

The Immunotherapeutic Role of Bacterial Lysates in a Mouse Model of Asthma

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Abstract

Introduction Asthma is the most common chronic lower respiratory disease in childhood throughout the world. Recurrent respiratory tract infections in young children, especially viral infections, are the major cause of acute asthmatic exacerbations and contribute to development of asthma. Bacterial extracts have been used to improve the immune defenses of the respiratory tract. However, seldom studies have examined the effect of bacterial lysates on childhood asthma. In this study, we examined whether bacterial lysates (OM-85) will improve symptoms of asthmatic mice via modulation of the immune response.

Methods Asthmatic mice models were established with OVA challenge and treated with oral administration of Broncho-Vaxom (OM-85). Next, infiltrations of inflammatory cells including eosinophil and neutrophils were examined. Pulmonary tissues in asthmatic mice models were analyzed by hematoxylin and eosin (HE) staining. The levels of Th1/Th2-typed cytokines in bronchoalveolar lavage fluid (BALF) of asthmatic mice models were examined by enzyme-linked immunosorbent assay.

Results Compared to control group, we found significant reduction of airway wall thickness, luminal stenosis, and mucus plug formation in asthmatic mice models after oral administration of OM-85. The infiltrations of eosinophil were also significantly decreased in BALF in asthmatic mice

models. Oral administration of OM-85 was shown to suppress Th2-type cytokine levels.

Conclusion Our findings provide evidence that oral administration of OM-85 is capable of attenuating airway inflammation in asthmatic mice models. Oral administration of OM-85 may have a positive impact in terms of asthma severity.

Keywords MICE · Asthma · Exacerbation · Bacterial lysates (OM-85) · Immunotherapy

Introduction

Asthma is the most common chronic lower respiratory disease throughout the world. The impact of asthma on quality of life of patients, as well as its cost, is very high. Unlike adults, children with asthma often presents with additional challenges due to ongoing maturation of the respiratory and immune systems. It is therefore not surprising that additional guidelines and/or consensus documents are available to support medical decisions on pediatric asthma. Asthma in childhood is strongly associated with allergy, especially in developed countries. Common exposures such as tobacco smoke, air pollution, and respiratory infections may trigger symptoms and contribute to the morbidity and occasional mortality. Asthma most often starts early in life and may progress or remit over time and can exhibit variability in severity over the course of disease. It is well known that a history of wheezing associated with respiratory viral infections early in life is one of the major risk factors for the later development of asthma [1–3], apart from sensitization to aeroallergens in early life and a family history of asthma and allergies. Respiratory tract infections, especially viral infections, are the major cause of acute asthmatic exacerbations and contribute to development of asthma in high-risk

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young children with susceptible genetic background [4–6]. The ties between respiratory viral infections in early life and the development of asthma in childhood have been the subject of many papers and reviews [7–9].

Respiratory tract infections (RTIs) represent one of the most common and important causes of human disease. There is a significant correlation between acute asthmatic exacerbations and recurrent respiratory tract infections (rRTI). There is also a significant correlation between development of asthma and recurrent respiratory tract infections (rRTI) [10–12]. rRTI in both upper and lower respiratory tracts are caused by a wide range of microorganisms. For viral infections, influenza viruses, parainfluenza viruses, respiratory syncytial virus, adenovirus, and rhinoviruses are the common causes of the disease. This could be superimposed by bacterial infection, including *Acinetobacter* spp., *Chlamydia pneumoniae*, *Enterobacteriaceae*, *Haemophilus influenzae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Nocardia asteroides*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (group A). Currently, primary prevention of asthma is not possible. However, control of asthma severity may be achieved and maintained with appropriate treatment in most children.

Several immunostimulants, including herbal extracts, bacterial extracts, and synthetic compounds, have been used to enhance the immune defenses of the respiratory tract. Since the 1970s, when the concepts of bacteria immunomodulators began, various products were developed and accepted mostly as supplementary treatment to patients who suffer from recurrent respiratory tract infections. Bacterial lysates are constituted of a mixture of bacterial antigens derived from different bacterial species that can vary depending on the formulation. OM-85 preparation contains lysates of eight bacterial pathogens, the most common microorganisms in respiratory tract infections: *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Klebsiella ozenae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus viridans*, *Streptococcus pyogenes*, and *Neisseria catarrhalis*. Each extract is prepared with billions of these bacterial antigens, which are obtained following a mass culture of reference bacterial strains, using an alkaline lysis method. OM-85 has been shown to increase the efficiency of immune system response, via both innate and adaptive immunity [13]. Previous studies have demonstrated that application of bacteria and their by-products can prevent development of allergic diseases. Bacterial lysate attenuates experimental food allergy by reducing allergen-specific IgE and IgG [14]. Also bacterial lysate can improve clinical response in atopic dermatitis patients by decreasing IL-4 cytokine

expression [15]. These responses are similar to the immune response to childhood asthma, which is also associated with antigen-specific CD4 T cell IL-4 production characteristic of a Th2 bias. It is from recent years that bacterial lysate was used as a combined treatment in asthma [16]. In this study, we have investigated whether bacterial lysates (OM-85) will improve symptoms of asthmatic mice via modulation of the immune response.

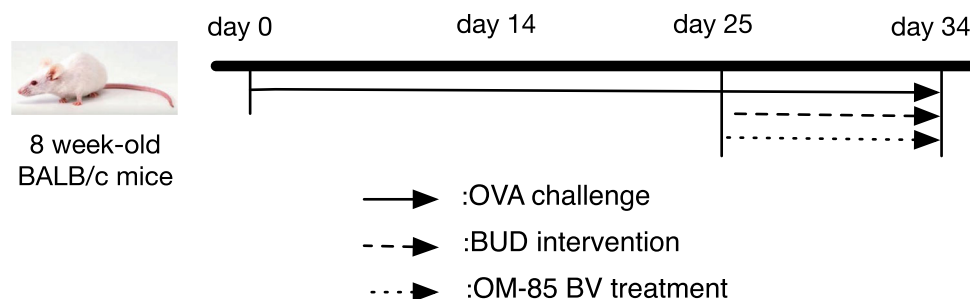
Methods

Mice and Preparation of an Asthma Model

Female BALB/c mice, 8 weeks old and weighing 23 ± 3.3 g, were purchased from the Experimental Animal Central South University (Changsha, China). This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The Institutional Animal Ethics Committee of Central South University XiangYa hospital approved experimental protocols.

BALB/c mice were divided into four groups (8 mice per group). Group A (negative control) mice were sensitized and challenged with normal saline. Group B (asthma control) mice were sensitized with ovalbumin (OVA) and aluminum hydroxide (Al (OH) 3), and challenged with OVA. Group C (BUD) mice were sensitized with OVA and Al (OH) 3, and aerosol inhalation budesonide suspension. Group D (BUD+OM-85) mice were sensitized with OVA and Al (OH)3, challenged with OVA, aerosol inhalation budesonide suspension, and 3.5 mg bacterial lysates intragastric administration.

OVA sensitization and challenge of BALB/c mice were performed as described previously with minor modifications [17]. Briefly, the BALB/c mice were sensitized by i.p. Injections of 100 µg of OVA (Sigma, Saint Louis, MO) and 10 mg of Al (OH) 3 suspended in 0.5 ml normal saline on day 0 and 14. On day 14, the BALB/c mice were challenged with 2% OVA aerosol. On days 25–34, the BALB/c mice were challenged with 1% OVA aerosol for 30 min each day to construct an asthmatic mouse model. On days 25–34, 1 mg BUD aerosol was inhaled by OVA-challenged mice 30 min before OVA challenge to construct the BUD intervention asthmatic mouse model. On days 25–34, 3.5 mg bacterial lysates and 1 ml saline were intragastrically administered by BUD intervention asthmatic mouse model 30 min before OVA challenge to construct the BUD+OM-85 intervention asthmatic mouse model (Fig. 1). An equal quantity of normal saline was administered to asthmatic and control mice. Control mice

Fig. 1 Protocol for experiment

were sensitized to normal saline and challenged with normal saline aerosols.

Preparation of BALF

Mice were anesthetized. Right lungs were lavaged four times with 1 ml of 37 °C normal saline using a 22-gauge needle inserted into the trachea. Cells in BAL fluid were washed with PBS (2000 r/min, 10 min, 4 °C), and the supernatant of the BAL fluid was stored at −70 °C for cytokine analysis. Cells were re-suspended in 150 µl of PBS. The total cell numbers in a 50 µl aliquot were determined with a hemocytometer. Eosinophils were identified based on standard morphology. At least 100 cells were counted and the absolute number of eosinophil was calculated.

Analysis of Lung Pathological Change

Twenty-four hours after the last challenge, mice were sacrificed and left lungs were distended by injecting 1 ml formalin (4% formaldehyde solution in PBS). Tissues were embedded in paraffin and cut into 3-µm-thick sections.

Lung sections were stained with hematoxylin and eosin (H&E) and examined under light microscopy. Bronchial inflammation was performed as previously described and modified [18]. The numerical scores for each view field were determined as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep. Every bronchus was measured on multiple lung lobes from 3 different depths of sectioned tissue. Each histology analysis was performed on 12 animals per treatment group.

Measurements of Cytokines

The levels of the cytokines interferon gamma (IFN-γ) and IL-4 were quantified in the supernatants of BALF by enzyme-linked immunosorbent assay (ELISA, Wuhan Boster Biological Technology, LTD. China). Supernatants

were analyzed by Multiplex bead array according to the manufacturer's protocol.

Statistical Analysis

Data were presented as mean ± SD in the text and figures. One-way ANOVA was used to detect differences between groups using SPSS13.0, and statistical significance was accepted at a *p* value. Group comparisons were made with the Kruskal–Wallis test; non-parametric data comparisons between groups were made with the Mann–Whitney U test, and unpaired *t* tests were used for parametric data.

Results

Effect of BUD+OM-85 on OVA-Induced Airway Inflammation

After the last OVA challenges, lung tissue was collected. Lung sections were stained with hematoxylin and eosin (H&E). The results showed that in comparison to the control PBS challenges, bronchial inflammation luminal stenosis, mucus plugs were found in the OVA-challenged mice. Meanwhile, there was a significant increase in cell infiltration in the peribronchiolar and perivascular connective tissues in the OVA-challenged group, including eosinophil, lymphocyte, monocyte, and polymorphonuclear cells. Among these infiltrated inflammatory cells, eosinophils were primary. After treatment with BUD, there was a significant decrease in bronchial inflammation. However, the BUD + OM-85 asthmatic mice group showed a marked improvement in bronchial inflammation (Fig. 2).

Effect of BUD+OM-85 on cytokine levels in BALF

There is clear evidence that BUD has a positive role in human and murine asthmatic models. Evidence suggests that asthma is caused by imbalance of Th2 and Th1 immune response and that some cytokines, such as IL-4 and IFN-γ, are involved in the asthmatic models [24, 25].

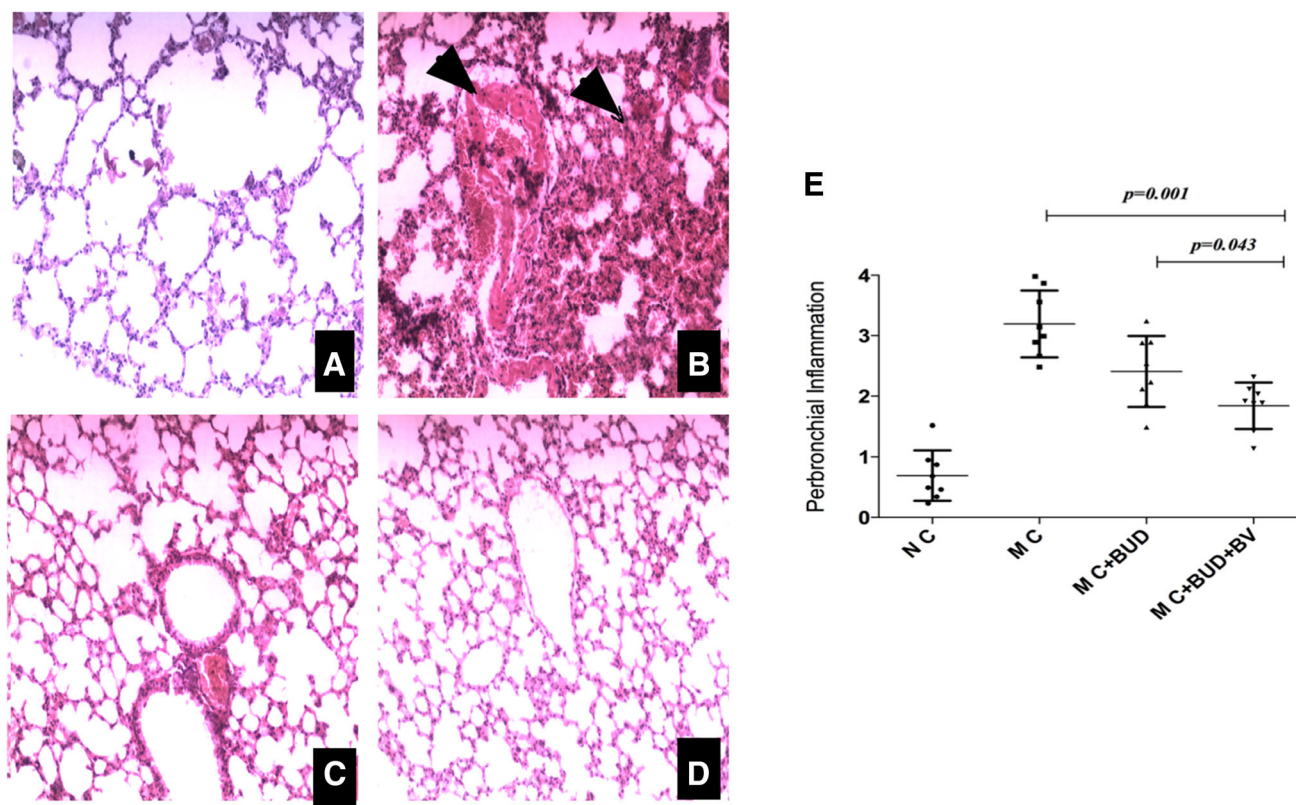


Fig. 2 BV decreased allergic airway inflammation in asthmatic mice models by HE stainings. **a** Representative histological results of pulmonary tissues in negative control mice. **b** Representative histological results of pulmonary tissues in asthmatic control mice. Bronchial inflammation, luminal stenosis, and mucus plugs were found. **c** Representative histological results of pulmonary tissues in

BUD-treated asthmatic mice. **d** Representative histological results of pulmonary tissues in BUD- and BV-treated asthmatic mice. **e** Peribronchiolar and perivascular inflammation. Data of individual mice are shown; medians are indicated as **bold lines**. Statistical analysis was conducted for the comparison between groups of mice. (magnification $\times 100$)

Thus, IL-4 and IFN- γ levels in BALF were measured using ELISA. OVA-challenged mice induced substantial IL-4 and decreased IFN- γ expression in BALF compared with the control group. After treatment with BUD, there was a significant decrease of IL-4 levels in BALF and a significant increase of IFN- γ levels. Treatment with BUD+OM-85 significantly enhanced the change of IL-4 and IFN- γ levels in BALF ($p < 0.05$)(Fig. 3).

BUD+OM-85 Decreases Airway Eosinophil Infiltration in OVA-Induced Asthma Mice

The eosinophil cell levels in the bronchoalveolar lavage fluid were significantly elevated in OVA-challenged mice versus control mice. BUD decreased the eosinophil number, as compared with the OVA-challenged mice. BUD+OM-85 further reduces the number of infiltrating eosinophil ($p < 0.05$) (Fig. 4). Compared with control group, the total cell numbers in the bronchoalveolar lavage fluid were significantly elevated after OVA challenge. However, there was no significant difference among OVA-

challenged group, BUD group, and BUD+OM-85 group (data not shown).

Discussion

Asthma is a complex multifactorial disease with vast heterogeneity in pathogenesis and severity [19, 20]. The different phenotypes of asthma have different immunological and pathological features. Mild asthma is characterized by chronic inflammation of the airways that is mostly eosinophilia in nature and allergic sensitization, and might be caused by immune response toward environmental antigens and leads to a T-helper type 2(Th2)-biased immune response [21]. As well as a Th2 immune response, neutrophil accumulation in the bronchial mucosa is an important feature of severe asthma and frequently includes a T-helper type 1(Th1) component [22]. Additionally, children with eosinophilia asthma can have bacterial bronchitis or viral infection of the lower respiratory airway. Children with asthma can also progress to a more severe neutrophil asthmatic phenotype. This heterogeneity

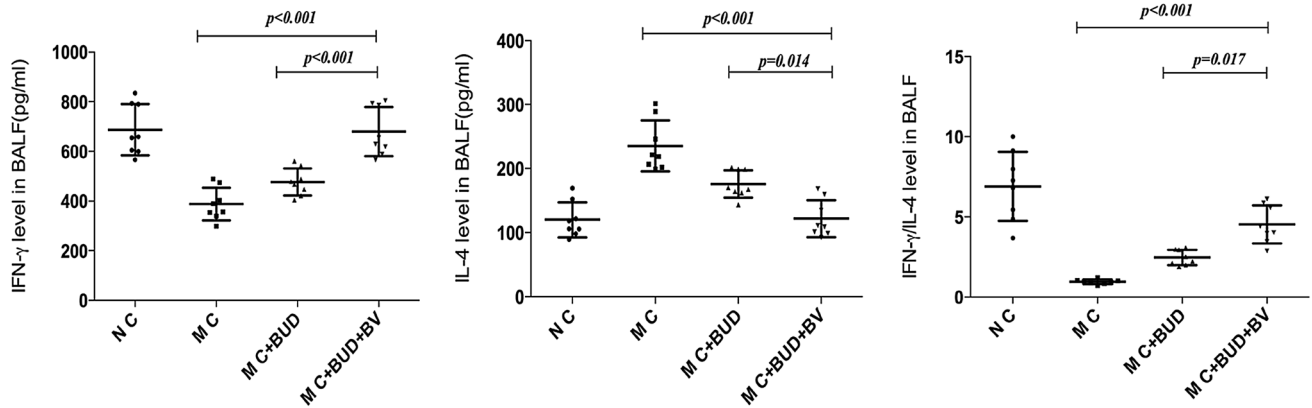


Fig. 3 Cytokine levels in BALF of asthmatic mice models (who had been treated by different protocol). The levels of IFN- γ , IL-4 in BALF of asthmatic mice models were examined by ELISA. Oral administration of BV significantly decreased the levels of IL-4, but increased the levels of IFN- γ . IFN- γ /IL-4 levels significantly

increased in BALF of asthmatic mice models in MC+BUD+BV group ($n = 8$). Each sample was tested for 3 times and averaged; all values were expressed as mean $\pm$ SD. (NC negative control, MC asthma control, MC+BUD asthma control+BUD, MC+BUD+BV asthma control + BUD + OM-85 BV

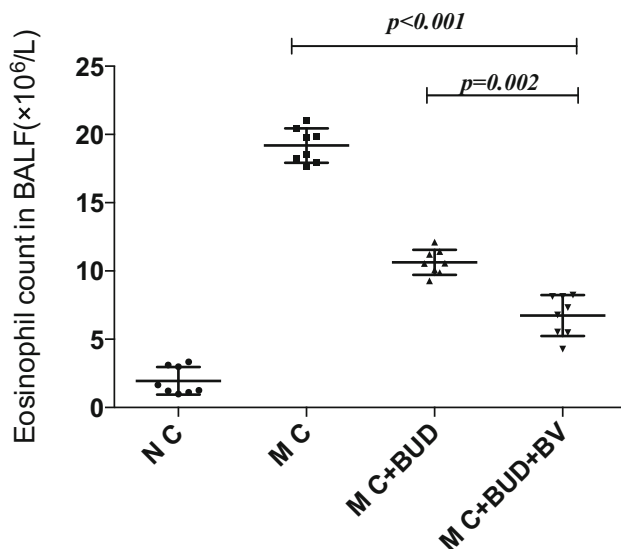


Fig. 4 BV decreased infiltrated Eosinophil in BALF of asthmatic mice models. Each sample was tested for three times and averaged; all values were expressed as mean $\pm$ SD

highlights the importance of more specific treatment approaches based on asthma pathogenesis.

Inhaled glucocorticoid is an accepted first-line therapy for asthma in adults and children. Therefore, we used BUD as a baseline treatment in this study. However, the use of glucocorticoids is limited due to the presence of corticosteroid resistance and potentially severe side effects in some patients, especially in children. So it is necessary to explore better treatment options in this disease.

Recently, significant progress has been made to analyze and understand the myriad of cytokines, chemokines, lipid mediators, and signaling cascades underlying asthmatic pathology. Given the numerous mediators that may play a

role in asthma, targeting a single cytokine or chemokine is unlikely to provide significant and prolonged clinical assistance [23–25].

Infection, including viruses and bacteria, is a usual cause of asthma exacerbation. RSV infection can lead to lung epithelium damage and release of a number of immunological factors. In children, RSV-induced severe respiratory tract inflammation is associated with a Th2-predominant immune response or a decreased Th1 immune response [26]. These mechanisms are associated with asthma [27, 28]. The potentiation of both specific and non-specific immune response has been considered one of a central role in the treatment of recurrences in respiratory tract infections. Specific immunity against viruses (such as influenza virus) and bacteria (*Streptococcus pneumoniae*) can be achieved with vaccination.

The use of bacterial lysate, as both innate and adaptive immunostimulating agent, has demonstrated prevention and treatment of respiratory infections and has been advocated for asthma treatment [29]. OM-85 is a typical bacterial immunostimulant that is extracted from eight common bacterial pathogens of the respiratory tract infection. It is more than 20 years long since OM-85 has been used [30]. A previous study indicated that OM-85 could reduce the number of acute respiratory infections by 25–50% compared with placebo in children with a history of rRTIs [31]. After treatment with OM-85, Children between one and six years of age with recurrent wheezing had a 40% reduction in the rates of viral-induced wheezing over the subsequent 12 months [32]. Moreover, the duration of each wheezing attack was two days shorter in the group given OM-85.

Previous studies reported that OM-85 could enhance both innate immunity and adaptive immune response by

influencing macrophage, neutrophils activity, pro-inflammatory cytokines production, lymphocytes regulation, and immunoglobulin synthesis [33]. In vitro, OM-85 effectively up-regulates oxidative metabolism and nitric oxide production by macrophages. In vivo, OM-85 boosted pathogen destruction by increasing macrophage activation and natural killer cell migration, activity, and antigen recognition. OM-85 also stimulated the expression of pro-inflammatory cytokines in macrophages and monocyte cells, therefore affecting indirectly natural and acquired immunity by stimulation of T and B-lymphocytes, granulocyte migration, and macrophage phagocytic activity. Expression of adhesion molecules, circulation monocytes and granulocytes-IFA-1, MAC-1 and protein p150 also is increased by OM-85.

That Th2-type- and Th1-type-biased immune response could be caused by environmental antigens exposure is an important feature of severe asthma [17, 19, 20]. In the past ten years, several studies explored mechanism of immunotherapy in asthma. OM-85 BV reduced airway inflammation by decreasing the numbers of eosinophils, neutrophils, and lymphocytes in BALF [34–36]; decreasing the levels of IL-1 β , IL-4, IL-5, and TGF- β 1; and increasing the levels of IFN- γ and IL-10 in BALF. In these studies, OM-85 BV treatment was enforced either before asthmatic model establishment or along with asthmatic model establishment. In our study, we strived to explore the effect of OM-85 BV after asthmatic model establishment, which is obviously closer to actual clinical situation. We studied the combined effect of OM-85 and BUD on the asthma mice model too. After all, BUD is the first-line therapy for asthma. Our data showed significant decrease in IL-4 level and increase in IFN- γ level after combined treatment of OM-85 and BUD, which indicated that OM-85 might modulate the bias of Th2-type and Th1-type immune response in asthma mice model. Eosinophil infiltration was also decreased in asthma model by OM-85 and BUD treatment. Parallel to its effect on the cellular response, the asthma model lungs, including airway wall thickness, luminal stenosis, and mucus plug formation, was improved after OM-85 and BUD treatment. However, a significant limitation in our study is that 8-week-old mice model were used; therefore, results in this study mainly showed the effect of OM-85 in adult asthma mice model. Our further studies using mice model mimic childhood asthma are warranted to explore the immunology effect of OM-85 in childhood asthma.

In conclusion, we have shown that oral administration of OM-85 reduced lower airway inflammation and decreased eosinophil infiltration in asthmatic mice. The imbalance of Th2-type and Th1-type immune response in asthma mice model was also modulated. These data suggest that oral administration of OM-85 could potentially be effective in

assisting treatment of asthma. Further studies on the underlying mechanism of action will contribute to the continued development and improvement of strategies for the prevention and treatment of acute asthma attacks.

Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This research involved animals. The study was approved by the Hospital Ethics Committee.

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