg differentiation [52]. In the present study, ESBP reversed the cytokine levels of Treg and Th17 cells induced by OVA challenge while E1P only decreased the level of IL-17. The treatment of E1P and ESBP suppressed TGFβ and RORγt expression but significantly increased Foxp3 expression in the lung tissue. These finding suggested the effects of *E. sibiricum* bulb polysaccharides on suppression of Th17 cells' function and the enhancement of Treg cells.

The improvement of polysaccharides solubility is beneficial to enhance absorption and bioavailability *in vivo*, which can confer polysaccharides with more diversified functions and wider application [53]. Therefore, from the perspective of solubility, E1P might have further development potential over ESBP. However, regard to the differences in pharmacological action between E1P and ESBP, the result differs from the reports suggesting that enzyme-assisted extraction could enhance the bioactivity of polysaccharides [54–56]. Compared with ESBP, E1P extracted with enzyme assistance was deficient in the effects of increasing IFNγ and IL-10 cytokines, RORγt mRNA and T bet protein. Different structures and physicochemical properties of polysaccharides contribute to changes in their bioactivities [57]. The amylase used to extract E1P might destroy the 1, 4-glycosidic bonds of polysaccharides, and more amylopectin or pectic polysaccharides can be released [58]. Thus, the structure and composition of E1P might be changed and different from ESBP. The structural differences and structure–effect relationship between E1P and ESBP deserve further study.

Conclusion

This study suggested that treatment with *E. sibiricum* bulb polysaccharides is a novel and selective method to improve asthma symptoms by balancing the Th1/Th2 and Th17/Treg. Therefore, *E. sibiricum* bulbs polysaccharides can be used as functional foods or adjuvant medicines for asthma sufferers. E1P and ESBP have their advantages. E1P has higher yield and solvency than ESBP, while ESBP exhibit more complete activities than E1P. The kind of *E. sibiricum* bulb polysaccharides that deserves further development should be determined by conducting more comparisons and evaluations.

Declarations

Acknowledgments

This work was supported by Ph.D. Research Setup Fund of Xinjiang Medical University of China (No. 2019-12) and Xinjiang Key Laboratory of Active components of Natural Medicine and Drug Release Technology (No. XJDX1713).

Conflict of interest

We declare that we have no conflict of interest.

Author Contribution

Shanshan Gao accomplished jobs including extraction of polysaccharides, *in vivo* experiments, statistical analysis and manuscript writing. **Yue Zhou c**ompleted the tests of lung tissue staining and RT-PCR. **Rongchang Liu** performed the tests of Western blot and provided assistance for the acquisition of relative data or images. **Xiangyun Xie** completed the tests of solubility and ELISA and provided assistance for relative data acquisition. **Xue Li** typed and quantified the infiltrated inflammatory cells in BALF. **Chunli Chen** and **Mei Wang** compiled theconcept and design of the study, supervised the experiment and revised the manuscript critically.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

References

1. juan, T.X., Bin, C., Tian, Z., Yong, X. & chang, Z.H. Advances in Clinical Featrues and New Drugs of Bronchial Asthma. Food and Drug **21**, 419–424 (2019).
2. Mays, L.E., *et al.* Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. J Clin Invest **123**, 1216–1228 (2013).
3. Qiu, Y.Y., *et al.* LncRNA-MEG3 functions as a competing endogenous RNA to regulate Treg/Th17 balance in patients with asthma by targeting microRNA-17/ RORγt. Biomed Pharmacother **111**, 386–

394 (2019).

4. Chen, X., *et al.* LAT alleviates Th2/Treg imbalance in an OVA-induced allergic asthma mouse model through LAT-PLC-γ1 interaction. Int Immunopharmacol **44**, 9–15 (2017).
5. Li, R.F. & Wang, G.F. JAK/STAT5 signaling pathway inhibitor ruxolitinib reduces airway inflammation of neutrophilic asthma in mice model. Eur Rev Med Pharmacol Sci **22**, 835–843 (2018).
6. Liang, P., *et al.* Huai Qi Huang corrects the balance of Th1/Th2 and Treg/Th17 in an ovalbumin-induced asthma mouse model. Biosci Rep **37**(2017).
7. Lee, G.R. The Balance of Th17 versus Treg Cells in Autoimmunity. Int J Mol Sci **19**(2018).
8. Wnuk, D., *et al.* Enhanced asthma-related fibroblast to myofibroblast transition is the result of profibrotic TGF-β/Smad2/3 pathway intensification and antifibrotic TGF-β/Smad1/5/(8)9 pathway impairment. Sci Rep **10**, 16492 (2020).
9. Zhang, K., *et al.* Anti-inflammatory properties of GLPss58, a sulfated polysaccharide from Ganoderma lucidum. Int J Biol Macromol **107**, 486–493 (2018).
10. Xie, J.H., *et al.* Advances on Bioactive Polysaccharides from Medicinal Plants. Crit Rev Food Sci Nutr **56 Suppl 1**, S60-84 (2016).
11. Barboríková, J., *et al.* Extracellular polysaccharide produced by Chlorella vulgaris - Chemical characterization and anti-asthmatic profile. Int J Biol Macromol **135**, 1–11 (2019).
12. Ding, F., Fu, Z. & Liu, B. Lipopolysaccharide Exposure Alleviates Asthma in Mice by Regulating Th1/Th2 and Treg/Th17 Balance. Med Sci Monit **24**, 3220–3229 (2018).
13. Maruthamuthu, V., Henry, L.J.K., Ramar, M.K. & Kandasamy, R. Myxopyrum serratulum ameliorates airway inflammation in LPS-stimulated RAW 264.7 macrophages and OVA-induced murine model of allergic asthma. J Ethnopharmacol **255**, 112369 (2020).
14. Lu, Y., Xing, Q.Q., Xu, J.Y., Ding, D. & Zhao, X. Astragalus polysaccharide modulates ER stress response in an OVA-LPS induced murine model of severe asthma. Int J Biol Macromol **93**, 995–1006 (2016).
15. bai, G.J.m., Chunxia, H., xi, G.S.p. & nursultan, G. Application value and factors affecting development and utilization of Erythronium sibiricum. Forestry of Xinjiang, 32–33 (2012).
16. Xu, X., Huangerhan & B.G. *Kazakh drug chi*, (The Ethnic Publishing House, Beijing, 2009).
17. Chen, C., Kasimu, R., Xie, X., Zheng, Y. & Ding, W. Optimised extraction of Erythronium sibiricum bulb polysaccharides and evaluation of their bioactivities. Int J Biol Macromol **82**, 898–904 (2016).
18. Chen, C., Xie, X. & Li, X. Immunomodulatory effects of four polysaccharides purified from Erythronium sibiricum bulb on macrophages. Glycoconj J **38**, 517–525 (2021).
19. Seo, J.H., Bang, M.A., Kim, G.Y., Cho, S.S. & Park, D.H. Erythronium japonicum suppresses histopathological lung abnormalities in an ovalbumin-induced asthma mouse model. INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE (2015).
20. Gao, S., *et al.* Optimisation of enzyme-assisted extraction of Erythronium sibiricum bulb polysaccharide and its effects on immunomodulation. Glycoconj J (2022).

21. Chen, J., Chen, L., Lin, S., Liu, C. & Cheung, P.C. Preparation and structural characterization of a partially depolymerized beta-glucan obtained from Poria cocos sclerotium by ultrasonic treatment. Food Hydrocolloids **46**, 1–9 (2015).
22. Guo, L., *et al.* Optimization of the Cellulase-Ultrasonic Synergistic Extraction Conditions of Polysaccharides from Lenzites betulina. Chem Biodivers **16**, e1900369 (2019).
23. Percie du Sert, N., *et al.* Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol **18**, e3000411 (2020).
24. Zhao, W. & Sun, G. Conversion of Drug Dosage between Different Experimental Animals. China Academic Journal of Electronic Publishing House, 52–53 (2010).
25. Xian, Z., *et al.* Imperatorin alleviates ROS-mediated airway remodeling by targeting the Nrf2/HO-1 signaling pathway. Biosci Biotechnol Biochem **84**, 898–910 (2020).
26. Jia, S., *et al.* Curcumol Ameliorates Lung Inflammation and Airway Remodeling via Inhibiting the Abnormal Activation of the Wnt/β-Catenin Pathway in Chronic Asthmatic Mice. Drug Des Devel Ther **15**, 2641–2651 (2021).
27. Hwang, Y.H., *et al.* The Protective Effects of Astaxanthin on the OVA-Induced Asthma Mice Model. Molecules **22**(2017).
28. Poitout-Belissent, F., Grant, S.N. & Tepper, J.S. Aspiration and Inspiration: Using Bronchoalveolar Lavage for Toxicity Assessment. Toxicol Pathol **49**, 386–396 (2021).
29. Shang, J., Liu, W., Yin, C., Chu, H. & Zhang, M. Cucurbitacin E ameliorates lipopolysaccharide-evoked injury, inflammation and MUC5AC expression in bronchial epithelial cells by restraining the HMGB1-TLR4-NF-κB signaling. Mol Immunol **114**, 571–577 (2019).
30. Anwar, M., Birch, E.J., Ding, Y. & Bekhit, A.E. Water-soluble non-starch polysaccharides of root and tuber crops: extraction, characteristics, properties, bioactivities, and applications. Crit Rev Food Sci Nutr, 1–33 (2020).
31. Rodrigues, D., *et al.* Impact of enzyme- and ultrasound-assisted extraction methods on biological properties of red, brown, and green seaweeds from the central west coast of Portugal. J Agric Food Chem **63**, 3177–3188 (2015).
32. Duan, M., Shang, H., Chen, S., Li, R. & Wu, H. Physicochemical properties and activities of comfrey polysaccharides extracted by different techniques. Int J Biol Macromol **115**, 876–882 (2018).
33. Wu, D., *et al.* Alpinetin prevents inflammatory responses in OVA-induced allergic asthma through modulating PI3K/AKT/NF-κB and HO-1 signaling pathways in mice. Int Immunopharmacol **89**, 107073 (2020).
34. Zhou, J.P., *et al.* Angiotensin-(1–7) Rescues Chronic Intermittent Hypoxia-Aggravated Transforming Growth Factor-β-Mediated Airway Remodeling in Murine and Cellular Models of Asthma. J Pharmacol Exp Ther **375**, 268–275 (2020).
35. Wang, L., *et al.* Critical roles of adenosine A2A receptor in regulating the balance of Treg/Th17 cells in allergic asthma. Clin Respir J **12**, 149–157 (2018).

36. Cui, F., *et al.* Lycium barbarum Polysaccharide Extracted from Lycium barbarum Leaves Ameliorates Asthma in Mice by Reducing Inflammation and Modulating Gut Microbiota. J Med Food **23**, 699–710 (2020).
37. Zhang, Z., *et al.* β-Glucan exacerbates allergic asthma independent of fungal sensitization and promotes steroid-resistant T(H)2/T(H)17 responses. J Allergy Clin Immunol **139**, 54–65.e58 (2017).
38. Malmström, K., *et al.* Airway hyperresponsiveness, remodeling and inflammation in infants with wheeze. Clin Exp Allergy **50**, 558–566 (2020).
39. Van Hulst, G., *et al.* Eosinophil diversity in asthma. Biochem Pharmacol **179**, 113963 (2020).
40. Orihara, K. & Matsuda, A. Pathophysiological roles of microvascular alterations in pulmonary inflammatory diseases: possible implications of tumor necrosis factor-alpha and CXC chemokines. Int J Chron Obstruct Pulmon Dis **3**, 619–627 (2008).
41. Fahy, J.V. & Dickey, B.F. Airway mucus function and dysfunction. N Engl J Med **363**, 2233–2247 (2010).
42. Ingram, J.L. & Kraft, M. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. J Allergy Clin Immunol **130**, 829–842; quiz 843 – 824 (2012).
43. Guihua, X., Shuyin, L., Jinliang, G. & Wang, S. Naringin Protects Ovalbumin-Induced Airway Inflammation in a Mouse Model of Asthma. Inflammation **39**, 891–899 (2016).
44. Gandhi, N.A., Pirozzi, G. & Graham, N.M.H. Commonality of the IL-4/IL-13 pathway in atopic diseases. Expert Rev Clin Immunol **13**, 425–437 (2017).
45. Kiwamoto, T., *et al.* Transcription factors T-bet and GATA-3 regulate development of airway remodeling. Am J Respir Crit Care Med **174**, 142–151 (2006).
46. Du, Q., Gu, X., Cai, J., Huang, M. & Su, M. Chrysin attenuates allergic airway inflammation by modulating the transcription factors T-bet and GATA-3 in mice. Mol Med Rep **6**, 100–104 (2012).
47. Aierken, A. & Xu, P. Bacterial respiratory tract inflammation in neonatal rat model is attenuated by benzofuran through inhibition of GATA3. Microb Pathog **125**, 313–317 (2018).
48. Hou, X., *et al.* Histone deacetylase inhibitor regulates the balance of Th17/Treg in allergic asthma. Clin Respir J **10**, 371–379 (2016).
49. Tumes, D.J., *et al.* Epigenetic regulation of T-helper cell differentiation, memory, and plasticity in allergic asthma. Immunol Rev **278**, 8–19 (2017).
50. Branchett, W.J., *et al.* A T cell-myeloid IL-10 axis regulates pathogenic IFN-γ-dependent immunity in a mouse model of type 2-low asthma. J Allergy Clin Immunol **145**, 666–678.e669 (2020).
51. Wei, Y., *et al.* Bu-Shen-Yi-Qi formulae suppress chronic airway inflammation and regulate Th17/Treg imbalance in the murine ovalbumin asthma model. J Ethnopharmacol **164**, 368–377 (2015).
52. Liu, F., Yu, J., Bai, L., Xue, Z. & Zhang, X. Pingchuan formula improves asthma via restoration of the Th17/Treg balance in a mouse model. BMC Complement Altern Med **15**, 234 (2015).
53. Zhensheng, L., *et al.* Methods to enhance the solubility of polysaccharides. FOOD AND FEï¼²MENTATION INDUSTï¼²IES **46**, 292–299 (2020).

54. Kim, J.H., Park, Y., Yu, K.W., Imm, J.Y. & Suh, H.J. Enzyme-assisted extraction of cactus bioactive molecules under high hydrostatic pressure. J Sci Food Agric **94**, 850–856 (2014).
55. Song, Y.R., *et al.* Effect of enzyme-assisted extraction on the physicochemical properties and bioactive potential of lotus leaf polysaccharides. Int J Biol Macromol **153**, 169–179 (2020).
56. Chen, H., Zhou, X. & Zhang, J. Optimization of enzyme assisted extraction of polysaccharides from Astragalus membranaceus. Carbohydr Polym **111**, 567–575 (2014).
57. Zeng, P., Li, J., Chen, Y. & Zhang, L. The structures and biological functions of polysaccharides from traditional Chinese herbs. Prog Mol Biol Transl Sci **163**, 423–444 (2019).
58. Sun, L., *et al.* α-Amylase-assisted extraction of polysaccharides from Panax ginseng. Int J Biol Macromol **75**, 152–157 (2015).

Figures

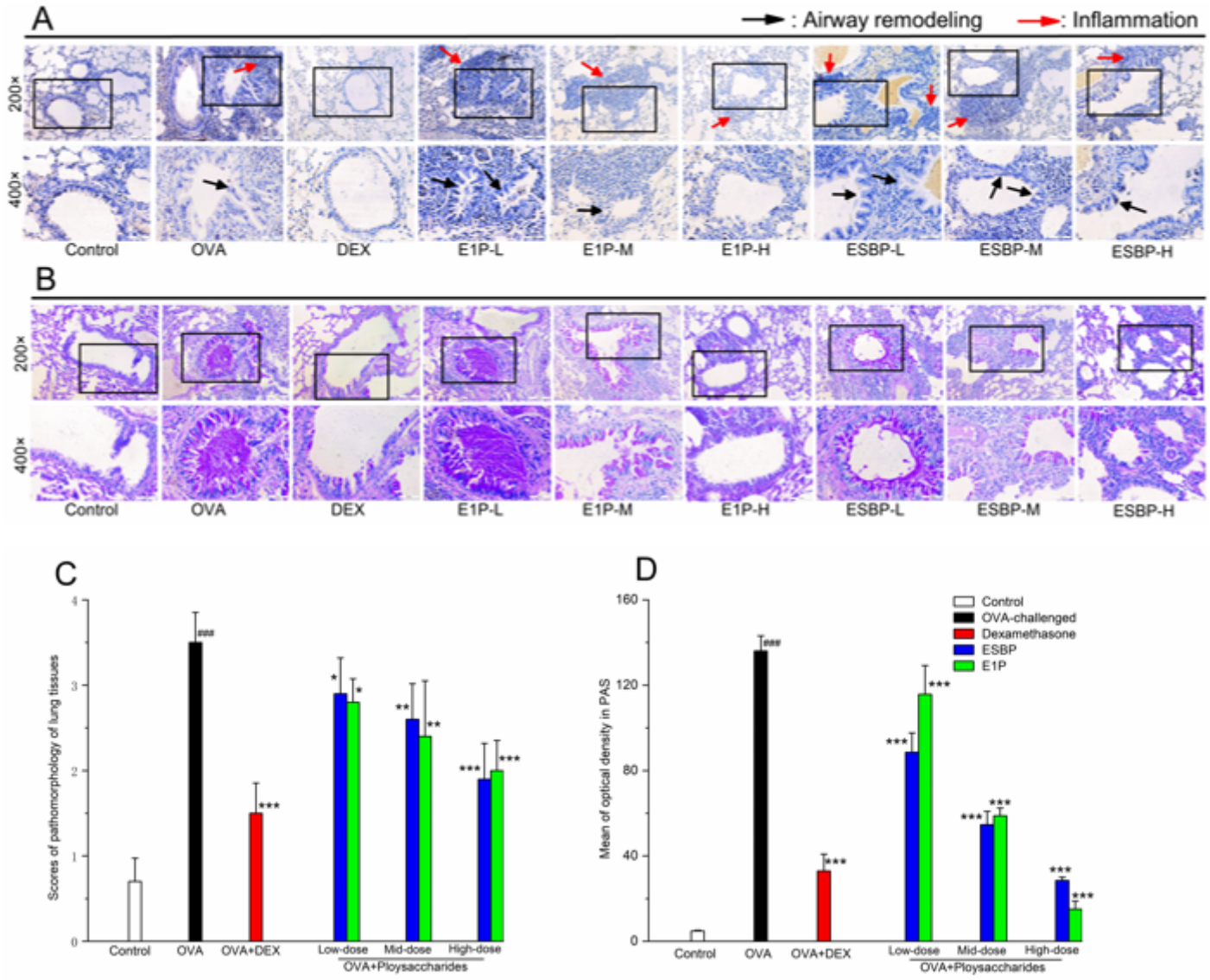

Figure 1

Effects of E1P and ESBP on pulmonary pathological states. A, representative images of HE. **B**, representative images of PAS. **C**, histopathological scores of lung tissues. **D**, mean of optical density in PAS. Data are expressed as mean ±SEM (n=6) "###" P <0.001 *vs* Control group. "*" P <0.05 *vs* OVA-challenged group. "**" P <0.01 *vs* OVA-challenged group. "***" P <0.001 *vs* OVA-challenged group.

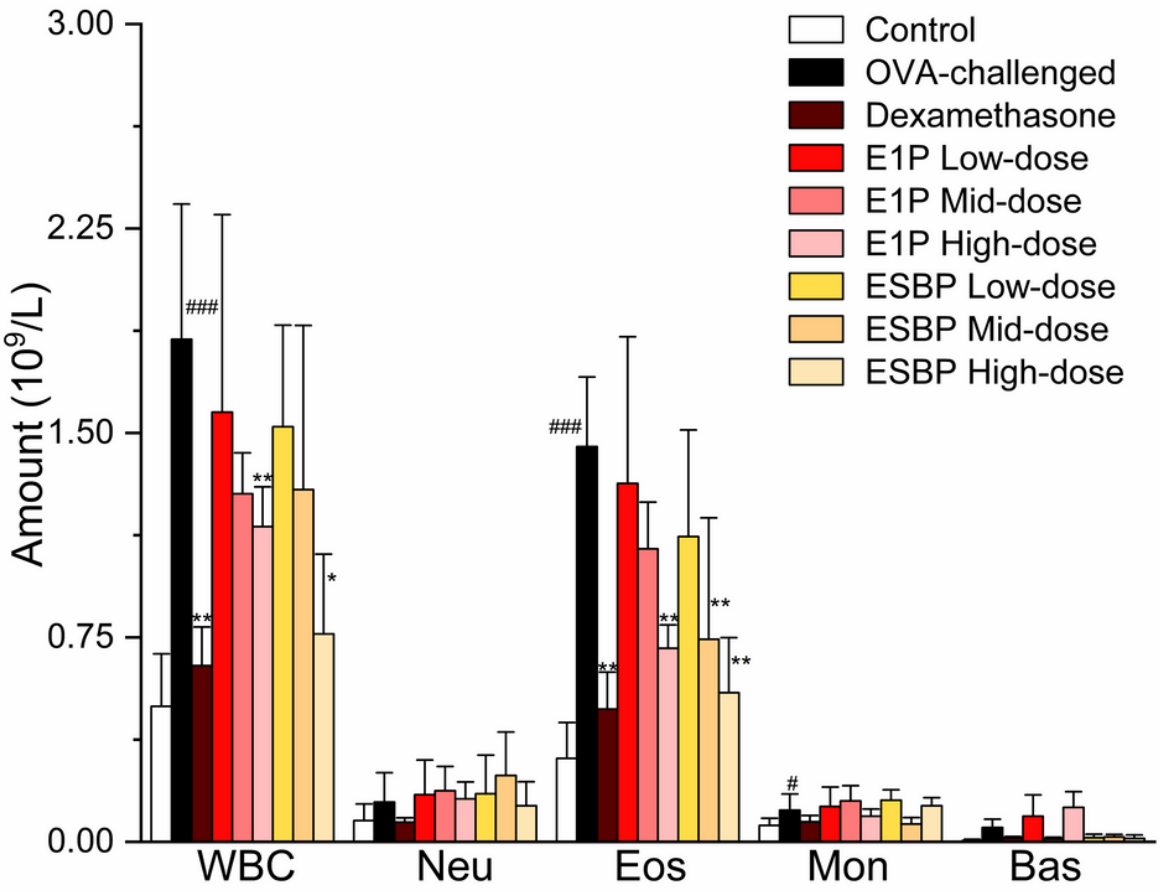

Figure 2

Effects of E1P and ESBP on recruitment of inflammatory cells in BALF. Data are expressed as mean ±SEM (n=5). "###" P <0.001 *vs* Control group. "*" P <0.05 *vs* OVA-challenged group. "**" P <0.01 *vs* OVA-challenged group.

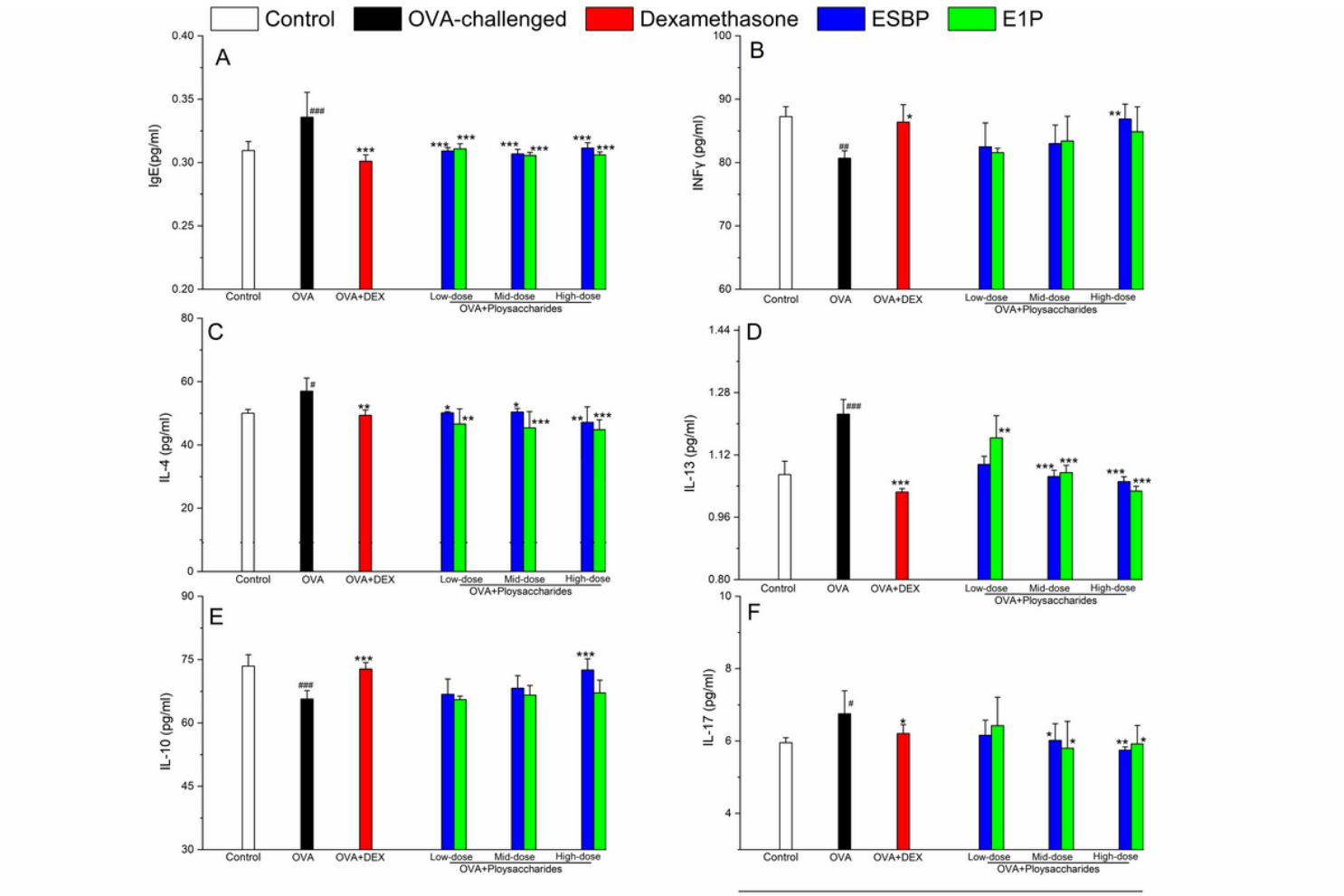

Figure 3

Effects of ESBP and E1P on inflammatory cytokine levels in BALF or serum of asthmatic mice. A, IgE levels in serum. **B**, INFγ levels in BALF. **C**, IL-13 levels in BALF. **D**, IL-4 levels in BALF. **E**, IL-10 levels in BALF. **F**, IL-17 levels in BALF. Data are expressed as mean ±SEM (n=5) "#" P<0.05 *vs* Control group. "##" P<0.01 *vs* Control group. "###" P <0.001 *vs* Control group. "*" P <0.05 *vs* OVA-challenged group. "**" P <0.01 *vs* OVA-challenged group. "***" P <0.001 *vs* OVA-challenged group.

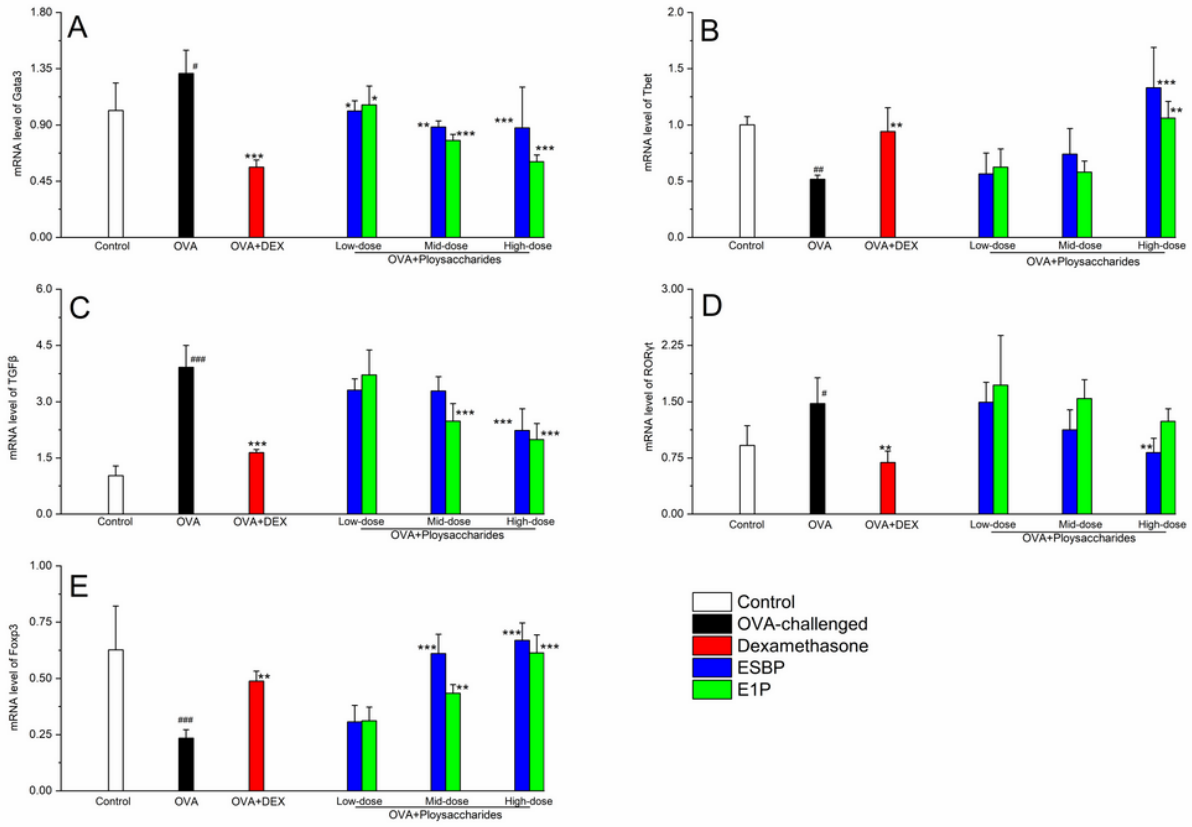

Figure 4

Effects of ESBP and E1P on mRNA levels of Gata3, T bet, TGFβ, RORγt and Foxp3. A, mRNA levels of Gata3 detected by RT-PCR. **B**, mRNA levels of T bet detected by RT-PCR. **C**, mRNA levels of TGFβ detected by RT-PCR. **D**, mRNA levels of RORγt detected by RT-PCR. **E**, mRNA levels of Foxp3 detected by RT-PCR. Data are expressed as mean ±SEM (n=5) "#" P <0.05 *vs* Control group. "##" P <0.01 *vs* Control group. "*" P <0.05 *vs* OVA-challenged group. "**" P <0.01 *vs* OVA-challenged group. "***" P <0.001 *vs* OVA-challenged group.

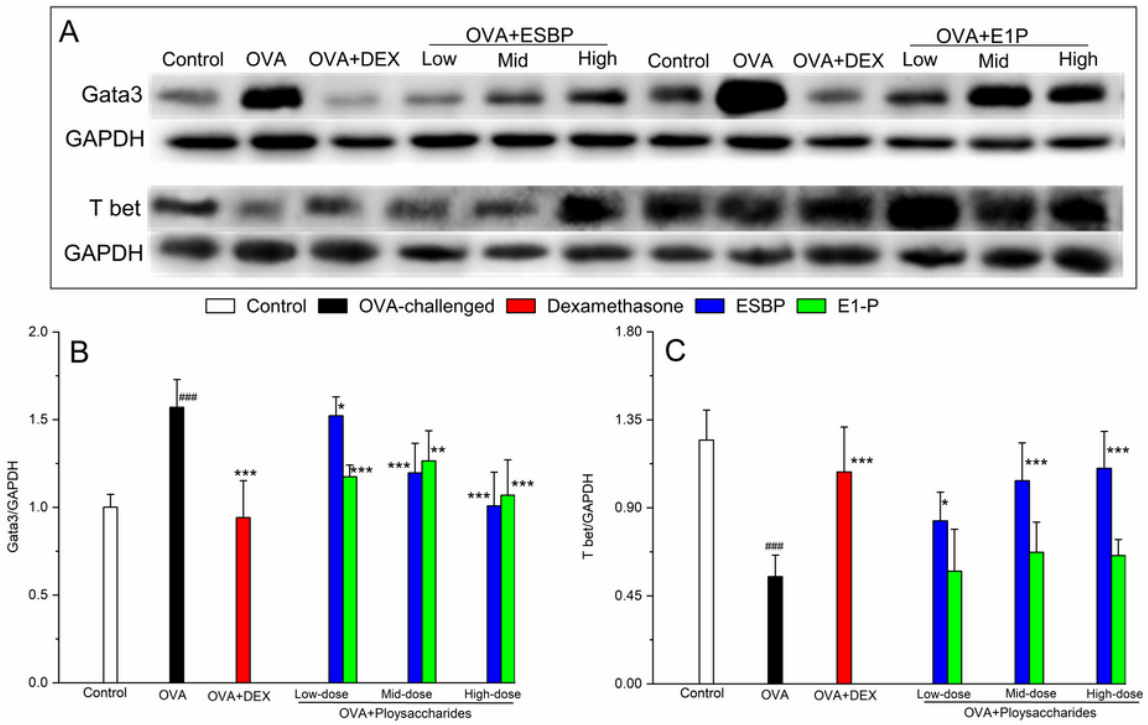

Figure 5

Effects of ESBP and E1P on levels of Th1/Th2 balance related protein. A, representative images of western blot of Gata3 and T bet. B, expression of Gata3 detected by western blot. **C**, expression of T bet detected by western blot. Data are expressed as mean ±SEM (n=6) "###" P <0.001 *vs* Control group. "*" P <0.05 *vs* OVA-challenged group. "**" P <0.01 *vs* OVA-challenged group. "***" P <0.001 *vs* OVA-challenged group.

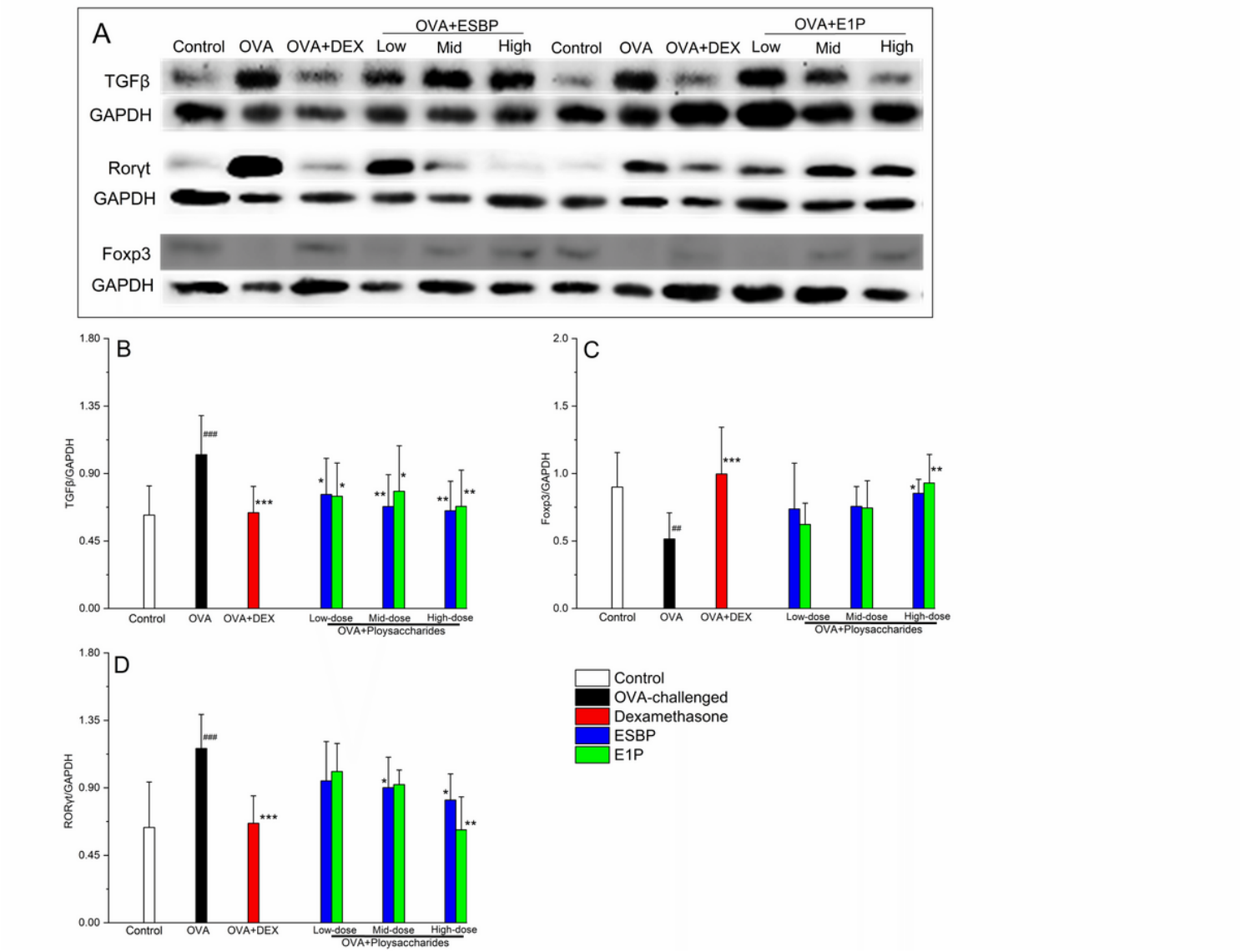

Figure 6

Effects of ESBP and E1P on levels of Th17/Treg balance related protein. A, representative images of western blot of TGFβ, RORγt and Foxp3. **B**, expression of TGFβ detected by western blot. **C**, expression of Foxp3 detected by western blot. **D**, expression of RORγt detected by western blot. Data are expressed as mean ±SEM (n=6). "#" P <0.05*vs* Control group. "##" P <0.01 *vs* Control group. "###" P <0.001 *vs* Control group. "*" P <0.05 *vs* OVA-challenged group. "**" P <0.01 *vs* OVA-challenged group. "***" P <0.001 *vs* OVA-challenged group.