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Running head: IL-17A autoantibody ameliorates airway inflammation

Autoantibody of interleukin-17A induced by recombinant *Mycobacterium smegmatis* attenuates airway inflammation in mice with neutrophilic asthma

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ABSTRACT

Objective: Previous studies have shown Interleukin (IL)-17A as an important contributor to the development of severe asthma, which is mainly characterized by neutrophilic inflammation and less response to corticosteroids. Consequently, the IL-17A-neutrophil axis could be a potential therapeutic target. Previously, we constructed a recombinant *Mycobacterium smegmatis* (rMS) expressing fusion protein Ag85A-IL-17A, and confirmed it could induce production of IL-17A autoantibody *in vivo*. This study uses a murine model of neutrophilic asthma to further investigate the effects of rMS on airway inflammation. **Methods:** DO11.10 mice were divided into four groups: phosphate buffered saline (PBS), asthma, rMS and MS. This murine model of neutrophilic asthma was established with ovalbumin (OVA) challenge, whereby PBS, rMS and MS were administered intranasally. Anti-inflammatory effects on inflammatory cell infiltration and expression of inflammatory mediators in bronchoalveolar lavage fluid (BALF) were evaluated, along with histopathological changes in lung tissues. **Results:** A sustained high-titer IL-17A autoantibody was detected in sera of the rMS group. Compared to the asthma group, the number of neutrophils, IL-17A, CXCL-1 levels and MPO activity in the rMS group were all significantly reduced ($p < 0.01$). Histological analysis showed rMS remarkably suppressed inflammatory infiltration around bronchia. The inflammation score and the mucus score in the rMS group were both significantly lower than those in the asthma group ($p < 0.001$). **Conclusion:** rMS ameliorated airway inflammation in mice with neutrophilic asthma caused by inducing IL-17A autoantibody and regulating the IL-17A-neutrophil axis, thus offering a possible novel treatment for neutrophilic asthma.

Keywords asthma; autoantibody; IL-17A; neutrophil; *Mycobacterium smegmatis*; airway inflammation

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 allergic asthma. Specifically, they are critical for eosinophil growth and differentiation, and IgE class switching, while participating in airway hyperresponsiveness and mucus hypersecretion [3, 4]. In addition to the Th2 cell, considerable attention has focused on the Th17 cell, as increasing evidence points to IL-17A as an important contributor to the development of asthma, particularly a specific moderate-to-severe asthma endotype, which usually presents with robust neutrophilic airway inflammation and less response to corticosteroids [5, 6]. In those asthmatic patients, IL-17A expression has been shown to significantly increase in sputum, bronchoalveolar lavage fluid (BALF), and peripheral blood [7, 8]. Recent studies have shown that IL-17A is involved in multiple aspects of asthma pathogenesis, including structural alterations of epithelial cells, airway inflammatory cell infiltration, and smooth muscle contraction [9, 10]. Most notably, the level of IL-17A expression in asthmatics is closely associated with disease severity [11]. Therefore, IL-17A has been suggested as a crucial regulator in the pathogenesis of asthma.

Despite decades of intensive research, only limited progress has been made in asthma treatment since the introduction of inhaled selective β_2 adrenoceptor agonists and glucocorticosteroids, leaving inhaled corticosteroids (ICS) as the backbone of current asthma therapy [1, 12]. Glucocorticosteroids are effective in atopic asthma with eosinophilic inflammation, but clinical data show that there are still many patients with uncontrolled asthma, and these cases may present with severe symptoms, as well as elevated levels of IL-17A in sputum, and neutrophil-predominant airway inflammation [13, 14]. Previous studies showed that neutrophils commonly express 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, leading to corticosteroid-resistance [15, 16]. At the same time, several studies have found that IL-17A is positively correlated with airway neutrophil infiltration and GR β expression in epithelial cells [17–19]. In addition, experimental studies have indicated that IL-17A recruits and accumulates neutrophils indirectly into the airway by inducing the transcription and release of chemotactic agents from airway structural cells [20]. Therefore, we hypothesize that whether there is a new therapeutic strategy designed to suppress IL-17A, and regulate the IL-17A-neutrophil axis, maybe it could effectively alleviate neutrophilic airway inflammation in asthma, including the glucocorticoid-resistant type.

In our previous study, we constructed a recombinant *Mycobacterium smegmatis* (rMS), and confirmed it could induce IL-17A autoantibody *in vivo* [21, 22]. This study aims to further explore the protective effects of 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 16–18 g, were obtained from Shanghai Model Organisms Center, Inc., in Shanghai, China. Mice were bred and housed in a specified pathogen-free (SPF) laboratory with 12-h of light-dark cycle. The physiological condition of the mice was checked three times a week before OVA sensitization, and every day after sensitization. Sodium pentobarbital was used before sacrifice in order to minimize suffering and distress. The experiment was approved by the Institutional Animal Care and Use Committee of the Shanghai Public Health Clinical Center (Permit Number: 2019–102).

Preparation of bacteria

rMS expressing fusion protein Ag85A-IL-17A was constructed in previous experiments [21]. Briefly, a recombinant plasmid pMFA42S-Ag85a-IL-17a was constructed by inserting fusion gene Ag85a-IL-17a into a shuttle vector pMFA42S, then transformed into *Mycobacterium smegmatis* (MS) by electroporation to obtain recombinant *Mycobacterium smegmatis*. MS and rMS were both preserved at -80°C in our laboratory and were recovered and diluted with sterile phosphate buffered saline (PBS) at 5×10^8 colony-forming unit (CFU)/ml before immunization.

Protocol of immunization and challenge

To dynamically monitor the expression of IL-17A autoantibody *in vivo*, mice were administered intranasally with rMS or MS bacterial solution at a dose 1.0×10^7 CFU in 20 μl of PBS at weeks 0, 2 and 4, then sera were collected once a week for ten consecutive weeks from the time of initial administration (**Figure 1A**). In addition, mice were divided randomly into four groups of six animals each (PBS, asthma, rMS and MS), and treated as shown in **Figure 1(B)**. Briefly, while under light anesthesia, mice in the rMS group and MS group were administered intranasally with rMS or MS at a dose 1.0×10^7 CFU in 20 μl of PBS on days 0, 14 and 28. Mice in the asthma group were administered intranasally with an equal volume of PBS. Except for the PBS group, all of the mice were challenged intranasally with 100 μg ovalbumin (OVA, Grade V, Sigma-Aldrich) in 20 μl PBS on days 35, 36 and 37. Mice in the 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 was centrifuged at 4,000 g for 10 min. IL-17A-specific IgG in sera was measured by enzyme-linked immunosorbent assay (ELISA). Plates were coated with commercial recombinant protein IL-17A (2.5 $\mu\text{g}/\text{ml}$, PeproTech), and antibodies were detected using goat anti-mouse IgG-horseradish peroxidase (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 the mean of optical density value at 450 nm.

Bronchoalveolar lavage collection

The trachea was dissected and cannulated. BALF was prepared by washing the lungs three times with 0.4 ml ice-cold PBS. One hundred (100) μl BALF was stored at -80°C for the myeloperoxidase (MPO) activity assay. The remainder was centrifuged, and the supernatant was stored for cytokine, chemokine and mucin analysis. The number of leukocytes was determined by 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, chemokine and mucin 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, CXCL-2 (eBioscience) and mucin 5AC (Abmart) in BALF were measured using specific mouse ELISA kits.

MPO activity analysis

The activity of MPO in BALF and lung tissues was measured using a commercially available MPO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and manufacturer's instructions were followed.

Histopathological analysis

After collecting BALF, the pulmonary lobe was dissected, fixed with 4% paraformaldehyde and embedded in paraffin. Lung tissues were sliced into 4 μ m thick sections, and were separately stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). Peribronchial inflammatory infiltration was assessed by three observers independently according to a published guideline [23]. A value of 0 was assigned when no inflammation was detectable. A value of 1 was assigned for occasional cuffing with inflammatory cells. A value of 2 was assigned for most bronchi or vessels surrounded by a thin layer (one to five cells thick) of inflammatory cells. A value of 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. The evaluation methods of goblet cell proliferation around the airway are based on follows: 0, normal; 1, less than 25% of the epithelium; 2, 25–50% of the epithelium; 3, 50–75% of the epithelium; and 4, > 75% of the epithelium [24]. In addition, the ratio of total bronchial wall area (WAt) to bronchial basement membrane perimeter (Pbm) (WAt/Pbm) was used for evaluating the thickness of the bronchial wall [25].

Statistical analysis

Data were analyzed using Prism software (version 8.3.1) and expressed as mean value $\pm$ 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-titer IL-17A autoantibody in sera

To dynamically monitor the expression and titer of IL-17A autoantibody, ELISA was performed, with IL-17A protein used as a coating antigen to detect the specific IgG in sera. There was no IL-17A specific IgG in sera of mice treated with MS, while IL-17A specific autoantibody began to appear in sera of rMS-immunized mice from the second week, and the autoantibody titer increased steadily and lasted until the tenth week (**Figure 2A**). As shown in **Figure 2(B)**, there was no IL-17A specific IgG in sera of mice in the PBS, asthma, and MS groups, while a high-titer IgG specific to IL-17A was detected in sera from the rMS group over time.

rMS decreases inflammatory cells in BALF

To investigate the effect of rMS on airway inflammation, total leukocytes and differential cell counts in BALF were calculated. As shown in **Figure 3**, total leukocyte cells, macrophages and neutrophils in the asthma group were all obviously elevated compared to the PBS group, and there was no statistically significant difference in the number of eosinophils between two groups. However, these increases in the number of total leukocytes ($p < 0.01$) and neutrophils ($p < 0.01$) were both markedly reversed by intranasal administration of rMS. There was no statistically significant difference in the number of total leukocytes, macrophages and neutrophils between the asthma group and the MS group.

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

In order to further study the effect of rMS on the 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 **Figure 4**, the levels of IL-17A, IL-6, IL-23, TNF- α , CXCL-1 and CXCL-2 in BALF from the asthma group were significantly higher than those in the PBS group, and rMS treatment markedly decreased the levels of these cytokines and chemokines. Although MS also decreased the levels of TNF- α and CXCL-2 compared to the asthma group, the effect of rMS was more marked, and there were statistically significant differences between two groups in the levels of TNF- α ($p < 0.001$) and CXCL-2 ($p < 0.05$). Meanwhile, compared to the PBS group, the activity of MPO in BALF and lung tissue were both significantly increased in the asthma group. In contrast, these increases noticeably declined in the rMS group. There was no statistically significant difference in the activity of MPO between the asthma group and the MS group.

rMS reduces airway inflammation infiltration and mucus hypersecretion

Finally, we examined whether rMS had an effect on pathological changes in lung tissue. A semi-quantitative score was used to assess peribronchitis in the airway. As shown in **Figure 5**, histopathology and ELISA analysis results manifested inflammatory cell infiltration, marked goblet cell hyperplasia and mucus secretion, and airway epithelium thickening in the asthma group. However, in the rMS group, mice showed dramatic reductions in thickening of airway epithelium, goblet cell hyperplasia, mucus secretion and infiltration of inflammatory cells in the peribronchiolar region. The inflammation score, the mucus score, the ratio of WAt to Pbm and the level of mucin 5AC (MUC5AC) in the asthma group were higher than those in the PBS group, however, these increases were all reversed by rMS. Although MS also decreased the mucus score-compared to the asthma group, the effect of rMS was more notable, and there was statistically significant difference between the rMS group and the MS group ($p < 0.05$).

Discussion

Asthma is typically characterized by airway inflammation, airway hyperresponsiveness and airway remodeling, where various inflammatory cells and cellular components are involved [26–28]. Previous studies suggested that Th17 cell-derived cytokines, such as IL-17A and IL-17F are important regulators of neutrophilic inflammation, and the aberrant IL-17A/IL-17F production may drive severe asthma [29, 30]. Recent studies demonstrate that individuals with severe asthma display not only severe airway hyperresponsiveness (AHR), but also intense neutrophilia and increased IL-17A production [31]. Consequently, blocking or suppressing the IL-17A-neutrophil axis may ameliorate airway inflammation in neutrophilic asthma.

Currently, a variety of monoclonal antibodies (mAb) and soluble receptors have been developed to neutralize the over-produced endogenous cytokines associated with severe disease. It has been reported that administration of monoclonal antibody or soluble receptor to block over-produced IL-13 has been proven to be effective in controlling airway inflammation in a murine model of asthma [32]. However, these approaches have disadvantages of high costs, frequent injections, short half-lives, and possible adverse effects [33, 34]. In order to overcome these disadvantages mentioned above, a new strategy that may offer long-term efficacy with fewer adverse effects has emerged. According to previous studies, a recombinant vaccine was prepared by inserting a peptide derived from the immunodominant epitope region of the targeted self-cytokine into the immunodominant epitope region of the carrier using gene recombination method to induce specific autoantibody [35, 36]. Based on the above theory, a recombinant *Mycobacterium smegmatis*-inducing IL-17A autoantibody was designed in our previous work [21], experimental results have shown that the recombinant *Mycobacterium smegmatis* had a long-term efficacy of generating IL-17A autoantibody after immunization with rMS, more importantly, these autoantibodies had high activity to neutralize IL-17A.

Since it has been reported that DO11.10 mice challenged by OVA exhibit an increase in neutrophil infiltration, instead of eosinophil [37], they were vaccinated with rMS to study its effects on the 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 neutrophils from peripheral blood into local bronchial tube via C-X-C chemokine [38–40]. Neutrophils themselves have potential for releasing cytokines and chemokines, such as IL-6, TNF- α , CXCL-1 and CXCL-2, to participate in airway inflammation [41, 42]. IL-6 and IL-23 are downstream cytokines of IL-17A, which can maintain and enhance IL-17A production [43–45]. Moreover, TNF- α can stabilize IL-17 mRNA expression, and has a chemoattractant effect on neutrophils [46, 47]. As a result, the IL-17A-neutrophil axis is amplified by a loop feedback.

In this study, experimental data showed that total leukocytes, neutrophils and macrophages were all elevated in BALF of OVA-challenged mice and were all significantly reduced by rMS. Histopathological examination also revealed an obvious decrease in inflammatory cells around bronchia in rMS vaccinated mice compared to asthmatic ones. Meanwhile, cytokines and chemokines related to the IL-17A-neutrophil axis, such as IL-6, IL-17A, CXCL-1 and CXCL-2, were all notably decreased in the rMS group, in contrast to the asthma group. These results indicate that rMS suppresses IL-17A expression and reduces inflammatory cells and mediators in non-eosinophilic asthma through dampening the IL-17A-neutrophil axis. In addition to inflammatory mediators being released from neutrophils, the neutrophil enzyme may be another main cause of airway injury. MPO generates reactive oxygen species, which directly contribute to airway epithelial damage. Also, neutrophil elastase, a protease enzyme, plays important roles in airway remodeling, as it can degrade structural proteins, and increase vascular permeability and metaplasia of bronchial mucus glands [48–50]. Experimental data showed the activity of MPO in both BALF and lung tissues were markedly elevated in asthma mice compared to control subjects, and these increases were reversed by rMS. Furthermore, PAS staining showed rMS effectively inhibited goblet cell hyperplasia, and ELISA analysis manifested that MUC5AC as one of the predominant gel-forming mucins in the airways, its secretion was also significantly suppressed by rMS. These results indicate that vaccination with rMS could suppress neutrophil enzyme activity and alleviate airway inflammation and impairment. Taken together, our data demonstrated that IL-17A autoantibody induced by recombinant *Mycobacterium smegmatis* has a significant inhibitory effect on neutrophilic airway inflammation in asthmatic mice by regulating the IL-17A-neutrophil axis (**Figure 6**). However, some limitation should be noted. We were unable to assess whether IL-17A autoantibody affects airway hyperresponsiveness, in addition, a more comprehensive assessment of immune response related to the autoantibody is necessary to further elaborate the roles of IL-17A autoantibody in asthma.

Conclusion

Vaccination with recombinant *Mycobacterium smegmatis* breaks the IL-17A-neutrophil axis by inducing the IL-17A autoantibody, followed by reduction of inflammatory cells and mediators, and suppression of the activity of neutrophil enzymes and mucus secretion. This sequence attenuates airway inflammation in mice with neutrophilic asthma, suggesting that rMS may offer protective effects on airway insults from asthma.

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Declaration of interest statement

The authors declare that they have no conflicts of interest.

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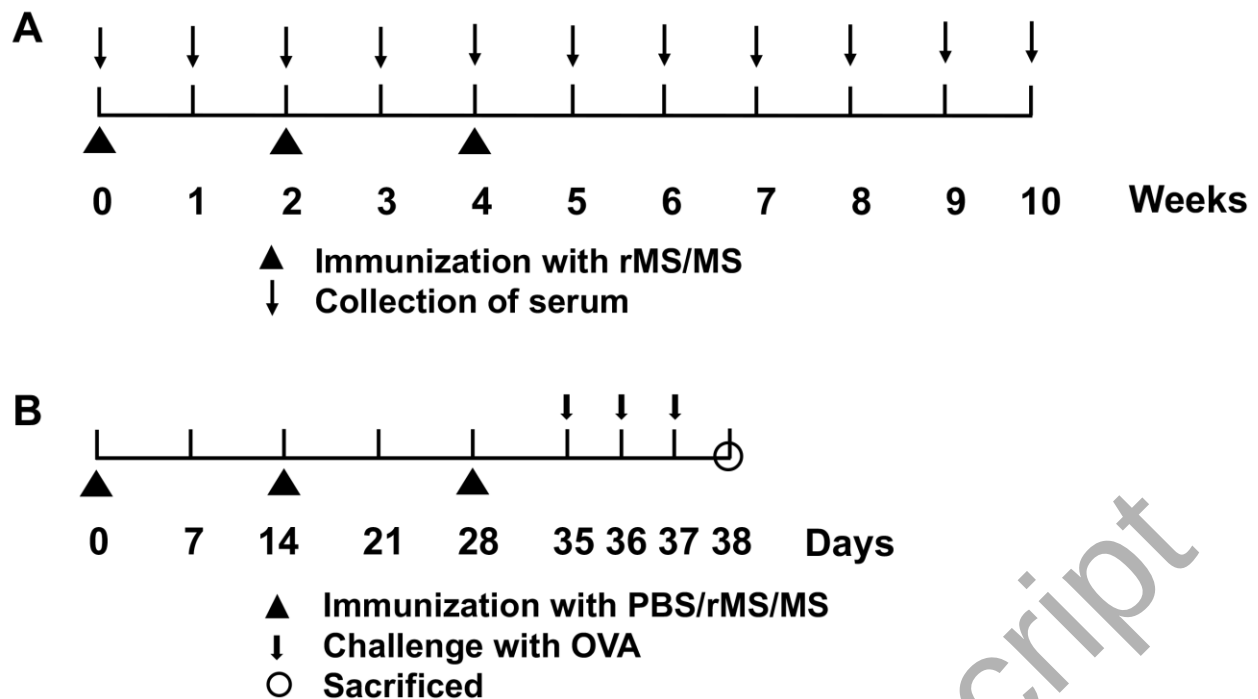


Figure 1. Protocol of immunization and challenge (A) rMS/MS immunization protocol. To dynamically monitor the expression of IL-17A autoantibody in vivo, DO11.10 mice were administered intranasally with rMS or MS bacterial solution at weeks 0, 2 and 4. Sera were collected once a week for ten consecutive weeks from the time of initial administration. (B) OVA-induced asthma model and treatment protocol. DO11.10 mice were divided randomly into four groups (PBS, asthma, rMS and MS), and treated as shown above. Mice in the rMS and MS groups were administered intranasally with rMS or MS bacterial solution on days 0, 14 and 28, then challenged intranasally with OVA on days 35, 36 and 37. Mice in the asthma group were administered intranasally with PBS on days 0, 14 and 28, then challenged intranasally with OVA on days 35, 36 and 37. Mice in the PBS group were administered and challenged with PBS as parallel manipulation.

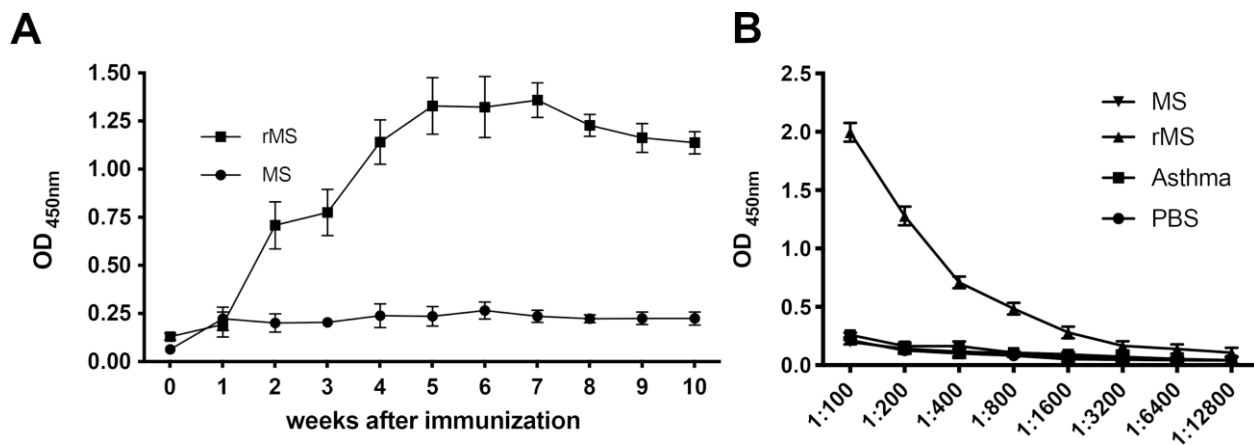


Figure 2. IL-17A autoantibody induced by rMS (A) Time course of IL-17A autoantibody production. Sera were collected for ten consecutive weeks, anti-IL-17A specific antibody was measured by ELISA. Results are presented as mean value $\pm$ SD, $n = 6$. (B) Titer of IL-17A autoantibody in sera. Sera were collected and serially diluted after the last challenge with OVA, and ELISA was applied to analyze IL-17A autoantibody. Results are presented as mean value $\pm$ SD, $n = 6$.

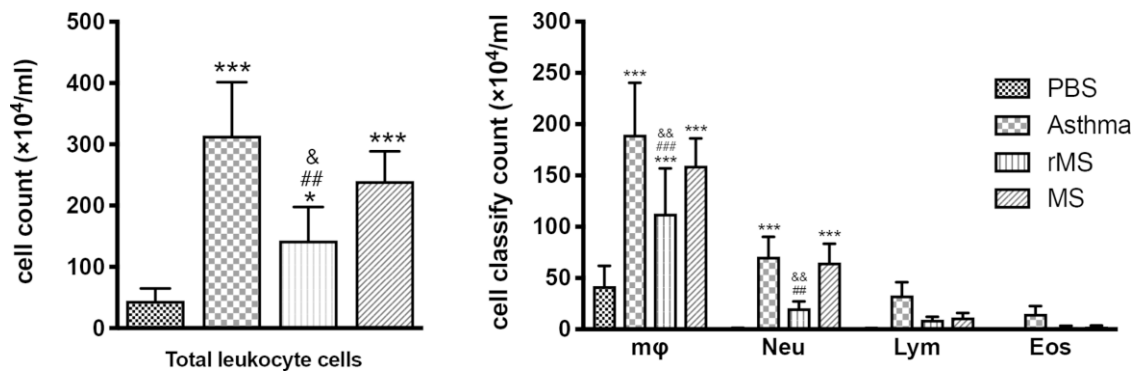


Figure 3. Effect of rMS on inflammatory cells in BALF mφ = macrophage, Neu = neutrophils, Lym = lymphocytes, Eos = eosinophils. Results are presented as mean value $\pm$ SD, $n = 6$. Significant difference was shown as * compared to the PBS group, $*p < 0.05$, $***p < 0.001$; # compared to the asthma group, $##p < 0.01$, $###p < 0.001$; & compared to the MS group, $&p < 0.05$, $&&p < 0.01$.

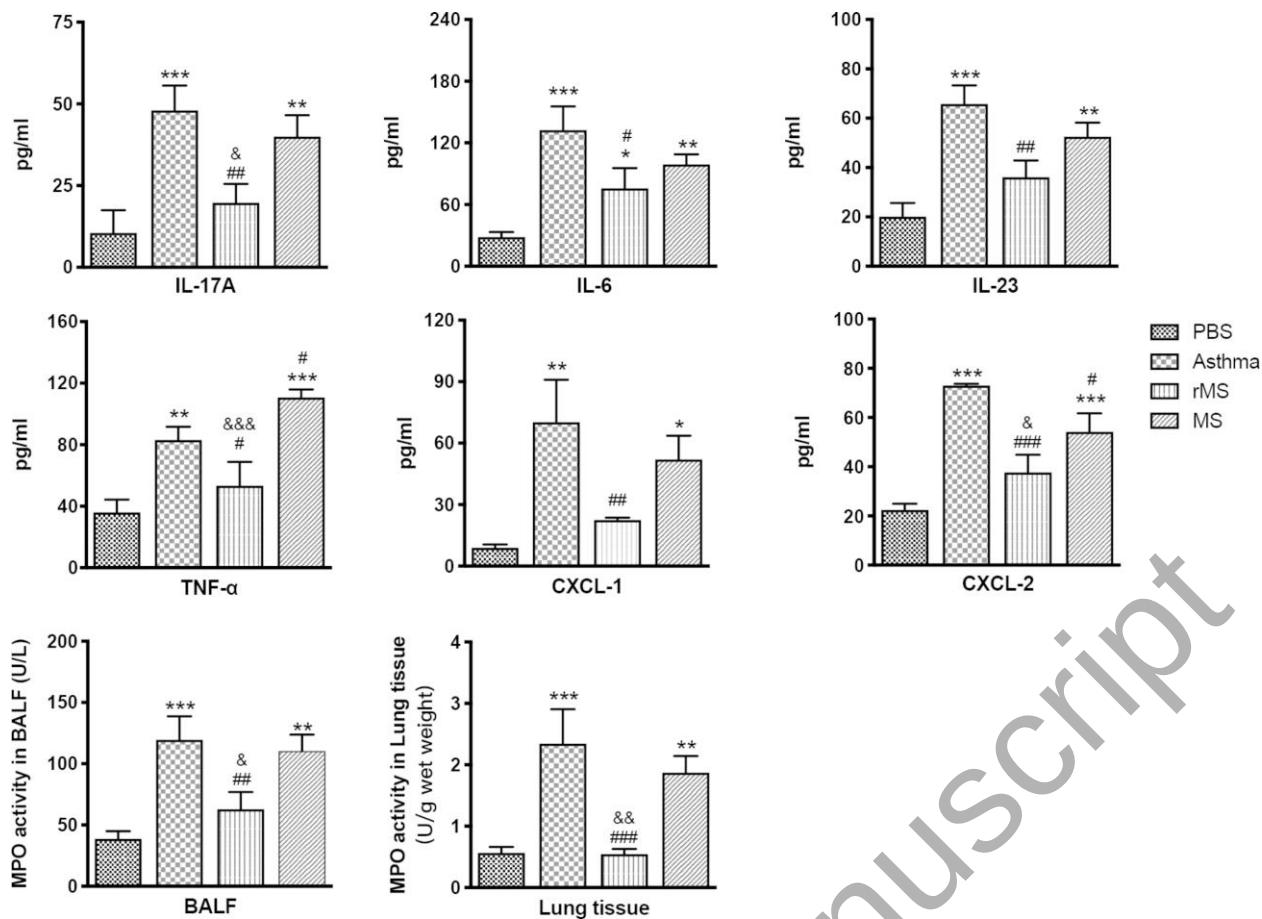


Figure 4. Effect of rMS on the expression of cytokines, chemokines and MPO activity Results are presented as mean value $\pm$ SD, $n = 6$. Significant difference was shown as * compared to the PBS group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # compared to the asthma group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$; & compared to the MS group, & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$.

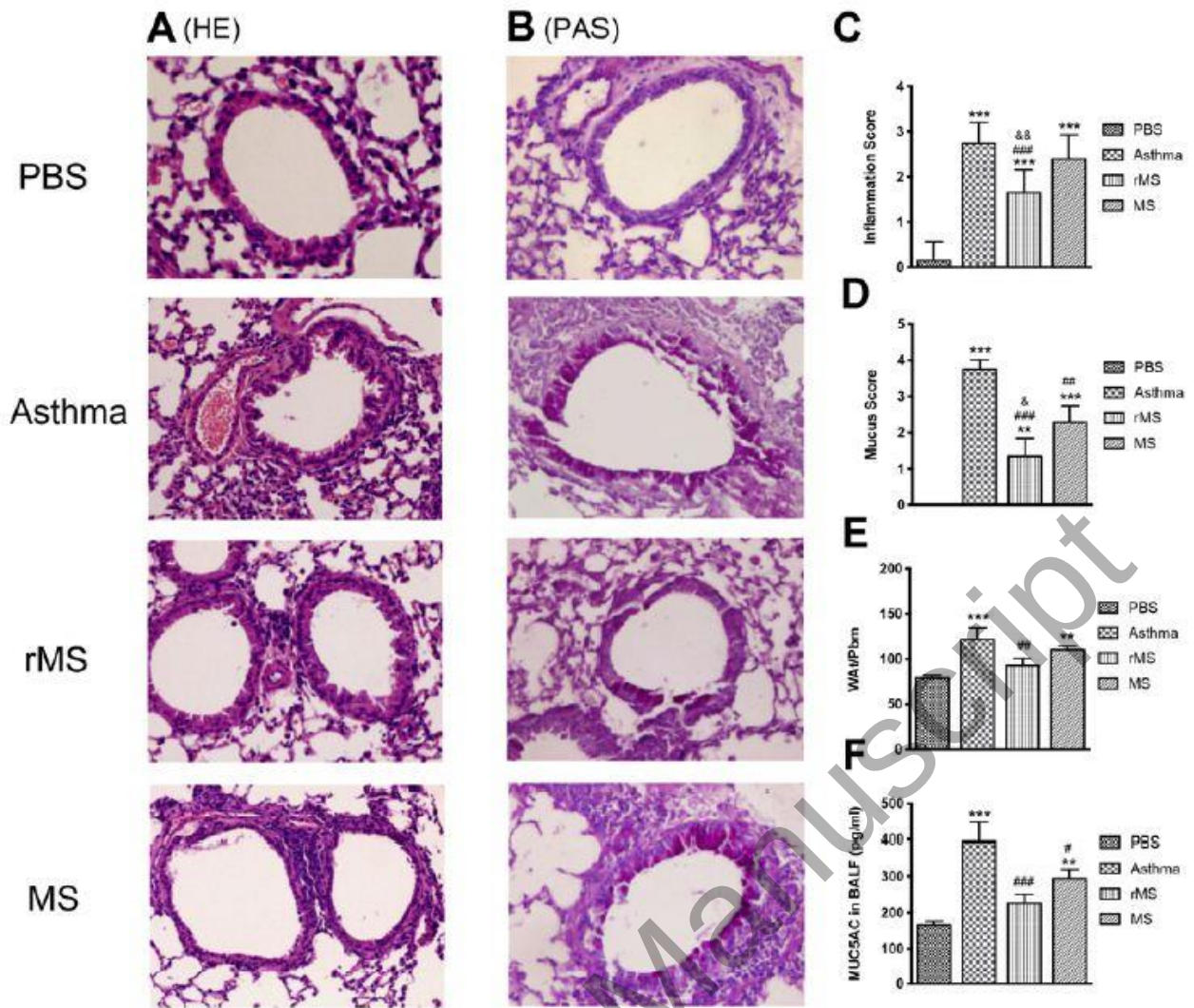


Figure 5. Effect of rMS on airway inflammation and mucus secretion (A) Lung sections stained with H&E ($\times 400$). (B) Lung sections stained with PAS ($\times 400$). Pictures show representative samples from the different group. (C) Inflammation score, (D) mucus score, (E) WAt/Pbm and (F) MUC5AC expression of every group was shown. Results are presented as mean value $\pm$ SD, $n = 6$. Significant difference was shown as * compared to the PBS group, $**p < 0.01$, $***p < 0.001$; # compared to the asthma group, $\#p < 0.05$, $##p < 0.01$, $###p < 0.001$; & compared to the MS group, $\&p < 0.05$, $\&\&p < 0.01$.

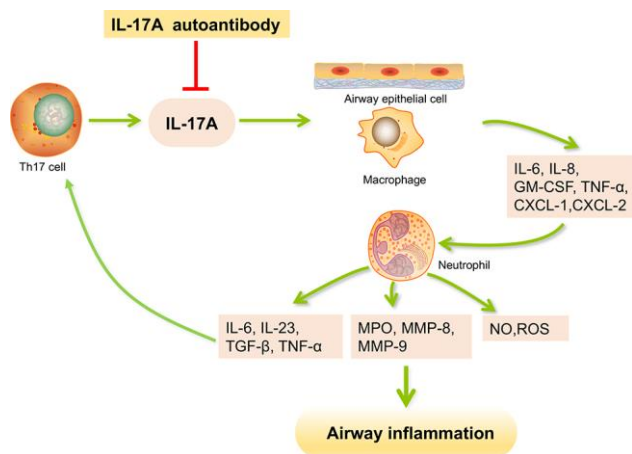


Figure 6. Mechanisms of IL-17A autoantibody regulating neutrophilic inflammation GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF, transforming growth factor; MMP, matrix metalloproteinase; ROS, reactive oxygen species.