

Cordyceps polysaccharide ameliorates airway inflammation in an ovalbumin-induced mouse model of asthma via TGF- β 1/Smad signaling pathway

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ARTICLE INFO

Keywords:

Airway inflammation
airway hyperresponsiveness
Asthma
Cordyceps polysaccharide
Smad
TGF- β 1

ABSTRACT

Allergic asthma is a chronic inflammatory disease characterized by airflow obstruction, airway hyperresponsiveness (AHR), airway inflammation, and mucus overproduction. Cordyceps polysaccharide (CPS) is one of the main bioactive compounds of *Cordyceps militaris*, a traditional Chinese medicine. In this study, we established a mouse model of asthma using ovalbumin (OVA) challenge and evaluated the potential regulatory effect of CPS (25, 50, and 100 mg/kg) on asthmatic mice. These results showed that the asthmatic mice treated with CPS suppressed the secretion of eotaxin, IL-4, IL-5, IL-13, and IFN- γ in the blood and bronchoalveolar lavage fluid (BALF), and decreased serum IgE levels compared to the vehicle-treated mice. CPS also alleviated inflammatory cell infiltration, goblet cell hyperplasia, and the increases of inflammatory cells in the mouse model of asthma. In addition, OVA-induced AHR was inhibited by CPS treatment. Further analyses of protein expression revealed that CPS inhibited the activation of transforming growth factor β 1 (TGF- β 1)/Smad pathway in mice with asthma. These findings indicated that CPS might serve as a potential therapeutic agent for the management of allergic asthma.

1. Introduction

Allergic asthma is a complex inflammatory syndrome in which exposure to allergens leads to bronchial inflammation, mucus hypersecretion, airway hyperresponsiveness (AHR) and airway remodeling (Mukherjee and Zhang, 2011). Recent epidemiological data have shown that the prevalence of allergic asthma increased from 5.0%–7.3% from 1996 to 2016, whereas the prevalence of non-allergic asthma remained unchanged (Backman et al., 2017). The pathogenesis of allergic asthma involves the development of a type 2 helper (Th2) lymphocyte-mediated immune response, which is characterized by the production of immunoglobulin E (IgE), bronchial hyperresponsiveness, inflammatory cell infiltration, mucus overproduction, and eventually airway remodeling (Maddox and Schwartz, 2002). The transforming growth factor β 1 (TGF- β 1)/Smad signaling pathway has been shown to be one of the key mechanisms associated with airway remodeling in asthma (Qu et al., 2012). The suppression of TGF- β 1 activation and the

subsequent phosphorylation of Smad proteins ameliorated airway inflammation and remodeling both *in vitro* and *in vivo* (Chen et al., 2015; Wang et al., 2018). Current treatment strategies for asthma are limited to bronchodilators (e.g. inhaled corticosteroids and β 2-adrenoceptor agonists) and anti-inflammatory drugs (Holgate and Polosa, 2008). The exploration of new therapeutic options, especially those targeting airway inflammation and remodeling, will be important for the management of allergic asthma.

Cordyceps militaris (*C. militaris*), a traditional caterpillar medicinal mushroom with multiple pharmacological properties, has been used for medication in China for over 3000 years (Li et al., 2006). Studies have shown that the main bioactive compounds in *C. militaris*, including cordycepin, adenosine, and polysaccharides, possess anti-tumor, anti-inflammatory, antioxidant, hypoglycemic, and hypolipidemic activities (Cui, 2015). In a mouse model of asthma, a traditional medicine composed of *Radix Astragali* and *C. militaris* efficiently ameliorated asthma airway remodeling via inhibiting the production of TGF- β 1 and

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<https://doi.org/10.1016/j.resp.2020.103412>

Received 5 November 2019; Received in revised form 17 January 2020; Accepted 6 February 2020

Available online 07 February 2020

1569-9048/ © 2020 Published by Elsevier B.V.

upregulating the expression of Smad7 (Gao et al., 2009). The extract of cordyceps was also shown to inhibit airway inflammation in ovalbumin (OVA)-challenged mice (Chiou and Lin, 2012). Although emerging evidences have demonstrated the beneficial effect of *C. militaris* in relieving asthmatic symptoms, further investigations are needed to identify the therapeutic role of individual bioactive constituents. A study in human mesangial cells showed that cordyceps polysaccharides (CPS) inhibited cell proliferation through the downregulation of TGF- β 1 and Smad3 in a dose-dependent manner, indicating the regulatory potential of CPS on the TGF/Smad signaling pathway (Wang et al., 2014).

In this study, we aimed to explore the regulatory role of CPS in airway inflammation, inflammatory cell infiltration, mucus secretion, and AHR in a mouse model of OVA-induced asthma. We hypothesized that CPS treatment would alleviate OVA-induced airway inflammation and AHR by targeting TGF/Smad signaling pathway.

2. Materials and methods

2.1. Preparation of CPS

Cultured *C. militaris* was purchased from Wanfong Pharmaceutical Factory (Hangzhou, Zhejiang, China) and extracted for CPS as previously mentioned (Li et al., 2001). In brief, *C. militaris* were boiled in water for 2 h, followed by centrifugation at 3000 rpm. The concentrated supernatant was then removed and precipitated using four volumes of 95 % ethanol. The precipitate was suspended in water, dialyzed, and lyophilized to obtain the enriched crude polysaccharide fraction. Then 500 mg of the sample was resuspended in water, centrifuged, and eluted with a salt gradient in Tris-HCl buffer. The purity of CPS in the extracted samples was 62 %, as determined by sulfuric acid-phenol detection method (Li et al., 2003, p. 12).

2.2. Animals and asthma model

Female BALB/c mice (6 weeks old, 18–20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd and maintained in a controlled environment (12 h alternating light-dark cycle, 22–24 °C, 50–60 % humidity) with *ad libitum* access to food and water. All animal care and experimental procedures were approved by the Zhejiang Chinese Medical University guide for the care and use of laboratory animals (ethical approval code: ZSLL-2018102). The mice were randomly divided into 6 groups ($n = 10$ per group): control, OVA, OVA + CPS 25, OVA + CPS 50, OVA + CPS 100, and OVA + DEX. The mouse asthma model was established by sensitization and antigen challenge with OVA as previously described (Tanaka et al., 2001). All mice, except those in the control group, received sensitization and OVA challenge. Briefly, the mice were immunized by subcutaneous injection of 2 mg/mL OVA (Sigma-Aldrich, St. Louis, MI, USA) absorbed in 4 % aluminum hydroxide adjuvant (Sigma-Aldrich) in a total volume of 0.5 mL. On day 14, the mice received an intraperitoneal injection of 0.2 mL OVA emulsified in aluminum hydroxide. On day 23 after the initial sensitization, mice were challenged with OVA (10 mg/mL in normal saline) 3 times per week for 8 weeks (30 min each time) using an ultrasonic nebulizer (Omron Co., Tokyo, Japan).

2.3. Administration of drugs

Starting on day 23, animals in the OVA + CPS 25, OVA + CPS 50, and OVA + CPS 100 groups received daily intragastric administration of CPS at the doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg, respectively, for 8 weeks. Dexamethasone was dissolved in dimethylsulfoxide and diluted into phosphate-buffered saline (PBS, Sigma-Aldrich). The mice in OVA + DEX group were intraperitoneally injected with dexamethasone (0.5 mg/kg/day) 3 times per week for 8 weeks. When OVA challenge was administered on the same day of drug intervention, drugs

were given 1 h prior to the OVA administration. The control and OVA groups received equal amounts of normal saline.

2.4. Assessment of airway hyperresponsiveness (AHR)

Twenty-four hours after the last OVA inhalation, all animals were tested for AHR using a Buxco noninvasive plethysmography system (EMKA Technologies, Paris, France) as previously described (Bao et al., 2007; Hamelmann et al., 1997). Conscious, unrestrained mice were placed into a whole-body chamber. The baseline lung dynamic compliance (C_{dyn}) and airway resistance (RI) were recorded for 5 min at steady respiration. Then mice were treated with aerosolized normal saline or methacholine (Mch, Sigma-Aldrich) at increasing concentrations (6.25, 12.5, 25, 50, and 100 mg/mL), and the RI and C_{dyn} were monitored.

2.5. Collection of blood and bronchoalveolar lavage fluid (BALF) and cell counting

Immediately following the detection of AHR, the mice were anesthetized with 40 mg/kg pentobarbital sodium and blood samples were collected from the orbits. After centrifugation at 3500 rpm for 10 min, the serum was separated and stored at –20 °C. Then, the mice were euthanized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), and the BALF was collected as previously reported (Daubeuf and Frossard, 2012). In brief, the mouse tracheas were exposed and inserted with an intravenous needle. The lungs were washed with normal saline 3 times. Then, the BALF samples were collected and centrifuged. The supernatants were stored at –80 °C for the assessment of cytokine levels. BALF cell pellets were resuspended in PBS, made into cell smears using a cytospin, and stained with Wright-Giemsa (BioVision, Milpitas, California, USA). The number of total cells, eosinophils, macrophages, lymphocytes, and neutrophils in the BALF were determined using a hemocytometer under an optical microscope (400× magnification).

2.6. Histopathological analyse

After the BALF was collected, the lung tissues from each mouse were harvested and fixed in a buffer solution containing 10 % formalin and embedded in paraffin. The paraffin-embedded tissues were then cut into 5 μ m sections and stained with hematoxylin and eosin (H&E) for general morphology. Alcian blue-periodic acid Schiff (AB-PAS) staining was used to measure mucus production and to identify goblet cells in the epithelium.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of eotaxin (MM-0195M2, Meimian, China), IL-4 (MM-0165M2, Meimian, China), IL-5 (MM-0164M2, Meimian, China), IL-13 (MM-0173M2, Meimian, China) and IFN- γ (MM-0182M2, Meimian, China) in the BALF samples and the serum concentration of OVA-specific IgE (MM-1014M2, Meimian, China) were measured using ELISA kits according to the manufacturer's instructions. The absorbance at 450 nm was measured on an ELISA plate reader (CMax Plus, Thermo Scientific).

2.8. Western blot

The same portion of the lung tissue samples in all mice was weighed and homogenized in RIPA buffer with protease and phosphatase inhibitors (Bio-Rad Laboratories, Hercules, CA, USA). The protein concentration was quantified using a bicinchoninic acid assay (Pierce, Rockford, IL, USA). Then an equal amount of protein (80 μ g) from each group lung tissues was separated on a 10 % SDS-PAGE gel and then transferred onto polyvinylidene fluoride (PVDF) membranes

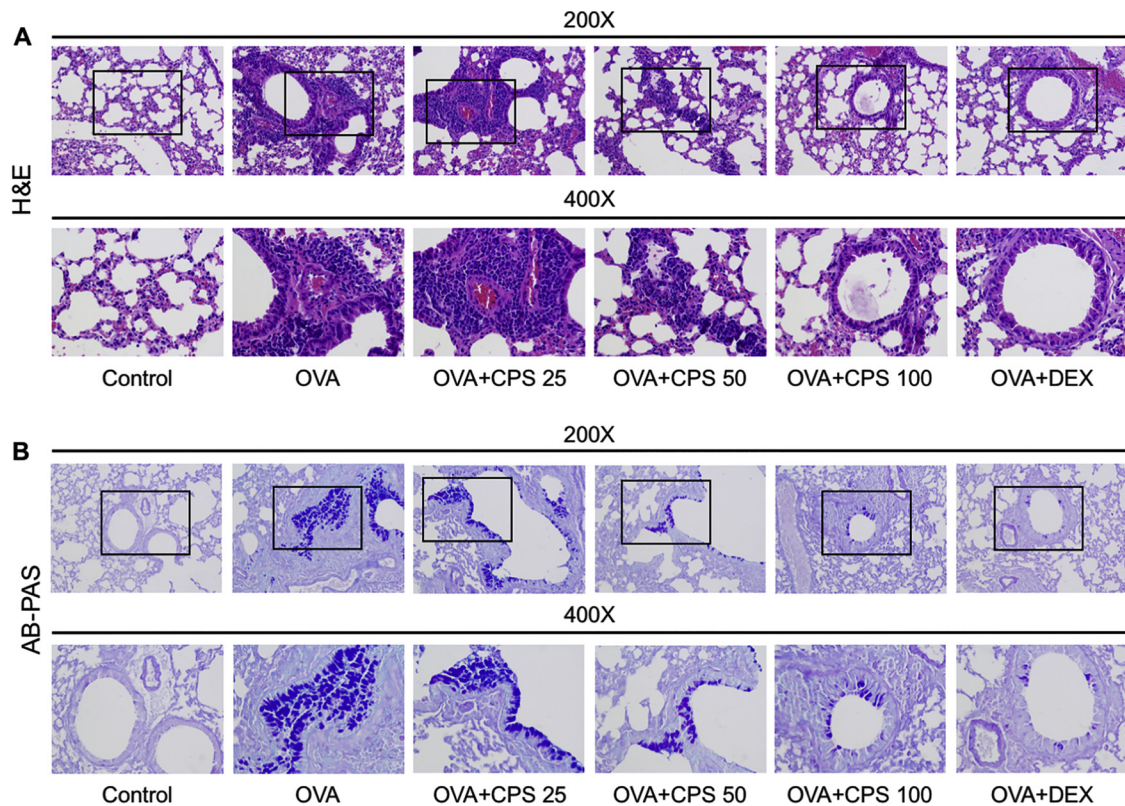


Fig. 2. Effect of CPS on the airway inflammation and mucus hypersecretion in the lung tissues of the asthmatic mice (H&E staining and AB-PAS staining). Lung tissues from all mice were fixed, sectioned into 5 μ m sections, and stained with (A) H&E, (B) AB-PAS for histological examination. A violet color indicated the over-production of mucus. Representative images obtained from different groups of mice are shown. (magnification 200 $\times$ and 400 $\times$).

and 100 mg/kg) or dexamethasone. CPS at all doses, as well as dexamethasone, also showed a robust inhibitory effect on the recruitment of eosinophils, macrophages, and lymphocytes. The number of neutrophils was reduced by CPS at middle and high concentrations, but not at 25 mg/kg, in the OVA-challenged mice. These findings indicated that CPS prevented the accumulation of inflammatory cells in OVA-induced asthma.

3.4. CPS suppressed the OVA-induced AHR in a mouse model of allergic asthma

To explore the effect of CPS on AHR, we assessed lung dynamic compliance (C_{dyn}) and airway resistance (RI) in all mice using a plethysmography system. The OVA-challenged group showed decreased C_{dyn} compared to the control mice. However, the impaired lung compliance was notably recovered by dexamethasone and CPS in a

dose-dependent manner (Fig. 4A). Moreover, the RI parameter in the mice following OVA exposure was substantially elevated compared to that of the controls, whereas the administration of CPS and dexamethasone effectively repressed airway resistance in the asthmatic mice (Fig. 4B). These data suggested that CPS relieved the OVA-induced AHR in mouse asthma model.

3.5. CPS impeded the activation of the TGF- β 1/Smad pathway in mice following OVA challenge

To investigate the regulatory effect of CPS on TGF- β /Smad pathway, the expressions of TGF- β 1 and Smad proteins in the lung tissue samples were analyzed. Induction of TGF- β 1, phosphorylated Smad2 and phosphorylated Smad3 was shown in the OVA-sensitized mice compared to the controls. Treatment with CPS at all doses and dexamethasone reduced the increase expression of TGF- β 1 and the

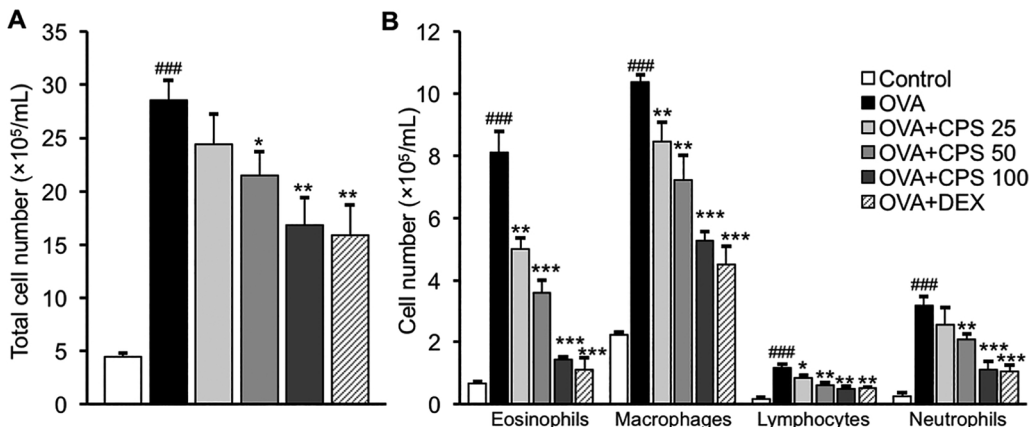


Fig. 3. Effect of CPS on the recruitment of inflammatory cells in the BALF following OVA challenge. The collected BALF cell pellets were made into cell smears using a cytospin and stained with Wright-Giemsa. (A) The number of total cells, (B) eosinophils, macrophages, lymphocytes and neutrophils in BALF were determined using a hemocytometer under an optical microscope (400 $\times$ magnification). # indicates a significant difference compared to the control group (### $p < 0.001$). * indicates a significant difference compared to the OVA group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

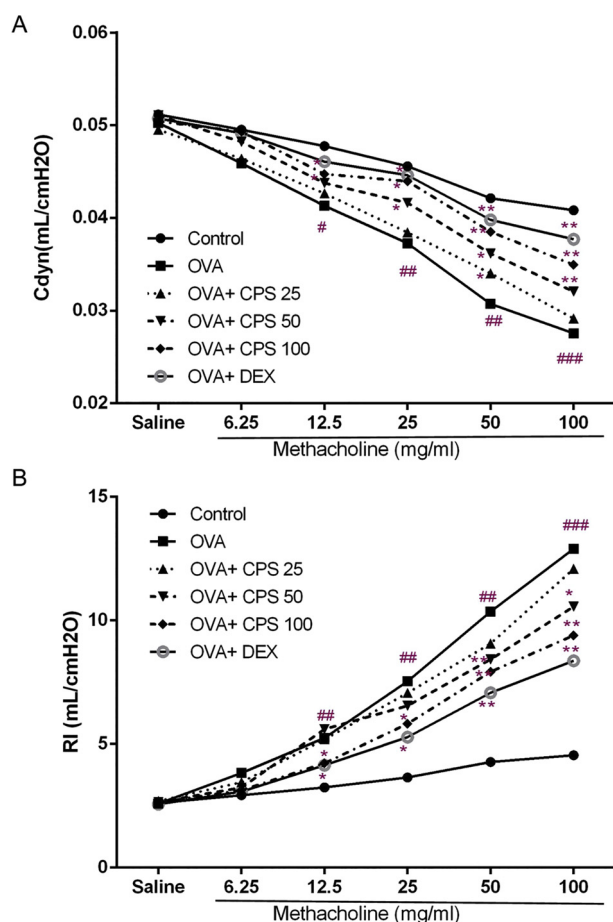


Fig. 4. Effect of CPS on AHR in response to aerosolized methacholine in the OVA-induced mouse model of allergic asthma. AHR was measured 24 h after the final OVA challenge using a Buxco noninvasive plethysmography system. Mice were administered by aerosolized normal saline or methacholine at 6.25, 12.5, 25, 50, or 100 mg/mL to evaluate the (A) lung dynamic compliance (Cdyn) and (B) airway resistance (RI). Cdyn represents pulmonary distensibility and RI indicates airflow obstruction. # indicates a significant difference compared to the control group (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). * indicates a significant difference compared to the OVA group (* $p < 0.05$, ** $p < 0.01$).

phosphorylation of Smad3 in asthmatic mice. The expression of phosphorylated Smad2 was also suppressed by dexamethasone and CPS (50 and 100 mg/kg) in the mice following OVA exposure. No significant difference was found in the Smad2 and Smad3 levels among all the groups. These data indicated that CPS restrained the activation of TGF- β 1/Smad pathway in the OVA-challenged mice (Fig. 5).

4. Discussion

Although advances have been made in treatment and therapeutic guidelines over the past decades, asthma remains a global health problem with a high burden of disability and health costs (D'Amato et al., 2016). Current available medications mainly include long-term preventive drugs that reduce airway inflammation and quick-relief inhalers that rapidly open swollen airways (Horak et al., 2016). Effective pharmaceutical options that can inhibit airway remodeling are still needed for the treatment of asthma exacerbation. Mouse allergen challenge models reproduce some clinical asthma features and have been widely used to investigate the pathophysiology of asthma (Lloyd, 2007). Chronic exposure to OVA, which induces robust and persistent allergic pulmonary inflammation in mice, enables the evaluation of potential therapeutic agents for asthma in a therapeutic setting (Nials

and Uddin, 2008). In this study, we showed that the polysaccharides extracted from *C. militaris* ameliorated the airway inflammation and bronchial hyperresponsiveness in OVA-challenged asthmatic mice. Most importantly, we showed that CPS alleviated airway inflammation in asthma via the inhibition of TGF- β 1/Smad activation.

Human allergic asthma is an inflammatory process characterized by airflow obstruction, AHR, airway inflammation, mucus overproduction, and airway remodeling (Bousquet et al., 2000). Eotaxin is a chemokine responsible for the recruitment of eosinophils and lymphocytes in the development of pulmonary allergic inflammation (Pease and Williams, 2001). Th2 cytokines, including IL-4, IL-5, and IL-13, are involved in asthma progression by promoting IgE synthesis and Th2 cell differentiation (Barnes, 2001). Inappropriate immunological responses to allergens have been shown to stimulate the production and release of IL-4, IL-5, and IL-13 (Kudo et al., 2013). Th1 cytokines such as IFN- γ also play a pivotal role in both acute and chronic asthma (Kumar et al., 2006). In this study, we showed that chronic OVA challenge in mice strongly increased the secretion of cytokines, including eotaxin, IL-4, IL-5, IL-13, and IFN- γ , in the BALF. Previous studies have also demonstrated that inflammatory cytokines contribute to the upregulation of IgE levels followed by inflammatory cell infiltration in the BALF, mucus hypersecretion, and finally bronchial remodeling (Barnes, 2001; Durrant and Metzger, 2010). In this study, we found that the plasma level of OVA-specific IgE was increased in the OVA-challenged mice and that the number of inflammatory cells in mouse BALF samples was decreased.

Airway remodeling in asthma consists of subepithelial fibrosis, increased smooth muscle fibers, goblet cell hyperplasia and myofibroblast hyperplasia (Durrani et al., 2011). In the current study, histopathological examination of the lung tissue samples showed severe infiltration of leukocytes and goblet cell hyperplasia in the OVA-challenged animals compared to the controls. Moreover, increased bronchial hyperresponsiveness has been found to be associated with airway remodeling in asthma (Bergeron et al., 2009). The recruitment of granulocytes and mononuclear cells in the BALF is also a critical step in the pathogenesis of allergic asthma (Hamid et al., 2003). Our findings showed that bronchial hyperresponsiveness and inflammatory cell counts were also induced by OVA exposure.

TGF- β 1 signaling contributes to airway remodeling via two mechanisms. First, TGF- β 1 acts as a stimulus for epithelial to mesenchymal transition (EMT) in bronchial epithelial cells (Doerner and Zuraw, 2009). Activation of the TGF- β 1 pathway contributes to airway remodeling, microvascular changes, and subepithelial fibrosis in bronchial asthma (Chen et al., 2011). Second, TGF- β 1 induces structural remodeling in bronchial asthma via TGF- β 1/Smad signaling pathway (Boxall et al., 2006). With the stimulation of TGF- β 1 signaling, Smad2 and Smad3 are phosphorylated and translocate to the nucleus to regulate the transcription of target genes (Groneberg et al., 2004). Previous studies have shown that OVA challenge enhances the expression of TGF- β 1 and the phosphorylation of Smad2/Smad3 in mice (Chen et al., 2015; Lee et al., 2017). Consistently, we observed elevated TGF- β 1 levels and the activation of Smad proteins in the mice following OVA challenge.

Cumulative evidence has shown the therapeutic impacts of *C. militaris* on allergic asthma. The administration of cultivated *C. militaris* fluid reduced the serum IgE levels, inflammatory cell infiltration, and BALF eosinophilia in asthmatic mice (Hsu et al., 2008). In patients with moderate-to-severe persistent asthma, treatment with capsules containing *C. militaris* for 3 months improved their health-related quality of life as measured by the Asthma Quality of Life Questionnaire, and decreased the serum levels of inflammatory markers compared to the control group (Wang et al., 2016). A study also showed that cordycepin, a nucleoside derivative extracted from *C. militaris*, attenuated airway hyperreactivity, mucus hypersecretion, and lung inflammation in a mouse asthma model via the blockage of p38-MAPK and NF- κ B pathways (Yang et al., 2015). In our work, we demonstrated that

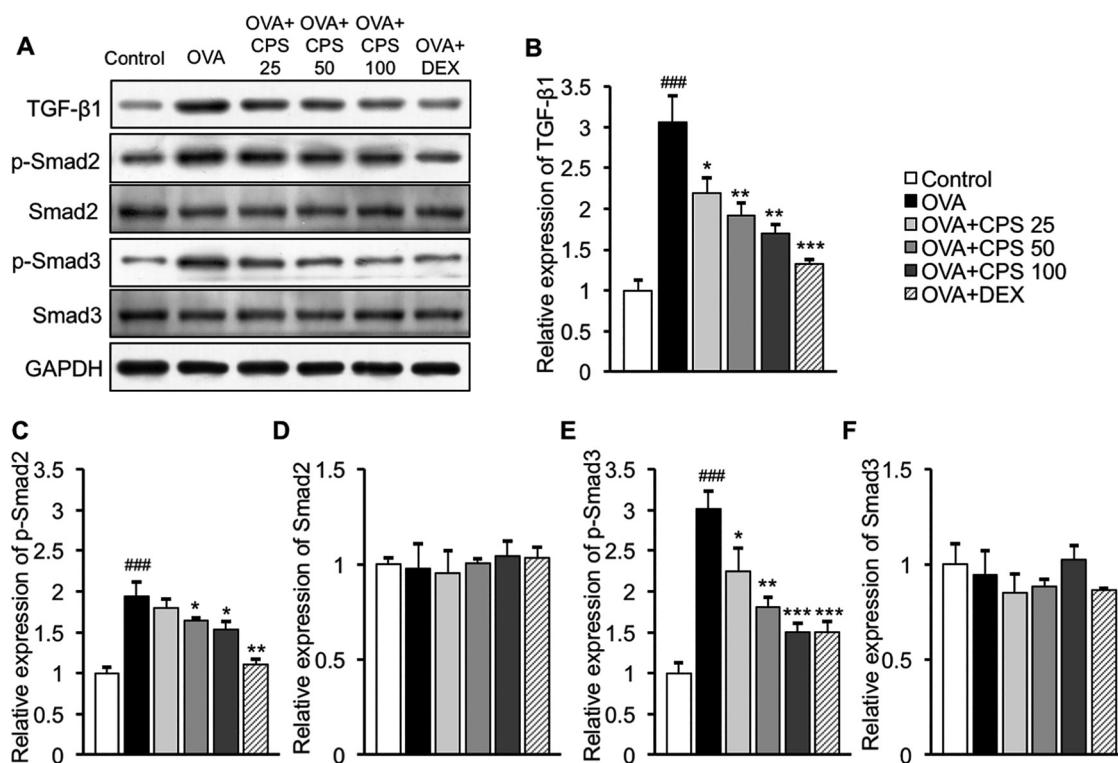


Fig. 5. Effect of CPS on OVA-induced activation of the TGF- β 1/Smad signaling pathway. The expression level of TGF- β 1, p-Smad2, Smad2, p-Smad3, and Smad3 in the lung tissue samples obtained from all mice were measured using Western blotting. (A) Representative blots and (B – F) relative expression levels normalized to GAPDH are shown. # indicates a significant difference compared to the control group (### $p < 0.001$). * indicates a significant difference compared to the OVA group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

polysaccharides extracted from *C. militaris* at increasing doses suppressed the secretion of inflammatory cytokines and serum IgE level in mice following OVA challenge. A previous study showed that *Cordyceps sinensis* (*C. sinensis*), which is also a traditional medicine with anti-inflammatory and antioxidant properties, suppressed the production of IL-1 β , IL-6, IL-8, IL-10 and TNF- α in LPS-activated human BALF samples, but increased the mRNA expression of IL-12 and IFN- γ (Kuo et al., 2001). With the differences in the levels of bioactive components, *C. sinensis* and *C. militaris* may result in varied immunomodulatory effects on airway inflammation in asthma (Yu et al., 2006). We further demonstrated that CPS ameliorated inflammatory cell infiltration, goblet cell hyperplasia, and OVA-induced AHR in a mouse model of asthma. By evaluating the protein expression in mouse lung tissues, we found that CPS decreased the levels of TGF- β 1 and phosphorylated Smad in the OVA-challenged mice, indicating the inhibitory effect of CPS on the activation of the TGF- β 1/Smad pathway in asthma.

5. Conclusion

In conclusion, this study showed that CPS played a potential beneficial role in ameliorating the airway inflammation and remodeling in OVA-challenged mice via suppressing the TGF- β 1/Smad signaling pathway, suggesting that CPS might serve as a promising therapeutic agent for the management of allergic asthma. Our results also provide support for the design of future protocols in preclinical studies.

Funding

This work was supported by the Zhejiang Provincial Natural Science Foundation of China [grant number LY15H270006].

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

Acknowledgement

Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.resp.2020.103412>.

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