Research Article

Achyranthes bidentata Polysaccharide along with Anti-IL-5 Antibody Inhibits Allergic Lung Inflammation and Airway Hyperresponsiveness Mice Induced by House Dust Mites

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Bronchial asthma is a chronic inflammatory disease which has become prevalent worldwide. There are millions of new patients and thousands of people die from asthma every year [2–4]. The number is also predicted to reach about 400 million over in the next decades [5].

Asthma is characterized by eosinophils infiltration into the pulmonary parenchyma and airways [6]. The products of eosinophils are considered as negative regulators that work on remodeling lung tissue, including airway thickening, fibrosis, and angiogenesis. Achyranthes bidentata polysaccharide (ABPS) is a kind of polysaccharide extracted from the dry root of Achyranthes plant Achyranthes bidentata. ABPS is reported to have multiple biological functions and acts on the human immune system. ABPS can induce production of TNF-α in macrophage, enhance the killing ability of NK cells, and promote the proliferation of B cells. Besides this, ABPS is reported that can induce apoptosis of eosinophils by upregulating the expression of proteins involved in apoptosis. In this study, we constructed chronic allergic asthma mice model induced by house dust mites (HDM) with airway hyperresponsiveness (AHR) and found that anti-IL-5 mAb and ABPS treatment can both decrease inflammatory cells infiltration especially eosinophils and decrease the level of serum IgE and HDM-specific IgG1. The level of IFN-γ is increased and AHR is improved, and a more significant phenomenon was observed in anti-IL-5 mAb and ABPS combined treatment.

1. Introduction

Asthma is characterized by eosinophil infiltration into the pulmonary parenchyma and airways [6]. The products of eosinophils are considered as negative regulators that work on remodeling lung tissue, including airway thickening, fibrosis, and angiogenesis, and asthma exacerbate [7, 8]. It is proved that asthma is a complex and heterogeneous disease which can be subdivided into several phenotypes due to clinical, physiological, and inflammatory markers [9, 10]. Eosinophilic asthma is one of these phenotypes characterized by increased serum or sputum eosinophils, which means the number of eosinophils correlates with disease severity [11]. Usually, patients with severe asthma and refractory eosinophilic phenotype will suffer frequent asthma exacerbations.

Inhaled glucocorticoids (GCs) were typically the gold-standard treatment to asthma patients because of their suppression of inflammatory mechanisms in parallel and being able to reduce AHR and degree of disease activity [12]. However, the side effects of GCs have been...
well documented, GCs require lifetime therapy for most of the asthma patients whose symptoms usually return if GCs are withdrawn [5]. Meanwhile, another common concern for GCs therapy is the various clinical response in the patients with asthma [13].

Current biological treatment strategies focused on specific targeting of Th2-associated cytokines [14]. Recent research revealed that periostin could be a novel biomarker for bronchial asthma in downstream of IL-13 signaling [15]. Also, it is well accepted that Th2-derived interleukin-5 (IL-5) plays a key role in developing and maturing of eosinophils, enhancing their adhesion to endothelial cells lining the postcapillary venules, activating, secreting, and prolonging survival through apoptosis inhibition in the tissues [16]. It has been well-known that IL-5 is upregulated within the Airways of both atopic and nonatopic asthmatic patients and that the expression of IL-5 negative related with pulmonary function [17, 18]. Therefore, IL-5 was considered a promising target to treat eosinophil-mediated inflammation [19–21]. Previous study showed dietary supplementation with the ABPS greatly increased the peripheral lymphocyte proliferation in a concentration-dependent manner in piglets. The main effect of ABPS was focused on strengthening muscles and bones remodeling. Pharmacological findings suggested ABPS is reported as an effective inhibitor of Th1/Th2 dysregulation; therefore, we considered ABPS a promising strategy to treat eosinophil-mediated inflammation in asthma.

Murine models are often used to study the delineation mechanisms and critical mediators of asthma with conflicting results which suggested that the dominant mechanism and mediators required for asthma induction differ on the use of model and the method of allergen sensitization [6]. Though animal models induced by ovalbumin (OVA) have been used in most studies, there are difficulties in evaluating recurrence and maintaining chronic inflammation [1]. And the study previously confirmed that when more closely resembles ambient allergen exposure in human subjects used in a murine model, the IgE will act as a more critical role in the pathogenesis of allergic asthma and mucosa pathology [6]. House dust mites (HDM) allergens are considered as the most important risk factor for the development of allergic diseases [22], and the chronic murine HDMs model has been considered as an improved preclinical in vivo model to assess the efficacy and mechanism of action of potential novel therapeutics for asthmatic disease which was recently shown to be effective in response to anti-interleukin-13 (anti-IL-13) treatment [23].

Here, in an HDM-induced chronic asthma murine model of lung inflammation, we assessed the effect of anti-IL-5 mAb and ABPS treatment, respectively, or combined in asthma. We analyzed the change of inflammatory cells especially eosinophils and cytokines in BALF as well as the change of IgE and HDM-specific IgG1 levels in serum.

2. Materials and Methods

2.1. Materials. Extraction of ABPS: take 50 g dry roots of plant *Achyranthes bidentata*, grind it into powder, and extract it with 10 volumes of 75% ethanol; then recover ethanol and sediment is obtained by lyophilization. The residue was extracted with 10 volumes of water, then concentrated, and finally added with 95% ethanol to precipitate. The precipitate obtained is dissolved in water and trichloroacetic acid is added to remove the protein and the supernatant is taken after centrifugation. The supernatant was added with 95% ethanol to obtain a precipitate. The precipitate was washed successively with absolute ethanol, acetone, and ether and freeze-dried to obtain a crude product of *Achyranthes polysaccharide*. The extracted Acetyltalatisamine Achyranth was reddish-brown powder. After acid hydrolysis, the ratio of hydrolysatse to fructose and glucose was determined by high performance liquid chromatography (HPLC).

2.2. Animals. Female C57BL/6 mice of SPF clean grade (6–8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., LTD. All animals were held in regular 12 h dark/light cycles at 25°C and were bred and maintained in the high-efficiency particulate air filter-exhausted ventilated cages. The mice received food and water ad libitum. All the animal procedures were conducted in concordance with the Institutional Ethical Committees for Use of Experimental Animals.

2.3. Allergen Sensitization of Murine Models. Fifty C57BL/6 mice were randomly divided into five groups, as control group, asthma group, anti-IL-5 mAb treated group, ABPS treated group, and anti-IL-5 mAb-ABPS combined group. Mice were sensitized to house dust mite (HDM) extracts (Greer, Lenoir, NC, USA) by subcutaneous injections of 50 μg HDM adsorbed to 2 mg of alum [Al(OH)₃] gel (Sigma Chemical Co., St. Louis, MO, USA) on days 0 and 7 and challenged intranasally with 25 μg of HDM in 50 μL of PBS for 4 consecutive days a week for 3 weeks. Controls received only alum and were challenged with saline (0.9% NaCl) instead of HDM. Necropsy was performed 24 h after the final allergen challenge.

2.4. Medicine Treatment. To assess the different effect of medicine in chronic allergic murine asthma model, different therapeutic agents were dosing to corresponding groups: anti-IL-5 mAb group were treated with 100 μg of rat anti-mouse IL-5 mAb (Abcam Shanghai Trade Co., LTD, Shanghai, China) 2 h before the first intranasal HDM application and thrice weekly before necropsy; ABPS group were treated with follow operations: after the second sensitization of HDM, intraperitoneal injection of 70 mg/kg per day, and in sensitized phase, ABPS were administered intraperitoneally 1 h prior until the mice were sacrificed.; anti-IL-5 mAb-APBS combined group were treated with both of this two organics.

2.5. Assess of Airway Hyperresponsiveness to Methacholine. Airway hyperresponsiveness (AHR) of mice to methacholine was measured before necropsy. Mice were placed in
2.6. Bronchoalveolar Lavage Fluid Collection and Cell Counting. Before dissection, the bronchoalveolar lavage fluid (BALF) was performed by twice instillation of 0.8 mL ice-cold PBS then centrifuged (1500 rpm, 5 min, 4°C). The supernatant was immediately aliquoted and stored at −70°C for subsequent measurement of cytokine content. The precipitation was resuspended in 0.5 mL of PBS, and the total number of cell was counted by Neubauer chamber. 100 cells in cytospin were prepared and stained by Rosenfeld’s stain to evaluate differential cell counts.

2.7. Lung Histology. After the collection of BALF, the left lung lobe was immersed in 10% phosphate-buffered formalin for 24 h, followed by 70% ethanol, and embedded in paraffin. Tissues sections of 5 μm were then stained with H&E (haematoxylin and eosin) to assess histopathological changes. The slides were manually examined under a light microscope ×100.

2.8. Cytokine and Immunoglobulin Assays. The concentration of IFN-γ (R&D system, Minneapolis, MN, USA) in the BALF and serum was measured by ELISA kits. The minimum detectable concentration was 2 pg/mL.

Serum IgE levels were detected by Mouse IgE ELISA kits (Becton, Dickinson and Company, New Jersey, USA) and manipulated as follows: 100 μL of rat anti-mouse IgE monoclonal antibody were added to each well, plated, and incubated at 4°C overnight. After being washed and blocked with PBS containing 1% skim milk mouse sera were incubated overnight at 4°C, and antibody binding was assessed by the addition of 100 μL of biotinylated anti-mouse IgE detection mAb and streptavidin-horseradish peroxidase conjugate (SAv-HRP) incubated at 37°C for 1 h. After the addition of the enzyme substrate, plates were read at 450 nm in an ELISA reader (Bio-Rad, Richmond, CA, USA). The purified mouse IgE antibody was used for the total IgE standard. The minimum detectable concentration was 2 ng/mL.

HDM specific IgG1 was measured by ELISA kit. HDM was coated overnight at 4°C with 100 μL of 5 μg/mL HDM in carbonate buffer (0.05 mol/L, pH 9.6) and by detecting HDM specific IgG1 binding with biotinylated rat anti-mouse IgG1 detection mAb and SAv-HRP. The other procedures are the same with detection serum IgE.

2.9. Flow Cytometry Assays. Centrifuge the BALF fluid and resuspend cells into cell suspension. Loading Percoll in different density (1.085, 1.080, 1.075, and 1.070) to a centrifuge tube. Pipetting the cell suspension under Percoll carefully and centrifuging (1500 rp, 25 min at room temperature). Eosinophils were loaded between 1.075 and 1.070 density ladder. Wash eosinophils with PBS twice and resuspend with binding buffer, dyeing with Annexin-V in the dark for 15 min; PI was added before flowing.

2.10. Statistics. The differences among the groups were analyzed using InStat 2.01 software (GraphPad, San Diego, CA, USA). Data are given as means + SEM for normally distributed. For comparison, multiple groups ANOVA or Kruskal–Wallis with parametric (Bonferroni) or nonparametric (Dunn’s) posttest, respectively, was performed. For all tests, P values less than 0.05 were considered significant.

3. Results

3.1. Extraction and Component Analysis of Acetyltalatisamine Achyranthan. After repeated extraction and precipitation, reddish-brown powder is obtained. After acid hydrolysis, a series of preparations are performed to detect the ratio of fructose to glucose by HPLC. Fructose was separated at about 13th minute (13.27th minute) and glucose was separated at one minute later (14.11th minute). The ratio of fructose and glucose was about 4 to 1 (fructose to glucose).

3.2. Effect of Therapeutic Agent on Airways Hyperresponsiveness. In order to investigate the effect of therapeutic agent on AHR in our murine chronic allergic inflammation model, plethysmography was conducted to detect changes in respiratory dynamics following exposure to methacholine with a concentration of 6.25 mg/mL. The results showed an increased sensitivity to methacholine challenge after HDM exposure. And Penh is significantly higher than the control group (P < 0.01) (Figure 2). And the AHR to methacholine was significantly decreased with the treatment of anti-IL-5 mAb (P < 0.05) and ABPS (P < 0.05), or the combined treatment group (P < 0.01) (Figure 1).

3.3. Effect of Anti-IL-5 mAb and ABPS Combined Treatment on Pulmonary Inflammation. The total inflammatory cells,
Figure 2: Effect of anti-IL-5 mAb and ABPS treatment on hyperresponsiveness induced by methacholine. Airway response of control, asthma group, or therapy group to methacholine (24 h after exposure to HDM). Data was presented as mean Penh ± SEM and was from one experiment, with n = 10 mice per group. ** P < 0.01, *** P < 0.001, compared to the control group. θ P < 0.05, θθ P < 0.01, compared to the asthma group. The significance of the data was evaluated by one-way ANOVA with Bonferroni’s multiple comparison test compared to control.

eosinophils, neutrophils, mononuclear/macrophages, and lymphocytes in BALF fluid was significantly elevated in HDM-challenge mice versus saline control mice as shown in Table 1 (versus control, P < 0.001, P < 0.01, P < 0.001, P < 0.01, P < 0.001, P < 0.01, separately). Anti-IL-5 mAb or ABPS treatment can significantly decrease the number of total cells, eosinophils, neutrophils, and lymphocytes (versus asthma group, P < 0.01, P < 0.001, P < 0.01, P < 0.01, P < 0.01, P < 0.01, separately under anti-IL-5 mAb group. P < 0.05, P < 0.01, P < 0.05, P < 0.05, P < 0.05, separately under ABPS group), especially in the combined group (versus asthma group, P < 0.05, P < 0.001, P > 0.05*, P < 0.01, separately).

Inhibition of lung inflammation infiltration by anti-IL-5 mAb and ABPS treatment, respectively, or combined was also confirmed by histology examination. The results showed the inflammatory cells infiltration into peribronchial and perivascular areas in asthma group as compared to the control using H&E staining. Treatment of anti-IL-5 mAb and ABPS can both reduce the magnitude of inflammatory cells infiltration into the peribronchial and perivascular connective tissues as compared to asthma group (Figure 3), and this phenomenon is more significant in combined treatment group (Figure 3).

3.5. Effect of Anti-IL-5 mAb and ABPS Combined Treatment on Serum IgE and HDM-Specific IgG1. Usually, Th2 cell immune responses are associated with the production of IgG1 and IgE antibody. The results of the assays showed that exposure to HDM resulted in the increase generation of HDM-specific IgG1 and IgE in serum (P < 0.001). And the treatment of anti-IL-5 mAb or ABPS can decrease the levels of both serum IgE and HDM-specific IgG1 (P < 0.01, P < 0.05 in anti-IL-5 mAb group, P < 0.05, P < 0.05 in ABPS group), similar before, anti-IL-5 mAb and ABPS combined group has significantly reduced on the levels of both serum IgE and HDM-specific IgG1 (P < 0.001, P < 0.01) (Figure 5).

3.6. Effect of Anti-IL-5 mAb and ABPS Combined Treatment on Apoptosis. To identify the function of inducing apoptosis of anti-IL-5 mAb and ABPS combined treatment, the proportion of eosinophils derived from BALF was analyzed. As the results, the proportion of eosinophils from asthma group was higher than control group (P < 0.01). After treatment, the proportion of eosinophils from treatment group was markedly elevated (P < 0.01), especially in anti-IL-5 mAb and ABPS combined treatment group (Figure 6).

4. Discussion

Animal models were an invaluable tool in translational research, especially mouse models with a large selection of specific reagents and techniques which have made major contributions to the understanding of asthma pathophysiology and the main characters of the disease [24]. The increasing focus on asthma research is severe asthma because of unmet medical need. And the current findings emphasise the necessity of developing reproducible asthma exacerbation models to provide the opportunities for pharmacological intervention studies.

OVA is the classical allergen for asthma models because of its low costs, useless and well-known immunological characteristics [22], and being able to produce an airway inflammation model exhibiting plentiful human asthma-like cellular and pathophysiological features. However, there is an increasing concern about the prolonged exposure to OVA by inducing tolerance in the animals [25]. Additionally, in human subjects, a big proportion of asthma is due to aeroallergens, and OVA-induced asthma is far from being a common event. Therefore, asthma animal models sensitized by common allergens might be more relevant tools to the study of human asthma [26]. To date, several kinds of allergen extracts or purified proteins which are more clinically relevant have become useful in animal models such as fungi, HDMs (i.e., Dermatophagoides farina (Der f) and Dermatophagoides pteronyssinus (Der p)), cockroach, ragweed, and pollen spores [27–31]. HDMs are one of the most common allergens in asthma patients. 50–85% asthma patients are typically allergic to HDMs with increased levels of HDM-specific IgE [32]. The exposure to HDM can overcome the
limitation of prolonged exposure to OVA models of tolerogenic response and allow us to investigate changes related to chronic, persistent allergen exposure. Remodeling of the airways with increased mucous cell density and AHR can be constructed by prolonged exposure to HDM and remains elevated for a while after discontinuation of HDM exposure [33]. Meanwhile, it is well known that chronic exposure to inhaled allergens can induce an inflammatory response in the lung leading to clinical symptoms of asthma [10]. Therefore, in this article we choose the HDM extracts to induce the chronic allergic asthma model in order to mimic the clinical process of human subjects better.

According to our data, we successfully constructed the chronic allergic lung inflammation model induced by HDM with a significant increase of inflammatory cells especially eosinophils, AHR, serum total IgE, and HDM-specific IgG1 and the decrease of IFN-γ, which verifies that chronic exposure to HDM is able to cause cellular infiltration of the airways by immune and inflammatory cells [10] and lays foundation for the research of anti-IL-5 mAb treatment of eosinophilic asthma.

Although, GCs are the gold-standard treatment for asthma. It still remains some disadvantage for those patients with GC-refractory eosinophilic asthma and requires unmet medical need. Given that airway inflammation is the key to asthma pathogenesis, new therapies targeting key cells and mediators that drive the inflammatory responses in the asthmatic lung is becoming hotspot [11]. Allergic inflammation is well marked by prominent infiltration of eosinophils and Th2 lymphocytes. It has been reported that IL-5 plays an important role for the regulation of eosinophil maturation, recruitment, survival, and their release into the blood, and eosinophils are a prominent feature in the pulmonary inflammation which is associated with allergic asthma. Thus, IL-5 has been proposed to be a potential molecular target in the treatment of asthma [34]. Similarly, there are reports which proved that ABPS also effectively works on the inhibition of dysregulation of Th1/Th2 in asthma. ABPS was

<table>
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<th>Group</th>
<th>n</th>
<th>Total cells (×10⁴/mL)</th>
<th>Eosinophils (×10⁴/mL)</th>
<th>Neutrophils (×10⁴/mL)</th>
<th>Mononuclear/macrophages (×10⁴/mL)</th>
<th>Lymphocytes (×10⁴/mL)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>4.261 ± 0.689</td>
<td>0.2052 ± 0.0319</td>
<td>1.7502 ± 0.1862</td>
<td>0.3782 ± 0.0322</td>
<td>2.8912 ± 0.2381</td>
</tr>
<tr>
<td>Asthma</td>
<td>10</td>
<td>19.35 ± 3.802***</td>
<td>8.9862 ± 1.0763***</td>
<td>4.2198 ± 0.7621**</td>
<td>4.2781 ± 0.5621***</td>
<td>7.2671 ± 1.9761**</td>
</tr>
<tr>
<td>ABPS</td>
<td>10</td>
<td>9.78 ± 3.66*</td>
<td>3.543 ± 0.856**</td>
<td>2.933 ± 0.659*</td>
<td>2.045 ± 0.4533*</td>
<td>6.0941 ± 1.543*</td>
</tr>
<tr>
<td>IL-5 Ab</td>
<td>10</td>
<td>11.44 ± 3.04**</td>
<td>2.76 ± 0.55***</td>
<td>3.37 ± 0.544**</td>
<td>3.04 ± 0.665***</td>
<td>5.4941 ± 1.333**</td>
</tr>
<tr>
<td>Combined</td>
<td>10</td>
<td>7.98 ± 1.5600*</td>
<td>1.6782 ± 0.0963***</td>
<td>2.0672 ± 0.3219</td>
<td>1.0621 ± 0.2875*</td>
<td>4.5912 ± 0.6782**</td>
</tr>
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Data was presented as mean ± SEM and was from one experiment. *P < 0.05; **P < 0.01; ***P < 0.001. The significance of the data was evaluated by one-way ANOVA with Bonferroni’s multiple comparison test compared to control.

Figure 3: Effect of anti-IL-5 mAb and ABPS, respectively, or combined treatment on airway inflammation (H&E, magnification ×100). Exposure to HDM increases airway inflammatory cell infiltration and treatment decreased it, which is more remarkable in the anti-IL-5 mAb and ABPS combined treatment group. (a, b, c, d, e) represents control, asthma group, ABPS group, anti-IL-5 and anti-IL-5 mAb, and ABPS combined group, respectively.
shown to markedly enhance DC maturation and differentiation in vitro through providing extra IL-12 and MHC II and upregulating antigen presentation, leading to activating CD4⁺ T cells [35]. Therefore, we tested the effect of ABPS on asthma and combined with IL-5 antibody in the treatment of asthma. Although this primary studies showed that anti-IL-5 mAb and ABPS treatment can improve disease status in asthma animal models, respectively, it has been proven inadequate in clinical patients with moderate, controlled asthma. Given the knowledge that asthma has several phenotypes, anti-IL-5 mAb and ABPS combined treatment is supposed to be effective in the patients with eosinophilic asthma [36]. In order to identify the hypothesis, we use HDMs to construct a chronic murine asthma model with large number of eosinophils infiltrated and assess the effect of anti-IL-5 mAb and ABPS, respectively, or combined treatment on eosinophilic asthma.

In order to determine the effect of anti-IL-5 mAb and ABPS combined treatment on eosinophilic asthma, we administrate anti-IL-5 mAb and ABPS combined treatment at the time of initial intranasal exposure to HDM three weeks until necropsy which shows dramatical inhibition to the infiltration of the lung tissue and airway by eosinophils. The results showed that anti-IL-5 mAb and ABPS combined treatment was found to reduce HDM-induced chronic asthma responses significantly. Anti-IL-5 mAb and ABPS combined treatment can improve the AHR and reduce inflammatory cell infiltration, especially eosinophils.

Asthma is caused by IgE-mediated hypersensitivity reactions, and the increase of serum IgE is the main character of asthma. Meanwhile, IFN-γ is the typical mediators secreted by Th1 cells which plays an important role in the development of asthma. It has been reported that IL-5 can help IL-4 to promote the production of IgE. When IL-5 and IL-4

**Figure 4:** The levels of IFN-γ in BALF (a) and serum (b) of control, asthma group, anti-IL-5 mAb, and ABPS, respectively, or combined treatment group were detected by ELISA. Data was presented as mean Penh ± SEM and was from one experiment, with n = 10 mice per group. **P < 0.01, ***P < 0.001, compared to the control group. # P < 0.05, ## P < 0.01, ### P < 0.001, compared to the asthma group. And the levels of IFN-γ detected from combined treatment group was significantly higher than individual treatment group. The significance of the data was evaluated by one-way ANOVA with Bonferroni’s multiple comparison test compared to control.

**Figure 5:** Effect of anti-IL-5 mAb and ABPS, respectively, or combined treatment on the levels of IgE and HDM-specific IgG1. Levels of HDM-specific IgG1 (a) and IgE (b) in serum were measured by ELISA data presented as mean ± SEM and are from one experiment, with n = 10 mice per group. ***P < 0.001, compared to the control group. † P < 0.05, ‡ P < 0.01, §§ P < 0.001, compared to the asthma group. The significance of the data was evaluated by one-way ANOVA with Bonferroni’s multiple comparison test compared to control.
In summary, we have shown that anti-IL-5 mAb and ABPS combined treatment results in significantly decreasing of eosinophil infiltration into the lung and improvement of airway function in HDM-induced mouse models of eosinophilic asthma. A combination of anti-IL-5 mAb and ABPS presented excellent therapeutic effects on asthma. These effects correlated with a significant improvement of AHR, a significant reduction of pulmonary inflammatory cells infiltration, an increase of IFN-γ, and a reduction of serum IgE and HDM-specific IgG1, indicating that anti-IL-5 mAb and ABPS therapy promoted Th1 cytokine secretion and suppressed Th2 cytokine secretion thus attenuated the accumulation of eosinophilic. Our study verified that anti-IL-5 mAb and ABPS combined treatment is a promising therapy for the improvement of eosinophilic asthma.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

References


