Autoantibody of Interleukin-17A Induced by Recombinant *Mycobacterium Smegmatis* Attenuates Airway Inflammation in Neutrophilic Asthma

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Research Article

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Abstract

Asthma is a chronic inflammatory disorder, previous studies have shown Interleukin (IL)-17A is an important contributor to the development of severe asthma characterized by intense neutrophilic inflammation and less responsive to corticosteroid. Hence, IL-17A-neutrophil axis may be a potential therapeutic target for asthma. In our previous work, we constructed a recombinant *Mycobacterium smegmatis* expressing fusion protein Ag85A-IL-17A (rMS) and confirmed that it could induce the production of IL-17A autoantibody in vivo. Here, in the present study, we aim to further investigate the effects of the rMS on airway inflammation and explore the underlying mechanisms in a murine model of neutrophilic asthma. The murine model of neutrophilic asthma was established with ovalbumin (OVA), and mice were intranasally vaccinated with rMS, then IL-17A autoantibody in sera was detected, and its anti-inflammatory effects on inflammatory cell infiltration and expression of inflammatory mediators involved in IL-17A-neutrophil axis in bronchoalveolar lavage fluid (BALF) as well as histopathological changes of lung tissues were evaluated. The data showed that sustained high-titer of IL-17A autoantibody was detected in rMS group, intranasal vaccination significantly decreased inflammatory cells, cytokines and chemokines related to IL-17A-neutrophil axis in BALF, suppressed the activity of neutrophil enzyme, and histological analysis manifested the rMS remarkably reduced inflammatory cell infiltration, mucus secretion and airway epithelial thickness. Overall, these results demonstrate the rMS ameliorated airway inflammation in mice with neutrophilic asthma via inducing IL-17A autoantibody and regulating the IL-17A-neutrophil axis, thus providing a possible novel treatment in neutrophilic asthma.

Introduction

Asthma is a chronic inflammatory disease with marked heterogeneity and complicated pathophysiology, affecting millions of individuals worldwide [1]. Research has been propelled forward by innovations stemming from the Th1/Th2 immune dysregulation, providing a molecular framework for understanding eosinophilic airway inflammation in asthma [2]. Th2 cytokines, such as IL-4, IL-5 and IL-13, are known to play a pivotal role in the pathogenesis of asthma, they are critical for eosinophil growth and differentiation and IgE class switching, meanwhile, they participate in airway hyperresponsiveness and mucus hypersecretion in asthma [3, 4]. In addition to Th2 cell, considerable attention has been paid to Th17 cell and increasing evidence demonstrates IL-17A is an important contributor to the development of asthma, particularly to severe asthma, which usually presents intense neutrophilic airway inflammation and less responsive to corticosteroids [5, 6]. Indeed, high levels of IL-17 are found in sputum and BALF of patients with severe asthma, and moreover there is a positive correlation between the level of IL-17A and the severity of disease. In addition, IL-17A has been confirmed to be involved in multiple aspects of asthma pathogenesis, including structural alterations of epithelial cells, inflammatory cell infiltration and smooth muscle contraction [7, 8].

Despite decades of intensive research, only limited progress has been made in asthma treatment since the introduction of inhaled selective β2 adrenoceptor agonists and glucocorticosteroids, therefore inhaled corticosteroids (ICS) still serve as the backbone of current asthma therapy [1, 9]. Glucocorticosteroids are

effective in atopic asthma with eosinophilic inflammation, however, clinical data show that there are still quite a few patients with uncontrolled asthma, and these cases usually manifest severe clinic symptoms as well as elevated level of IL-17A in sputum and neutrophil-predominant airway inflammation [10, 11]. Research has established that neutrophil commonly expresses glucocorticoid receptor (GR) -β in nature rather than GR-α, the former is an isoform of the latter and lacks the glucocorticoid binding domain to interact with the glucocorticoid response element, thus leading to corticosteroid-resistance [12, 13]. Meanwhile, IL-17A is positively related to airway neutrophil infiltration and GR-β expression in epithelial cell [14–16]. Therefore, we speculate that if there is a new therapeutic strategy designed to suppress IL-17A and regulate the IL-17A-neutrophil axis, maybe it could effectively alleviate airway inflammation in asthma, including glucocorticoid-resistant asthma.

In previous study, we have constructed a recombinant *Mycobacterium smegmatis* and confirmed it could induce IL-17A autoantibody in vivo [17, 18]. And this study aims to further explore the protective effects of the rMS on airway inflammation and the underlying mechanisms involved in regulating the IL-17A-neutrophil axis in the murine model of neutrophilic asthma.

Materials And Methods

Animals

Female DO11.10 mice on a BALB/c background at 6–8 weeks of age and weighing 16-18g were obtained from Shanghai Model Organisms Center, Inc., Shanghai, China. Mice were bred and housed in a specified pathogen-free (SPF) laboratory with 12-hour of light-dark cycle. The physiological condition of mice was checked three times a week before OVA sensitization and every day after sensitization. Sodium pentobarbital was used before scarifice in order to minimize suffering and distress. The experiment was approved by Institutional Animal Care and Use Committee of Shanghai Public Health Clinical Center (Permit Number: 2019 − 102).

Preparation of bacteria

Recombinant *Mycobacterium smegmatis* expressing fusion protein Ag85A-IL-17A was constructed in previous experiment [17]. *Mycobacterium smegmatis* (MS) and rMS were both preserved at -80℃ in our laboratory. rMS and MS were recovered and diluted with sterile phosphate buffered saline (PBS) at 5×10^8CFU/ml before immunization.

Protocol of immunization and challenge

Mice were divided randomly into 4 groups of 6 animals (PBS, asthma, rMS and MS) and treated as shown in **Fig. 1**. Briefly, mice in rMS group and MS group under light anesthesia were administered intranasally with rMS or MS at a dose 1.0×10^7 colony-forming unit (CFU) in 20 μl of PBS on days 0, 14 and 28, and mice in asthma group were administered intranasally with an equal volume of PBS. Except of PBS group, all of mice were challenged intranasally with 100 μg ovalbumin (OVA, Grade V, Sigma-

Aldrich) in 20μl PBS on days 35, 36 and 37. Mice in PBS group were administered intranasally with PBS as parallel manipulation. All mice were sacrificed on day 38.

IL-17A autoantibody assay in sera

Blood was allowed to clot at room temperature and centrifuged at 4,000 g for 10 min. Sera IL-17A specific IgG was measured by enzyme-linked immunosorbent assay (ELISA). In brief, Plates were coated with commercial recombinant protein IL-17A (2.5 μg/ml, PeproTech) and antibodies were detected using goat anti-mouse IgG-HRP (1:5,000, Santa Cruz). Substrate solution (eBioscience) was added to each well and incubated for 10 min and reactions were stopped with 2 M H_2SO_4. Data are expressed as mean of optical density value at 450 nm.

Bronchoalveolar lavage collection

The trachea was dissected and cannulated, BALF were prepared by washing the lungs three times with 0.4 ml ice-cold PBS. 100 μl BALF was stored at -80 ^{o}C for myeloperoxidase (MPO) activity assay, the rest was centrifuged, the supernatant was stored for cytokine and chemokine analysis. The number of leukocytes was determined using a hemocytometer and differential cell counts were conducted by using Wright-Giemsa stained cytospin preparations in a blinded manner. At least 100 cells were counted twice and differentiated according to standard morphologic criteria under light microscopy.

Cytokine and chemokine levels in BALF

ELISA was performed according to the manufacturer's instructions. The concentrations of IL-6, IL-23, IL-17A, TNF-α, CXCL-1 and CXCL-2 in BALF were measured using specific mouse ELISA kits (eBioscience).

MPO activity analysis

The activity of MPO in BALF and lung tissues was measured using commercially available MPO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following manufacture's instruction.

Histopathological analysis

After the collection of BALF, pulmonary lobe was dissected, fixed with 4% paraformaldehyde and embedded in paraffin. Lung tissues were sliced into 4 μm thick sections and then were stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS), respectively. Peribronchial inflammatory infiltration was assessed using a semiquantitative score (0–5) by 3 observers independently, and goblet cell hyperplasia with mucus hypersecretion was assessed with integrated optical density (IOD).

Statistical analysis

Data were analyzed using the Prism software (version 8.3.1) and expressed as mean value ± standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed with the Tukey post hoc test. The significance level was defined as $p < 0.05$.

Results

rMS induces high-titter IL-17A autoantibody in sera

To dynamically monitor the expression and titer of IL-17A autoantibody, ELISA was performed and IL-17A protein was used as coating antigen to detect the specific IgG in sera. As shown in **Fig. 2**, sera of mice in PBS group, asthma group and MS group had no IL-17A specific IgG, while mice treated with rMS had high-titter of IgG specific to IL-17A over time.

rMS decreases inflammatory cells in BALF

To investigate the effect of rMS on airway inflammation, total leucocytes and differential cell counts in BALF were calculated. As shown in **Fig. 3**, total leukocyte cells, macrophages and neutrophils in asthma group were obviously elevated compared to PBS group, and these increases were markedly reversed by rMS.

rMS suppresses the expression of cytokines, chemokines and the activity of neutrophil enzyme related to IL-17A-neutrophil axis

In order to further study the effect of rMS on IL-17A-neutrophil axis, we examined IL-17A, IL-6, IL-23, TNF-α, CXCL-1 and CXCL-2 levels and measured the activity of MPO in BALF and lung tissues. As shown in **Fig. 4**, compared to PBS group, cytokines and chemokine and the activity of MPO in BALF and lung tissue were significantly increased in asthma group and these increases were observably declined in rMS group.

rMS reduces airway inflammation infiltration and mucus hypersecretion

Finally, we examined whether rMS had effect on pathological changes of lung tissue. A semi-quantitative score was used to assess peribronchitis in airway [19]. Histological analysis manifested numerous inflammatory cell infiltration, marked goblet cell hyperplasia and mucus secretion and airway epithelium was thickened in asthma group. However, these pathological changes were dramatically reduced in rMS group (**Fig. 5**).

Discussion

Asthma is known as a chronic inflammatory disease and typically characterized by airway inflammation, airway hyperresponsiveness and airway remodeling, where inflammatory cells and various cellular components are involved [20–22]. Previous research has shown that IL-17A recruits a series of cytokines and chemokines by activating downstream cells, thereby acting on neutrophils [23, 24], therefor, blocking or suppressing IL-17A-neutrophil axis may alleviate airway inflammation in neutrophilic asthma.

Nowadays, antibodies are widely used in cell signal pathway's blocking. Monoclonal antibodies and soluble receptors have high specificity and efficacy, however, high cost, frequent injections and side effects such as allergic risk and infusion reaction often along with it [25, 26]. To overcome these disadvantages, a new strategy was emerged. Vaccine was established by reconstructing the peptide of targeted self-cytokine's immunodominant epitope region with foreign Th epitopes region into a carrier to induce specific autoantibody [27, 28]. Under this theory, a recombinant *Mycobacterium smegmatis* inducing IL-17A autoantibody was designed in previous study [17]. Experimental results showed a persistent effect of generating IL-17A autoantibody after immunisation with rMS and confirmed that these autoantibodies had high activity to neutralize IL-17A.

Since it has been reported that DO11.10 mice challenged by OVA exhibits an increase in neutrophil infiltration instead of eosinophil [29], rMS was vaccinated into DO11.10 mice to study the effects on neutrophilic asthmatic airway. Airway inflammation is a prominent hallmark in pathogenesis of asthma, and IL-17A is a pivotal mediator in neutrophilic inflammation, as it recruits neutrophil from peripheral blood into local bronchial tube via C-X-C chemokine [30–32]. Neutrophil itself has potential to release a bunch of cytokines and chemokines such as IL-6, TNF-α, CXCL-1 and CXCL-2 to participate in airway inflammation [33, 34]. IL-6 and IL-23 are downstream cytokines of IL-17A, which can maintain and enhance IL-17A production [35–37]. Moreover, TNF-α not only can stabilize IL-17 mRNA expression, but also have a chemoattractant effect on neutrophils [38, 39]. Thus, IL-17A-neutrophil axis is amplified by a loop feedback. In our experiment, data showed that neutrophils and macrophages were elevated in BALF of OVA-challenged mice, and which were significantly reduced by rMS. Histopathological examination also revealed an obvious decrease of inflammatory cells around bronchia in rMS vaccinated mice compared to asthmatic ones. Meanwhile, cytokines and chemokines related to IL-17A-neutrophil axis were all notably decreased in rMS group contrast to asthma group. These results indicate that rMS suppresses inflammatory cells and mediators in non-eosinophilic asthma through dampening IL-17A-neutrophil axis. Besides inflammatory mediators released from neutrophil, neutrophil enzyme may be another main cause of airway injury. MPO generates reactive oxygen species, which directly contribute to airway epithelial damage, and neutrophil elastase also plays important roles in airway remolding, as it can degrade structural proteins, increase vascular permeability and metaplasia of bronchial mucus glands [40–42]. Experimental data showed the activity of MPO in both BALF and lung tissues were obviously elevated in asthma mice compared to control subjects, and these increases were reversed by rMS. Further, PAS staining showed rMS effectively inhibited goblet cell hyperplasia and mucus secretion. These results indicate that vaccination with rMS could suppress neutrophil enzyme activity to alleviate airway inflammation and impairment.

In conclusion, vaccination of recombinant *Mycobacterium smegmatis* breaks IL-17A-neutrophil axis via inducing IL-17A autoantibody followed by reduction of inflammatory cells and mediators and suppression of the activity of neutrophil enzymes and mucus secretion, thereby attenuating airway inflammation in mice with neutrophilic asthma. Thus, recombinant *Mycobacterium smegmatis* may have protective effects on airway insults of asthma.

Declarations

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Conflicts of Interest The authors declare no competing interests.

Data Availability There will be data transparency for the present research work.

Code Availability Not applicable

Authors' Contributions LC and JHZ conceived and designed the study. LC and WTX analyzed and interpreted the data and wrote the manuscript. SM and RCZ executed the experiments and analyzed the data. All the authors read and approved the manuscript.

Ethics Approval This article does not contain any studies with human participants and animal experiment was approved by Institutional Animal Care and Use Committee of Shanghai Public Health Clinical Center.

Consent to Participate All the authors have given the consent to participate in the present research concept.

Consent for Publication All authors have read the final manuscript and gave the consent for publishing the manuscript.

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Figures

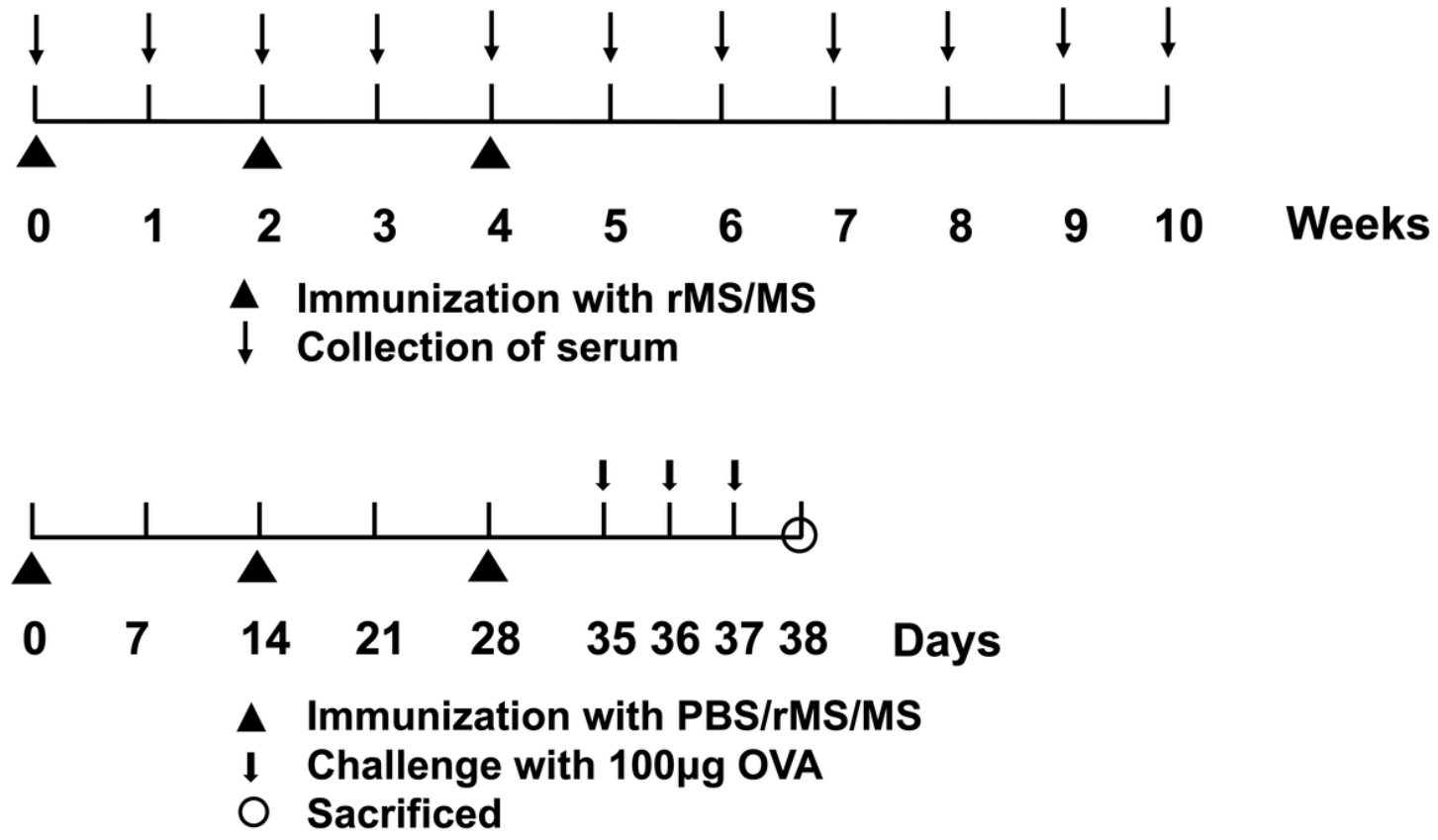

Figure 1

Mice were divided randomly into 4 groups of 6 animals (PBS, asthma, rMS and MS) and treated as shown in Fig 1

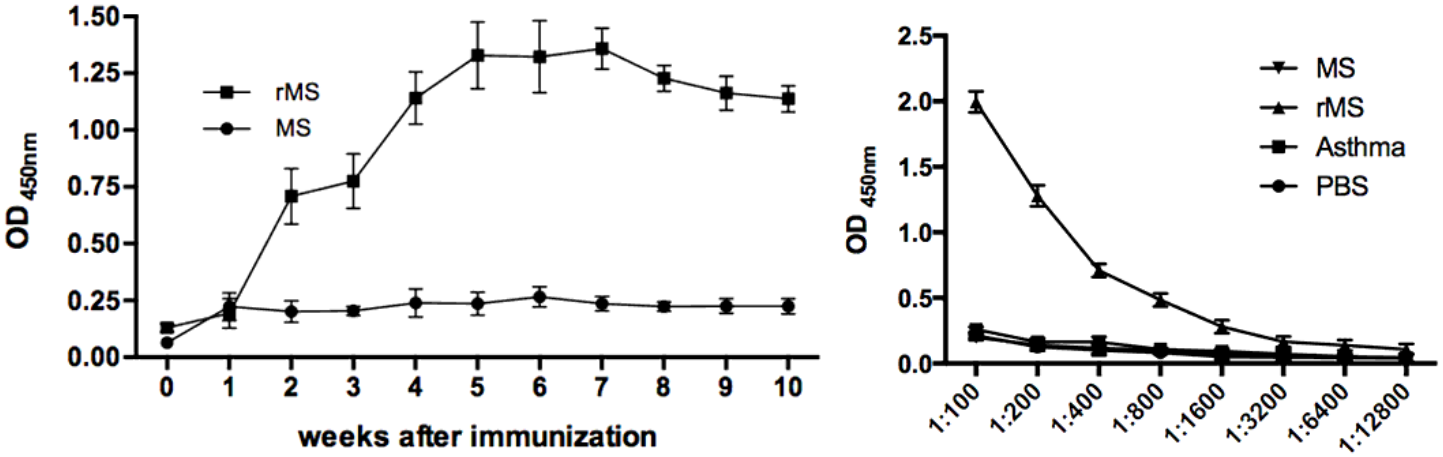

Figure 2

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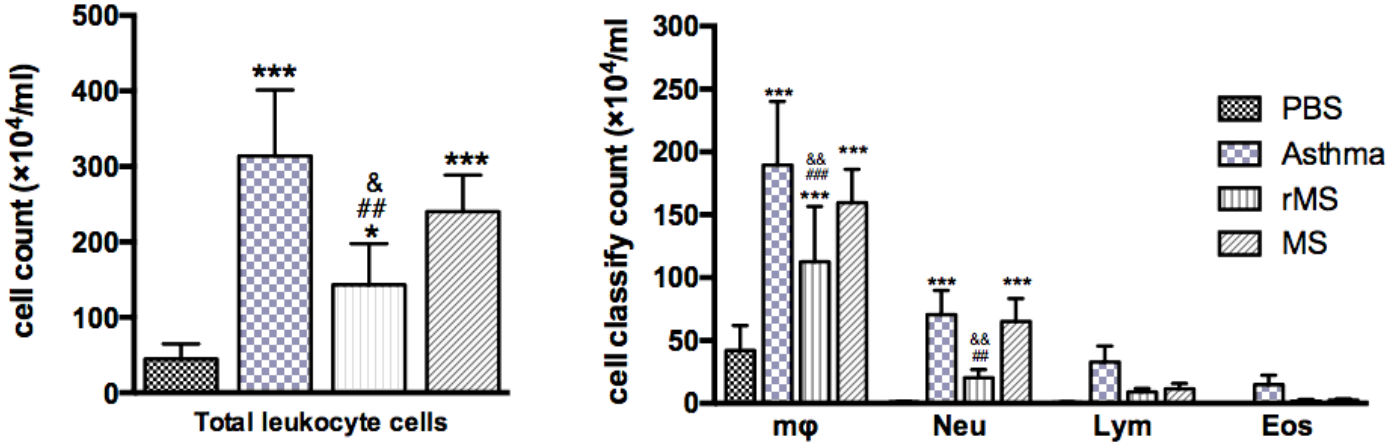

Figure 3

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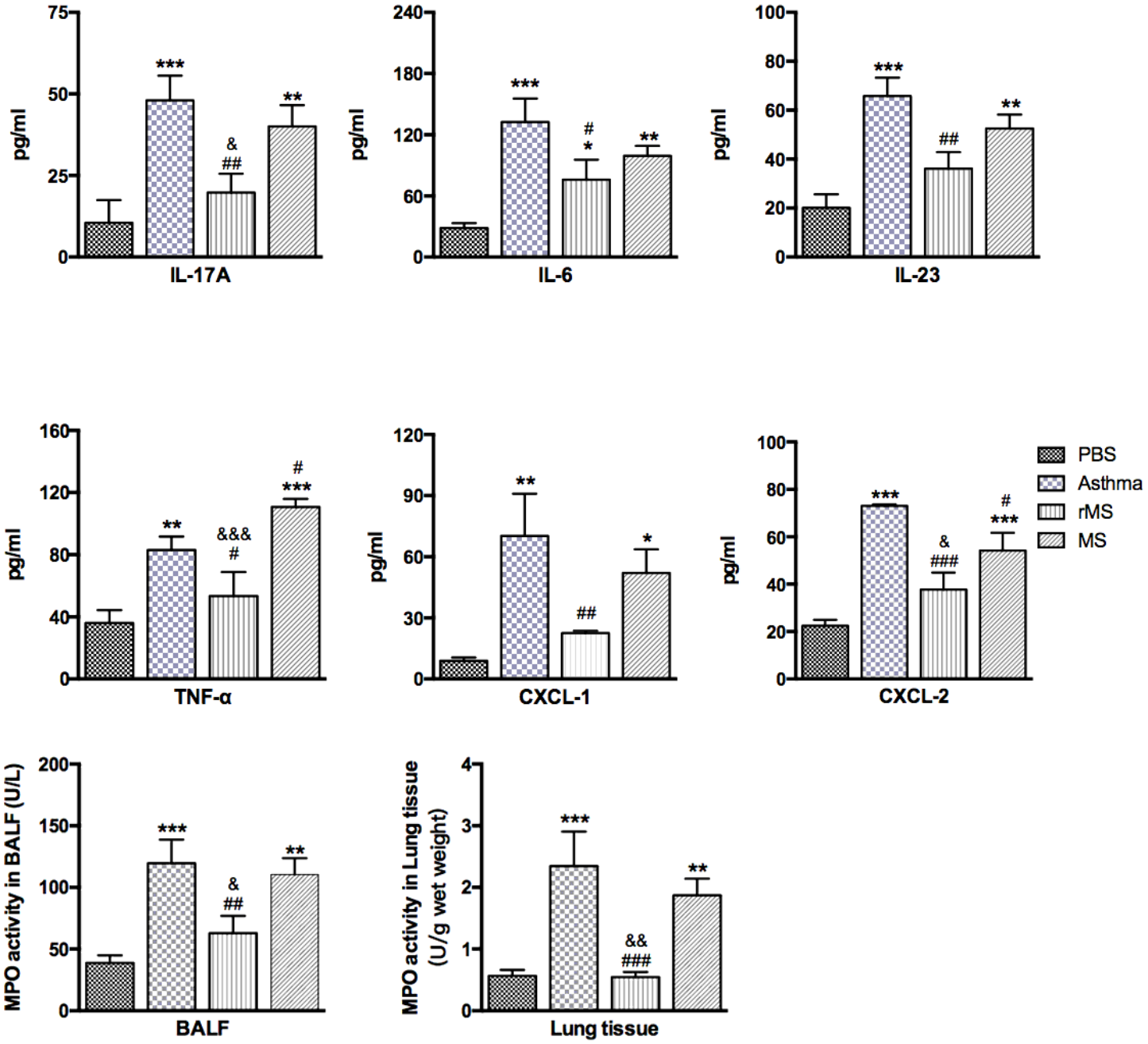

Figure 4

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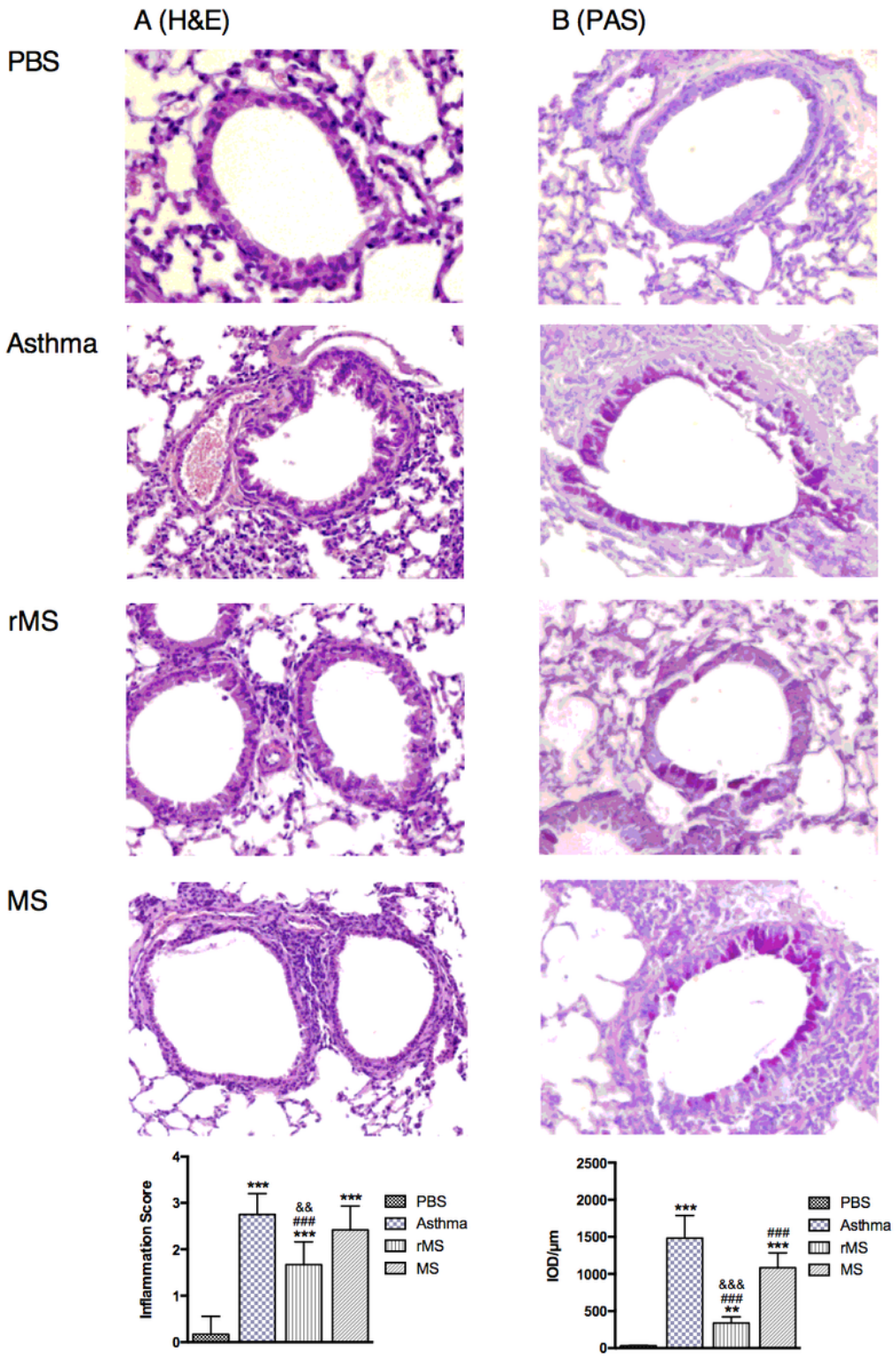

Figure 5

Histological analysis manifested numerous inflammatory cell infiltration, marked goblet cell hyperplasia and mucus secretion and airway epithelium was thickened in asthma group. However, these pathological changes were dramatically reduced in rMS group.