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IL-9 exacerbates the development of chronic obstructive pulmonary disease through oxidative stress

S.-C. ZOU, L.-L. PANG, Q.-S. MAO, S.-Y. WU, Q.-F. XIAO

[1]Department of Pneumology, Yantai Yuhuangding Hospital, Yantai, China
[2]Department of Pneumology, The People's Liberation Army (PLA) 184th Hospital, Yingtan, China
[3]Department of Pneumology, the First Hospital of Jiaxing, Jiaxing, China

Shenchun Zou and Lingling Pang contributed equally to this work

Abstract. – OBJECTIVE: To investigate the role of IL-9 in chronic obstructive pulmonary disease (COPD), and to explore its potential mechanism.

MATERIALS AND METHODS: A mouse COPD model was established by exposure to cigarette smoke. COPD mice were then randomly assigned into two groups, including: the PBS group and the IL-9 antibody group. The above two groups were treated with phosphate-buffered saline (PBS) or IL-9 injection, respectively. The histopathological changes in lung tissues of mice were observed by hematoxylin-eosin (H&E) staining. Immunohistochemistry was performed to detect IL-9-positive (IL-9+) cells in lung tissues. Expression levels of IL-9, sIL-9R, STAT3, and p-STAT3 in peripheral blood of mice were determined by quantitative Real time-polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA), and Western blot, respectively. In addition, the expression levels of superoxide dismutase (SOD), malondialdehyde (MDA), and reactive oxygen species (ROS) were detected.

RESULTS: H&E staining results showed that the airway wall structure of COPD mice in the PBS group was irregular. Ciliated columnar epithelium exhibited marked degeneration, necrosis and shedding. Besides, numerous inflammatory cell infiltration, narrowing and rupture of the alveolar septa, and larger cysts fused by adjacent alveoli were observed. H&E staining also indicated that the structure of alveolar epithelium was severely impaired in COPD mice. However, the pathological changes in lung tissues of mice in the IL-9 antibody group were much milder than those of the PBS group. Immunohistochemistry results showed a significant deposition of IL-9+ cells in the lung tissues of the PBS group. Meanwhile, the mRNA and protein levels of IL-9, sIL-9R, and p-STAT3 in the PBS group were also remarkably higher than those of the IL-9 antibody group. In addition, SOD content in the PBS group was significantly decreased, whereas the levels of MDA and ROS were significantly increased than those of the IL-9 antibody group.

CONCLUSIONS: IL-9 activated STAT3 and aggravated lung injury in COPD mice by increasing inflammatory and oxidative stress.

Key Words:
IL-9, Chronic obstructive pulmonary disease (COPD), Oxidative stress.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent irreversible airflow limitation, whose condition is progressively worsening. Harmful particles or gases that chronically stimulate airways and lungs may cause abnormally enhanced inflammatory responses, eventually leading to COPD. Due to the severity of COPD, lesions can simply involve the lungs or even affect the entire body. The life quality of COPD patients is closely related to the disease condition and the severity of comorbidities. Previous studies have indicated that COPD is the result of interaction between genetic susceptibility and external environmental factors. With increased social industrialization and aging of population, the incidence of COPD in China has increased remarkably. It is reported that the prevalence of COPD in people over than 40 years old is about 8.2% in China. COPD now ranks the fourth leading cause of deaths over the world[1]. Moreover, it will be the third leading cause of deaths worldwide by 2020, just secondary to cardiovascular diseases and cerebrovascular diseases[2]. Pathological changes of COPD mainly include epithelial inflammatory cell infiltration,

Corresponding Author: Qinfeng Xiao, BM; e-mail: qf868686@sina.com

goblet cell hyperplasia and mucinous gland hypertrophy. Besides, airway remodeling in peripheral airway, airway stenosis and obstruction are also frequently observed in these patients. The above changes are caused by repeated repair of airway wall. Central lobe emphysema is another main pathological characteristic of COPD in lung parenchyma. Late emphysema can be diffusely distributed throughout the lungs, and is accompanied by destruction of the pulmonary capillary bed. There are multiple complications of COPD, including cardiovascular disease, lung cancer, osteoporosis, anxiety and depression[3]. As a common and frequently occurring respiratory disease, COPD is a public health issue of great concern. In addition, COPD has brought heavy burden to society and economy. Cytokines are a group of secretory proteins produced by immune effector cells, such as T lymphocytes, B lymphocytes, macrophages and vascular endothelial cells. Meanwhile, cytokines are capable of regulating and determining the nature of immune response. Recent studies have found that they are involved in various aspects of immunity and inflammation, including antigen presentation, cell differentiation, adhesion molecule expression and inflammatory cell infiltration and activation[4]. Th1 and Th2 are two subpopulations of $CD4^+$ T helper cells. Cytokines secreted by the above two cells are called Th1 and Th2 cytokines, respectively. Researches have been performed on investigating the role of cytokines in COPD. As one of the major functional genes, Th2 cytokine IL-9 has been well studied in the pathogenesis of bronchial asthma. However, few studies have explored the effect of IL-9 on COPD pathogenesis. IL-9 receptor (IL-9R) is widely presented on the membrane surface of cells, such as neutrophils and alveolar macrophages. Meanwhile, IL-9 is a pleiotropic cytokine secreted by $CD4^+$ Th2 cells, which has a variety of immunomodulatory effects in immune response[5]. Due to its important potential clinical value, IL-9 has attracted more and more attention in recent years[6,7]. The role of oxidative stress in the pathogenesis of COPD has been well recognized[8]. Oxidative stress is the imbalance between oxidants and antioxidants, which is caused by production of excessive oxidants or reduction of antioxidants in the body. Long-term exposure to endogenous (e.g., phagocytic release) or exogenous (e.g., air pollution and CS) oxidants, combined with decreased anti-oxidative enzyme activity and reduced non-enzymatic antioxidants ultimately lead to enhanced oxidative stress[9]. Oxidative stress not only directly damages lung tissues, but also inactivates anti-protease oxidation. This may eventually accelerate the occurrence and development of COPD. The aim of this study was to detect the expression levels of oxidative stress markers in peripheral blood of COPD mice, and to analyze the correlation between IL-9 and oxidative stress in COPD.

Materials and Methods

Animal Model

Male C57BL/6 mice (Model Animal Research Center of Nanjing University) were exposed to CS twice a day, with 5 cigarettes per time (i.e. 10 cigarettes/day). Smoking duration was more than 1 h each time for 5 consecutive weeks. One hour before CS exposure every time, mice were intraperitoneally injected with 100 μg IL-9 antibody. All mice were sacrificed 36 days after CS exposure, and lung tissues were collected for subsequent experiments. This study was approved by the Ethics Committee of Jiaxing First Hospital.

Hematoxylin-Eosin (H&E) Staining

Paraffin sections were dewaxed with xylene solution and placed in alcohol solution for 2 min. After normal dehydration, the sections were transparently processed and sealed. Finally, the tissues were stained with hematoxylin and eosin. Finally, the staining results were observed under an optical microscope.

Immunohistochemistry

Paraffin-embedded lung tissues were cut into 3-μm thick slices and paraffined. The sections were then subjected to xylene and ethanol, followed by incubation with blocking solution at room temperature for 30 min. Subsequently, the tissue samples were incubated with primary antibody at 4°C overnight, followed by incubation with secondary antibody at room temperature for 1 h. The sections were sealed and photographed using a Nikon Eclipse 80i microscope.

Western Blot

Total protein was extracted using cell lysates (RIPA) (Beyotime (Shanghai, China) containing protease and phosphatase inhibitors. Extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were incubated with primary antibodies of IL-9, sIL-9R, STAT3

and p-STAT3 at 4°C overnight. After washing with PBS, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 2 h. Immuno-reactive bands were exposed by the enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) method. The integrated optical density (IOD) values of each band were determined by a gel imaging analysis system.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was then performed according to the instructions of PrimeScript RT Kit (TaKaRa, Otsu, Shiga, Japan). Diluted complementary deoxyribose nucleic acid (cDNA), as well as a certain amount of primers, premix and ultra-pure water was mixed into a 20-μL reaction system. Specific cDNA amplification was performed using an ABI 7500 FAST real-time PCR machine. Primers used in this study were as follows: IL-9, forward: 5'-ATGTTGGTGACATACATCCTTGC-3', reverse: 5'-TGACGGTGGATCATCCTTCAG-3'; STAT3, forward: 5'-CAATACCATTGACCTGCCGAT-3', reverse: 5'-GAGCGACTCAAACTGCCCT-3'; Beta-actin, forward: 5'-ATCTACGAGGGCTATGCTCT-3', reverse: 5'-TACTCCTGCTTGCTGATCCA-3'.

Detection of SOD and MDA

Superoxide dismutase (SOD) was obtained using the WST-1 method. Blank wells, standard wells, treatment wells and control wells were prepared

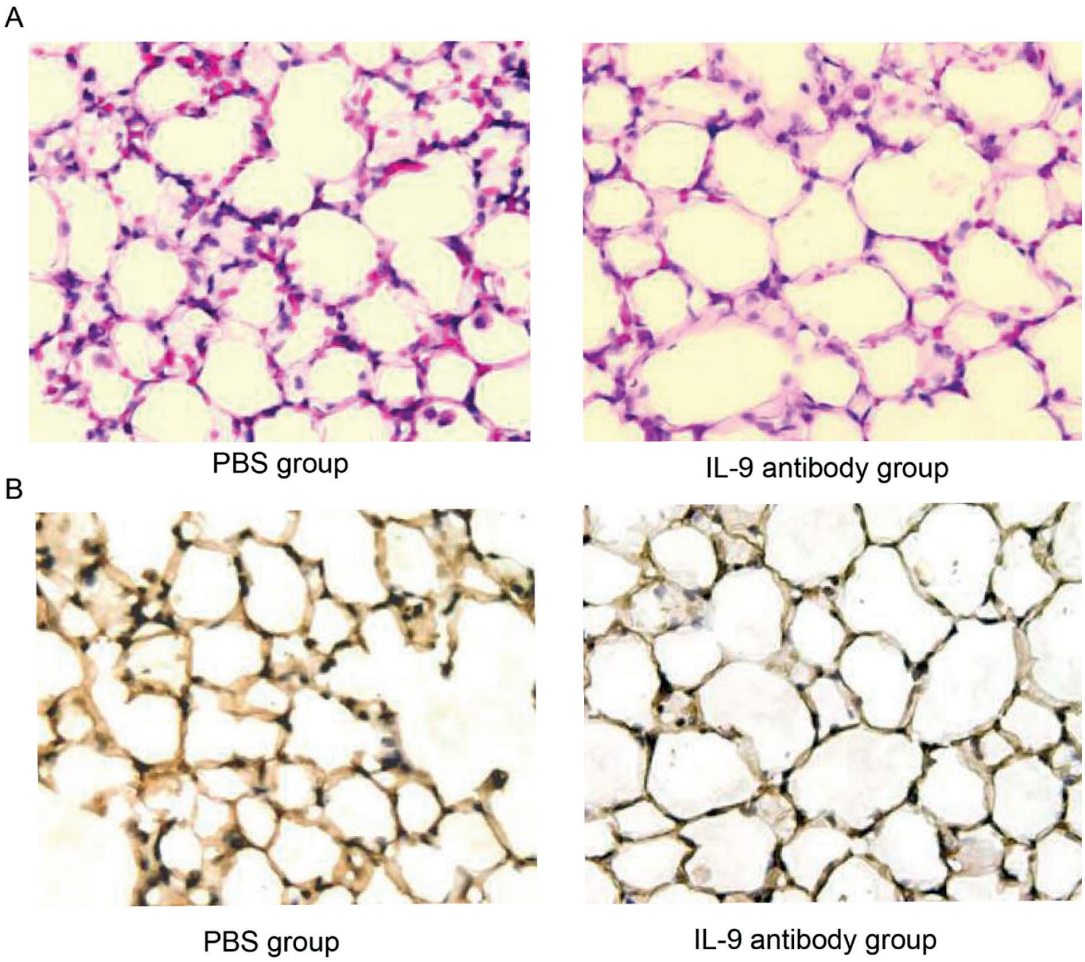

Figure 1. Inhibition of IL-9 expression significantly inhibited the structural destruction of lung tissue in COPD mice (Magnification*40). ***A,*** H&E staining of lung tissues in COPD mice. ***B,*** IL-9 expression in the lung tissues of COPD mice was measured by immunohistochemistry. Representative microphotographs of IL-9$^+$ cells (scale bars = 100 μm) were shown. The qualification of IL-9$^+$ cells were calculated from three randomly selected fields. For all graphs, error bars indicated mean ± SD (*p<0.05, **p<0.01, ***p<0.001).

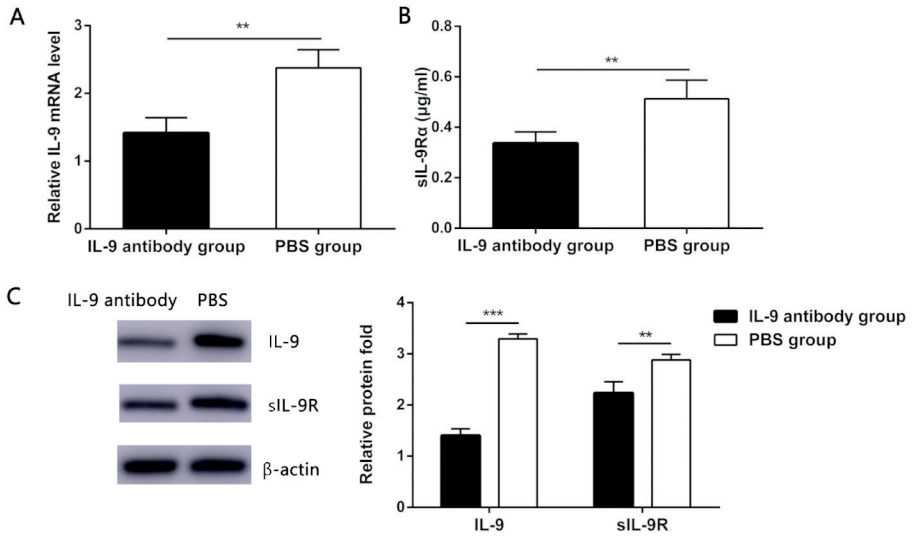

Figure 2. IL-9 positively regulated the expression of sIL-9R in peripheral blood. ***A,*** Assessment of IL-9 transcript abundance by qRT-PCR. ***B,*** Detection of protein level of sIL-9R by ELISA. ***C,*** Determination of protein levels of IL-9 and sIL-9R by immunoblotting. β-actin was used as a loading control. For all graphs, error bars indicated mean ± SD (*p<0.05, **p<0.01, ***p<0.001).

as required, respectively. The cells were incubated at 37°C for 20 min. Absorbance value of each well at 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA). Malondialdehyde (MDA) was measured using the thiobarbituric acid method. Blank wells, standard wells, treatment wells and control wells were prepared as required, respectively. The absorbance of each well at 532 nm was detected by a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS 22.0) (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (La Jolla, CA, USA) were used for all statistical analysis and image processing, respectively. Survival analysis was performed using Kaplan-Meier survival curves. Independent sample t-test was introduced to analyze the differences between two groups. x^2-test was used for comparison of classification data. All quantitative data were expressed as mean ± standard deviation. p-values < 0.05 were considered statistically significant.

Results

Inhibition of IL-9 Expression Improved Structural Destruction of Lung Tissue in COPD Mice

To investigate the role of IL-9 in COPD, we established a mouse COPD model by CS exposure. The lung tissues of mice were fixed with paraformaldehyde and then sectioned. H&E staining showed that there were a large number of inflammatory cells infiltrating into the vascular wall and surrounding vascular tissue in mice of the PBS group. In addition, PBS-treated mice exhibited significant airway structural disturbances, degeneration, necrosis and loss of ciliary columnar epithelium. Meanwhile, severe damage to alveolar epithelium, narrowing of alveolar septum, and larger cysts fused by some alveoli were also observed. However, lung structure of mice in the IL-9 antibody group was significantly less disrupted than that of the PBS group (Figure 1A). Further immunohistochemistry results showed that the level of IL-9 in lung tissues of the PBS group was remarkably higher when compared with that of the IL-9 antibody group (Figure 1B). The above results indicated that inhibition of IL-9 expression significantly improved the pathological changes of lung tissue in COPD mice.

Inhibition of IL-9 Decreased the Level of sIL-9R in Peripheral Blood of COPD Mice

To explore the role of IL-9 and its receptor sIL-9R in the pathogenesis of COPD, peripheral blood samples were collected from the internal iliac vein. QRT-PCR results showed that the mRNA level of IL-9 in the PBS group was remarkably higher than that of the IL-9 antibody group (Figure 2A). Next, we detected the expression of sIL-

9R by ELISA, and found that sIL-9R level in the IL-9 antibody group was remarkably decreased when compared with that of the PBS group (Figure 2B). Similar results were obtained from Western blot (Figure 2C). In general, the expression of serum free receptors was markedly reduced in COPD mice after IL-9 inhibition.

IL-9 Promoted Inflammation By Activating Phosphorylation of STAT3

Previous studies have shown that IL-9 can bind to IL-9 receptor, thereby activating STAT3 and its associated downstream genes. To verify the effect of IL-9 on STAT3 activation in COPD, we first examined the mRNA level of STAT3 by qRT-PCR. Results illustrated that inhibition of IL-9 expression could not affect the mRNA level of STAT3 (Figure 3A). However, Western blot results indicated that the protein expression level of p-STAT3 was remarkably increased in the PBS group when compared with that of the IL-9 antibody group (Figure 3B). These data revealed that IL-9 might promote the development of inflammation in COPD mice by activating STAT3 phosphorylation.

IL-9 Intensified Oxidative Stress In Lung Tissue of COPD Mice

Oxidative stress is reported to be one of the important mechanisms leading to the development of COPD. Meanwhile, COPD patients experience systemic and local oxidation-reduction imbalance. In this study, we used SOD and MDA commercial kits to detect the level of oxidative stress in the two groups, respectively. SOD eliminates free radicals in the body and fights oxidative stress. MDA level reflects the degree of membrane lipid peroxidation, and indirectly indicates the degree of oxidative damage. Our data suggested that compared with the IL-9 antibody group, SOD level in the PBS group was significantly decreased, while the MDA level was significantly increased ($p<0.05$) (Figure 4A-4B). In addition, the amount of ROS in the PBS group was also remarkably higher than that of the IL-9 antibody group (Figure 4C).

Discussion

COPD is a chronic inflammatory disease involving a variety of cells and cellular components. COPD lesions mainly affect airways, lung parenchyma and pulmonary vessels. Persistent and progressive increase of irreversible airflow restriction is the major characteristic of COPD. The inflammatory response of airway in COPD patients mainly involves neutrophils, monocyte macrophages and lymphocytes. In addition, airway epithelial cells, eosinophils and mast cells are reported to participate in this process. Key link of this process is the activation and aggregation of neutrophils. Current studies have suggested that excessive chronic inflammatory response of COPD is due to its immune-regulatory disorders. The mortality and morbidity of

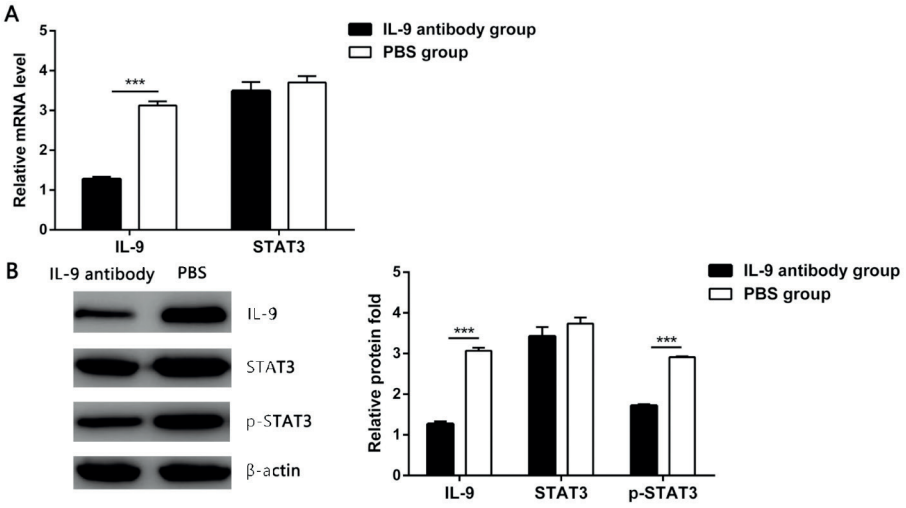

Figure 3. IL-9 promoted inflammation by promoting phosphorylation of STAT3. ***A,*** Assessment of STAT3 transcript abundance by qRT-PCR. ***B,*** Protein levels of STAT3 and p-STAT3 were determined by immunoblotting. β-actin was used as a loading control. For all graphs, error bars indicated mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

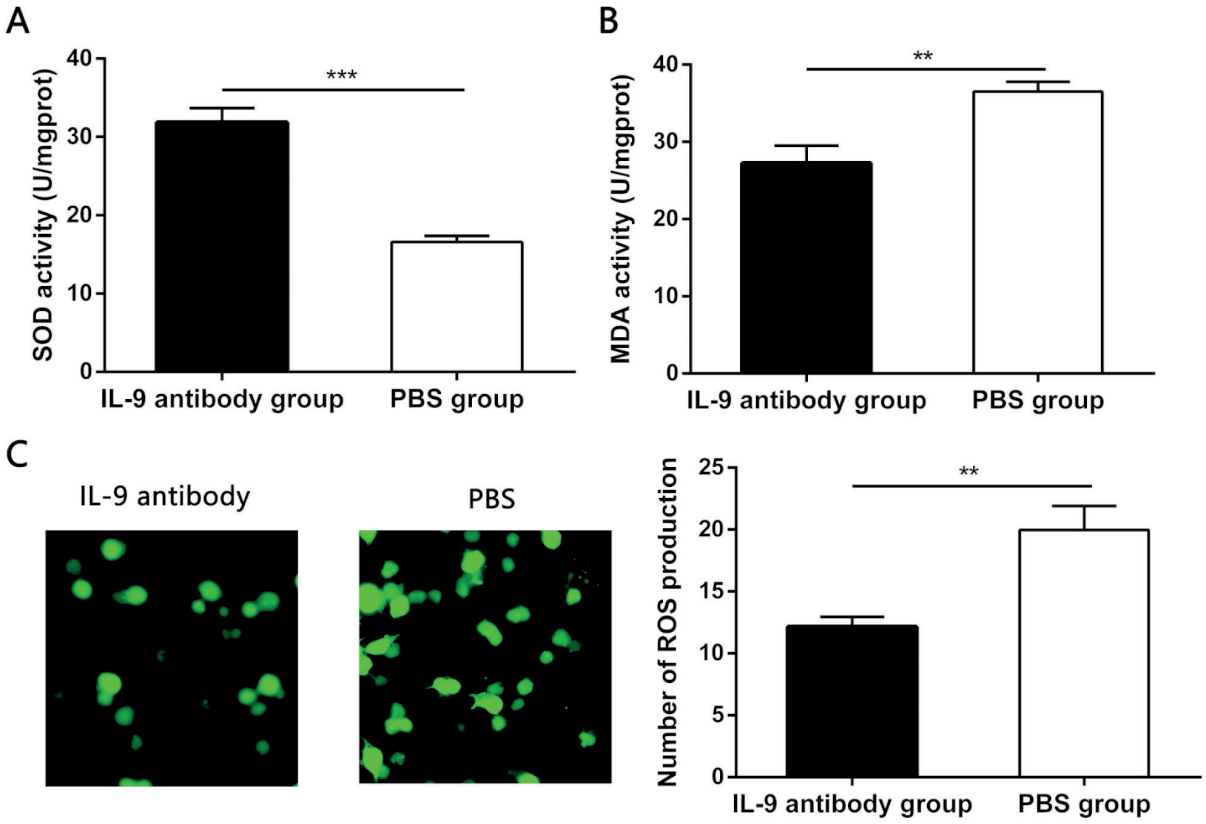

Figure 4. IL-9 aggravated oxidative stress in COPD mice. ***A,*** SOD activity of mice in the PBS group was significantly reduced. ***B,*** MDA activity of mice in the PBS group was significantly increased. ***C,*** The number of ROS^+ cells in the PBS group was significantly larger. The identification results were calculated from three randomly selected fields. For all graphs, error bars indicated mean ± SD (*p<0.05, **p<0.01, ***p<0.001).

COPD remain high, making it a serious threat to public health[10]. It is reported that there are about 3 million people die from COPD every year in the world. The global economic burden caused by COPD accounts for about 70% annually[11,12]. In China, COPD ranks third in the overall death cause, which ranks first of death cause in rural areas[13]. Therefore, prevention and treatment of COPD is particularly important.

The exact pathogenesis of COPD is not yet clear. It is believed that inflammatory reactions, oxidative stress, protease-resistant protease disorders and autoimmunity all contribute to the development of COPD. Oxidative stress is considered as the main cause of smoking-induced COPD[14]. Under normal circumstances, the oxidative and antioxidant systems can maintain a dynamic balance. Once this balance is broken, oxidative stress is stimulated, eventually leading to relative diseases[15]. The production of MDA is believed to be consistent with oxygen free radicals. Hence, the determination of MDA reflects the level of lipid peroxidation. Previous studies have demonstrated that lipid peroxidation is one of the mechanisms that lead to lung injury. Therefore, MDA was used as an indicator to assess the level of lung oxidation in this study[16]. Besides, antioxidants deficiency is also an important cause of oxidative enhancement and antioxidant imbalances. Abnormalities in enzymatic and non-enzymatic antioxidant systems have also been observed in COPD patients[17]. In this study, we detected the levels of SOD and MDA to reflect oxidative stress in COPD mice. Persistent chronic inflammation is the main pathological feature of COPD. Many inflammatory chemokines secreted by macrophages and epithelial cells induce neutrophils, monocytes and lymphocytes, and transfer the circulatory system into the lungs. These inflammatory cells are activated to release large amounts of inflammatory mediators. This may destroy the lung structure and further promote inflammation. IL-9 is a pleiotropic cytokine secreted by $CD4^+$ Th2 cells.

IL-9 has a variety of immunomodulatory effects on the body's immune response, particularly on T lymphocytes, eosinophils and mast cells[18]. It is reported that IL-9 elevates the mRNA expression of high-affinity IgE receptor H-chain on the surface of mast cell membranes. IL-9 is also capable of stimulating mast cells to produce Th2 cytokines, such as cytokine IL-6, thus participating in inflammatory reactions[19]. Meanwhile, IL-9 stimulates the proliferation and differentiation of mast cells[20]. Various studies[21,22] have detected the expression of IL-9R on the surface of human peripheral blood poly-nuclear neutrophils (PMN). Results have found that IL-9R also exists in PMN. In addition, IL-9 induces the release of IL-8 *via* IL-9R located on the surface of PMN membrane, whereas anti-IL-9 neutral antibody can prevent this process. In short, there is still a lack of effective prevention and treatment methods for COPD. We found that IL-9 exerted a crucial role in the inflammation and oxidative stress of COPD. Furthermore, IL-9 might be a potential target for the prevention and treatment of COPD.

Conclusions

We demonstrated that IL-9 activated STAT3 and aggravated lung injury in COPD mice by up-regulating inflammatory and oxidative stress.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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