

IL-33 induced inflammation exacerbated the development of chronic obstructive pulmonary disease through oxidative stress

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Abstract. – **OBJECTIVE:** We aimed at exploring the role of IL-33 in mouse chronic obstructive pulmonary disease and its potential molecular mechanism.

MATERIALS AND METHODS: The chronic obstructive pulmonary disease (COPD) mice model was established by cigarette smoking (CS). COPD mice were randomly assigned into PBS group and IL-33 antibody group. The peripheral blood and lung tissues of mice from two groups were collected for the following experiments. Pathological changes of the lung tissues in both groups were analyzed by hematoxylin and eosin (HE) staining. IL-33 positive cells in lung tissues were detected by immunohistochemistry. Then, the mRNA and protein levels of IL-33, sST2, ERK and TNF- α in the mice peripheral blood of the two groups were accessed by Real-time polymerase chain reaction (RT-PCR) and Western blot. Finally, the indicators related to oxidative stress, including superoxide dismutase (SOD), malondialdehyde (MDA) and reactive oxygen species (ROS) in the mice serum of two groups were measured.

RESULTS: After successful construction of COPD mouse model by CS, HE staining illustrated that the structure of airway wall of lung tissue in mice from PBS group was irregular. The ciliated columnar epithelium presented significant degeneration, necrosis and shedding. A large amount of inflammation cell infiltration was observed in vascular tissues. The alveolar epithelial structure was severely damaged and alveolar septum was narrowed and ruptured. Adjacent alveoli were found to be fused into larger cysts. The above pathological changes were relatively better in mice from IL-33 antibody group. Immunohistochemical results demonstrated that IL-33 was remarkably deposited in the lung tissue of PBS group. The mRNA and protein levels of IL-33, sST2, ERK and TNF- α in peripheral blood of PBS group were much higher than those of IL-33 antibody group. At the same time, SOD level in PBS group decreased, while MDA level and ROS production increased.

CONCLUSIONS: IL-33 aggravates lung injury in COPD mice by increasing inflammation response and oxidative stress, which may serve as a target for predicting and treating COPD.

Key Words:

IL-33, COPD, Oxidative stress, Inflammatory response.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized as chronic bronchitis and/or emphysema with airflow obstruction. It can further develop into pulmonary heart disease and respiratory failure¹. COPD is considered to be related to harmful gases and particles, thereby leading to the abnormal inflammatory response with a high morbidity and mortality². COPD is projected to become the third leading cause of death in the world by 2020, with the global financial burden rising to the fifth in all diseases³. Studies⁴ have suggested that the factors associated with the occurrence of chronic bronchitis and obstructive pulmonary emphysema may be involved in the pathogenesis of COPD. The risk factors that have been found include smoking, dust, chemical inhalation, air pollution and respiratory infections, etc.⁵. Among them, smoking is the most common risk factor causing COPD worldwide⁶. Recent studies⁷ have shown that immune response of COPD is activated by CS. Activated immune cells release a variety of inflammatory factors, causing pathological changes, such as small airway remodeling, destruction of the alveolar wall and local atelectasis⁸. It is generally believed that chronic airway inflammation, progressive pulmonary function decline and oxidative stress

damage are the main manifestations of COPD^{9,10}. Currently, multiple studies have pointed out that inflammatory cells, cytokines and inflammatory mediators may participate in the regulation of COPD¹¹. Among them, IL-33 is mainly involved in allergic diseases such as allergic rhinitis, asthma and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus¹². IL-33 is located at p13.3, which binds to ST2, an IL-1 receptor family member. Binding of IL-33 and its receptor can activate NF- κ B and MAPK signaling pathways, contributing to the production of Th2 cytokines involved in allergic reactions^{13,14}. IL-33 is thought to be expressed predominantly in the endothelial cells and smooth muscle cells. Importantly, it is found that IL-33 is up-regulated in these cells upon inflammatory stimuli¹⁵. IL-33 and its receptor exert an essential function in the inflammatory process and host immune regulation, thereby becoming a hot research topic. Meanwhile, about 90% of COPD patients have a history of smoking, which is considered the most common cause of COPD¹⁶. A large number of studies¹⁷ have pointed out that the chemical substances in cigarette smoke, including hydroxyl radicals, hydrogen peroxide, reactive oxygen species, etc. will cause oxidative stress damage, thereby increasing the disease development of COPD. In summary, the study of cytokines and oxidative stress to the progression of COPD has a very crucial clinical significance in the prediction and treatment of COPD.

Materials and Methods

Animal Model

C57BL/6 male mice were exposed to smoke twice a day with 10 cigarettes, 5 cigarettes at 8:00 am and 4:00 pm, respectively. Smoking duration was more than 1 h for consecutive 5 weeks. The IL-33 antibody group and PBS group were intraperitoneally injected with 100 μ g of IL-33 antibody (diluted to 200 μ L with PBS) and/or 200 μ L of PBS, respectively, 1 h prior to smoking every week. Mice were sacrificed 36 days after CS and lung tissues were harvested for pathology, gene and protein analysis. Animal experiments were approved by Ethics Committee.

HE Staining

After paraffin slices were treated by dewaxing into xylene solution, slices were placed into the alcohol solution for 2 min, and washed with dis-

tilled water. Paraffin slices were then stained with hematoxylin and eosin (HE), respectively. After dewaxing and washing with water for 2 min, conventional dehydration, transparent treatment, sealing slices were performed. Staining results were observed under a light microscope.

Immunohistochemistry

Paraffin-embedded lung tissue was cut into 3 μ m slices, paraffinized, and rehydrated by xylene, ethanol and purified water, then blocked with blocking solution for 30 min at room temperature. Primary antibodies were used for incubation overnight, and secondary antibodies were used for incubation for 1 h at room temperature. Next, slices were sealed, and nikon Eclipse 80i microscope was used for taking pictures.

Western Blot

Total protein from serums was extracted using a cell lysate (RIPA) containing protease. Protein samples were then separated by conventional electrophoresis and incubated with primary antibodies overnight at 4°C (IL-33, sST2, ERK and TNF- α , CST, 1:1000, Danvers, MA, USA). After washed with phosphate-buffered saline (PBS), the membranes were incubated with horseradish peroxidase (HRP) labeled secondary antibody (goat anti-rabbit IgG, CST, 1:5000, Danvers, MA, USA) for 2 h at room temperature. Enhanced chemiluminescence (ECL, Beyotime, Shanghai, China) was performed to imaging, and the integral optical density (IOD) value of each band was determined by Gel imaging analysis system. β -actin was taken as the internal reference.

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

We used TRIzol to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). Diluted cDNA and a certain amount of primers, premix, and ultrapure water, were mixed into a 20 μ L reaction system. ABI 7500 FAST Real-time PCR instrument was used for cDNA amplification. The expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. Primers used in RT-PCR were as follows: IL-33, forward 5'-GGAATTCCATATGACATTGAGCATTGAGCATCCAAGGAAC-3', reverse 5'-CCGCTCGAGGATTTTCGAGAGCTTAAACA-3'; sST2, forward 5'-AAAACAAGCTTGCCCGCCACCATGATTGACAGACAGAG-3', reverse 5'-AAAACGGATCCGAGCAATGTGTGAGG-

GACTCCT-3'; ERK, forward 5'-CTAAAC-CACATCGGGAACCT-3', reverse, 5'-TACTTG-GGGGCTTTGATGGA-3' and TNF- α , forward 5'-AACTAGTGGTGCCAGCCGAT-3', reverse 5'-CTTCACAGAGCAATGACTCC-3'; β -actin, forward 5'-ATCTACGAGGGCTATGCTCT-3', reverse 5'-TACTCCTGCTTGCTGATCCA-3'.

Detection of SOD and MDA

Superoxide dismutase (SOD) was accessed using WST-1 method. Blank wells, standard wells, determination wells and control wells were prepared according to the requirements. Cells were incubated at 37°C for 20 min. Absorbance value of each well at 450 nm was detected by a microplate reader.

Malondialdehyde (MDA) was determined by thiobarbituric acid method. According to the instructions provided by the company, blank wells, standard wells, determination wells and control wells were prepared. Absorbance value of each well at 532 nm was detected by a microplate reader.

Statistical Analysis

We used statistical product and service solutions (SPSS 22.0, IBM, Armonk, NY, USA) software for data analysis. GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) was introduced

for image processing. Survival analysis was performed using Kaplan-Meier survival curves. Independent-sample *t*-test was performed to analyze the difference between two groups and χ^2 -test was performed to analyze the classification data. All data were expressed as mean \pm standard deviation. $p < 0.05$ was considered statistically significant.

Results

Inhibition of IL-33 Expression Significantly Suppressed the Structure Destruction of Lung Tissue in COPD Mice

CS was successfully introduced for the establishment of COPD mouse model. After treatment, the lung tissues of mice in each group were prepared into slices after fixation by paraformaldehyde, and pathological changes of lung tissues were observed. HE staining illustrated that the airway structure in lungs of mice injected with PBS was irregular, and the ciliated columnar epithelium presented significant degeneration, necrosis and shedding. A large amount of inflammatory cell infiltration was also observed in the vascular wall and surrounding vascular tissues. Furthermore, for the PBS group, the alveolar epithelial structure was severely damaged

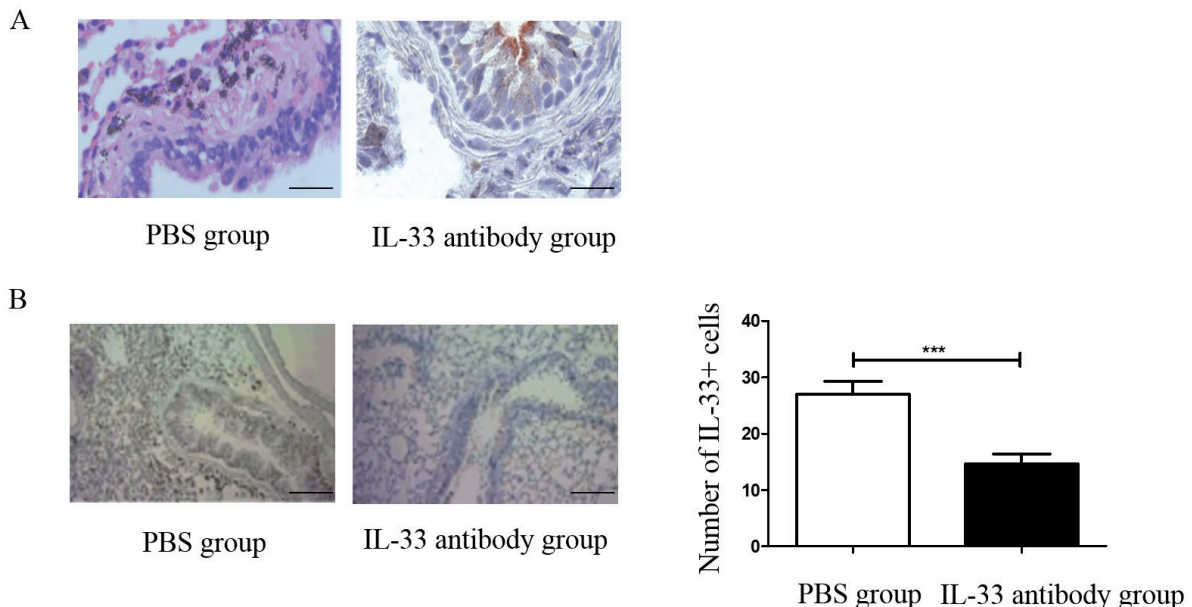


Figure 1. Inhibition of IL-33 expression significantly suppressed the structure destruction of lung tissue in COPD mice. **A**, HE staining of pathological change of lungs in COPD mice (40 \times). **B**, Immunohistochemistry results demonstrated IL-33 expression in lungs of COPD mice. The represented microphotographs of IL-33-immunopositive cells and the qualification of IL-33 positive cells calculated from three random fields were shown (40 \times). For all graphs, error bars indicate mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

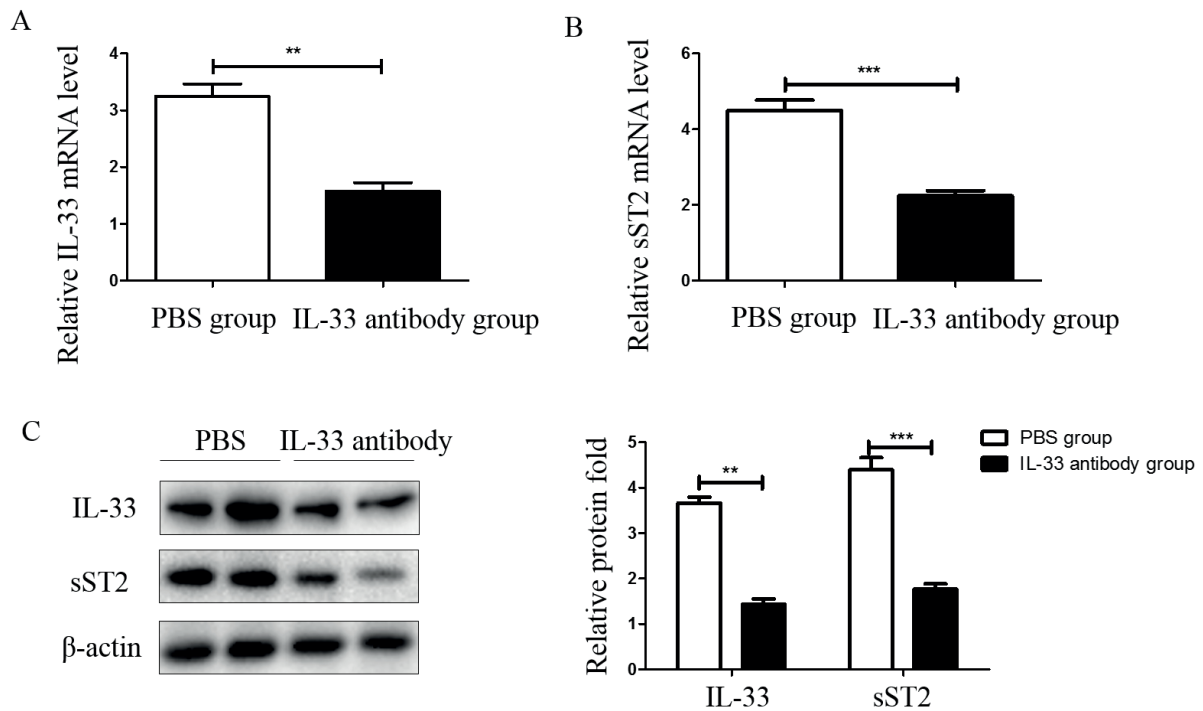


Figure 2. IL-33 positively regulated the expression level of sST2 in peripheral blood. **A**, The transcriptional abundance of IL-33 was evaluated by qPCR. **B**, The transcriptional abundance of sST2 was evaluated by qPCR. **C**, Protein levels of IL-33 and sST2 were determined by immunoblotting. β -actin was used as the loading control. For all graphs, error bars indicate mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001.

and alveolar septum were narrowed and ruptured. The adjacent alveoli were found to be fused into larger cysts. In contrast, the above pathological changes were relatively better in mice from IL-33 antibody group (Figure 1A). Immunohistochemistry results demonstrated that IL-33 expression was significantly precipitated in the mouse lung tissue of the PBS group (Figure 1B). The above data indicated that inhibition of IL-33 by IL-33 antibody can significantly reduce the pathological changes of lung tissues in COPD mice.

IL-33 Positively Regulated the Expression Level of sST2 in Peripheral Blood

Peripheral blood samples of mice were harvested from the angular vein for total RNA extraction after centrifugation. We found that the mRNA level of IL-33 in peripheral blood of mice injected with PBS was remarkably higher than that of IL-33 antibody group (p <0.05) (Figure 2A). Subsequently, we examined the expression level of sST2, a member of the IL-33-coupled receptor family, whose change in mRNA level was in line with the changing trend of IL-33 (Figure 2B). Western blot revealed similar results in the protein levels of IL-33 and sST2 (Figure 2C).

IL-33 Promoted Inflammation Through NF- κ B/MAPK Signaling Pathway

Numerous studies have shown that IL-33 as a pro-inflammatory cytokine, activates the nuclear factor NF- κ B and MAPK signaling pathways after binding to sST2. We hypothesized that NF- κ B/MAPK signaling may be involved in mouse COPD. First, we determined the expression levels of TNF- α and ERK in the NF- κ B/MAPK signaling pathway by RT-PCR. The data implied that the mRNA levels of TNF- α and ERK were upregulated by IL-33 expression (Figure 3A). Western blot also confirmed that protein levels of TNF- α and ERK were significantly elevated in the PBS group compared to the IL-33 antibody group (Figure 3B). The above experimental results showed that IL-33 promote inflammation in COPD mice through the NF- κ B/MAPK signaling pathway.

IL-33 Aggravated Oxidative Stress in COPD Mice

Recent studies have shown that oxidative stress is one of the important mechanisms leading to the development of COPD. COPD patients in the exacerbation and stable stage exert imbalance of systemic and local redox reactions. Here we used

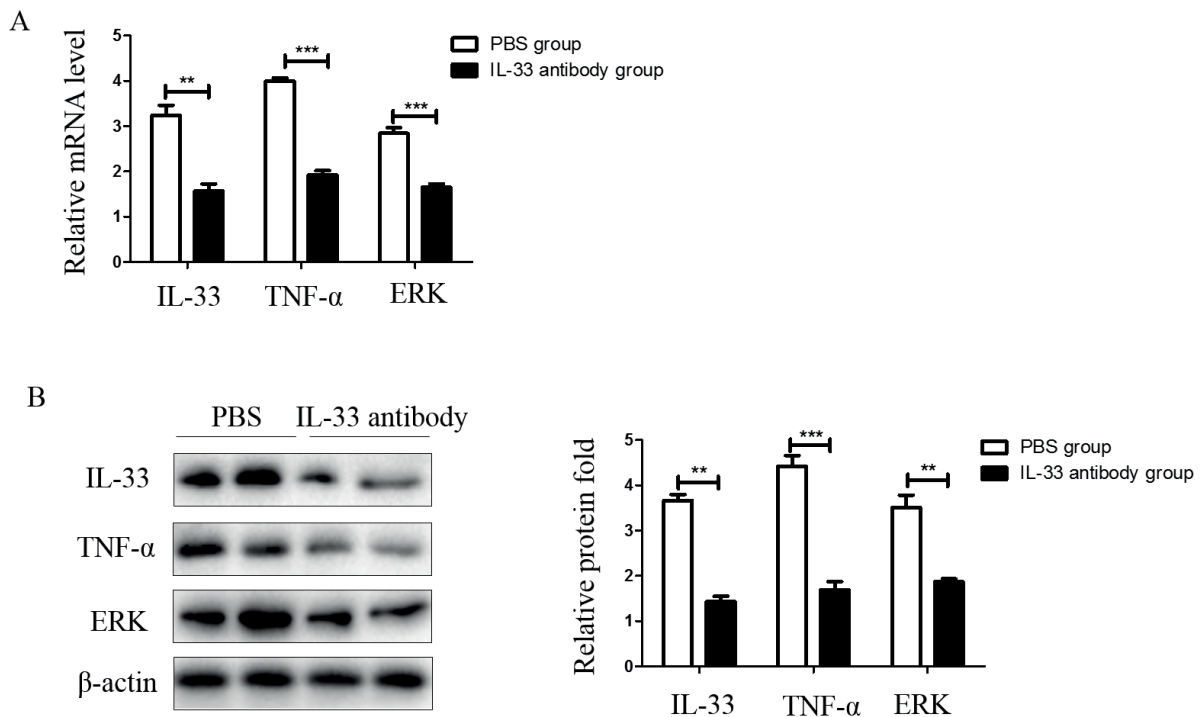


Figure 3. IL-33 promoted inflammation through NF- κ B/MAPK signaling pathway. **A**, The transcriptional abundance of TNF- α and ERK were evaluated by qPCR. **B**, Protein levels of TNF- α and ERK were determined by immunoblotting. β -actin was used as the loading control. For all graphs, error bars indicate mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001.

SOD and MDA kits to examine the extent of oxidative stress in COPD mice from the two groups. SOD can scavenge free radicals in the body and antagonize oxidative stress. MDA level reflects the degree of membrane lipid peroxidation, indirectly representing the extent of oxidative damage. Our data showed that compared with the IL-33 antibody group, the level of SOD in PBS group was significantly decreased (Figure 4A) and MDA level was significantly increased (Figure 4B), the differences of which were statistically significant (p <0.05, respectively). ROS level in the lungs of both groups also suggested that the amount of ROS produced in the PBS group was remarkably greater than IL-33 antibody group (Figure 4C).

Discussion

Chronic obstructive pulmonary disease (COPD) is an umbrella term used to describe progressive lung diseases including emphysema, refractory asthma and chronic bronchitis¹⁸. The pathological changes of COPD are mainly manifested by degeneration and necrosis of bronchial epithelial cells, adhesion and lodging of the cil-

ium, and infiltration of inflammatory cells. Although it is considered that COPD was related to the abnormal inflammatory response against CS and/or other harmful gases, the exact mechanism of COPD has not been clearly elucidated¹⁹.

Smoking is a major risk factor for COPD. Cosio et al²⁰ suggested that CS can directly damage pulmonary epithelial cells, which in turn produce a large number of chemokines for innate immune response. In addition, effect of T cells and B cells infiltration may lead to adaptive immunity, thus resulting in airway and lung tissue necrosis, apoptosis, airway remodeling and other pathological changes of COPD²¹. Additionally, inflammatory response caused by CS will persist even though the patients quit smoking. We first used a CS exposure method to construct the COPD mouse model. HE staining illustrated that the pathological changes in COPD mice, such as enlarged alveoli, partial alveolar septum breakage, alveolar fusion, emphysema formation and inflammatory cell infiltration were observed. The histopathological changes of the lungs in the PBS group were more pronounced (Figure 1A). IL-33 was first reported present in endothelial cells. Subsequent studies²² found that IL-33 can serve as a warning

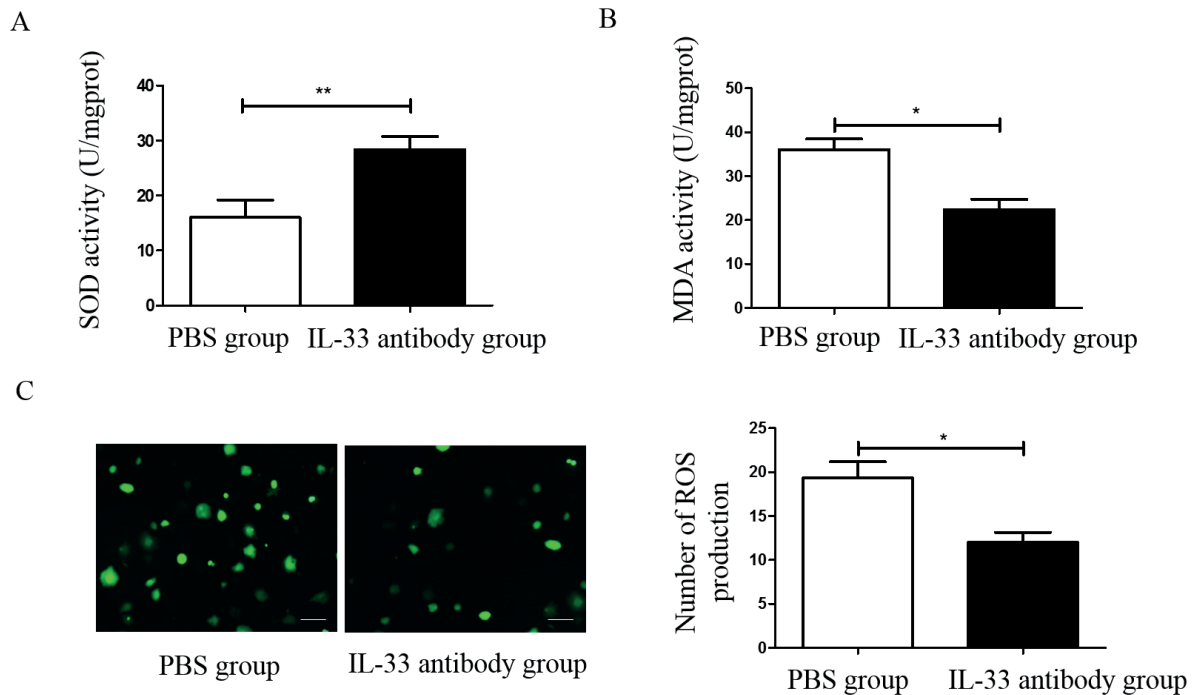


Figure 4. IL-33 aggravated oxidative stress in COPD mice. **A**, The activity of SOD in mice from PBS group was significantly decreased. **B**, The activity of MDA in mice from PBS group was significantly increased. **C**, ROS number in the PBS group was remarkably larger (20 \times). Qualification result was calculated from three random fields. For all graphs, error bars indicate mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001.

factor, which is released when inflammation or cell necrosis occurs. Pastorelli et al²³ have found that, in patients with active ulcerative colitis, IL-33 expression was significantly enhanced in mucosal epithelial cells. Here, immunohistochemistry results suggested that IL-33 was significantly deposited in the lung tissue of COPD mice, while the amount of protein deposition was significantly decreased after injection of anti-IL-33 antibody, indicating successful construction of this model (Figure 1B). The above data were consistent with the immunofluorescence results in detecting elevated protein level of IL-33 in bronchial epithelial cells of COPD patients²⁴. As a result, we believed that IL-33 exerts an essential role in the development and progression of COPD. By literature reviews^{25,26}, we observed that asthma-related studies have shown that inflammation induced by IL-33 depends on the regulation of NF- κ B/MAPK signaling pathway. We, therefore, suggested that in the development of COPD, whether IL-33 exacerbated inflammation of lung tissue depends on these signaling pathways. Therefore, we examined the expression levels of TNF- α and ERK in peripheral blood, the key molecules of NF- κ B/

MAPK signaling. Both Western blot and RT-PCR results showed that IL-33 significantly changed the protein and mRNA levels of TNF- α and ERK (Figure 3), suggesting that IL-33 induced inflammation also depends on NF- κ B/MAPK signaling pathway. Further studies have also found that IL-33 plays a crucial role in causing COPD inflammation and oxidative stress (Figure 4). The above studies suggested that IL-33 may serve as a molecular target for the prediction and treatment of COPD, which provides the basis for future study of COPD targeted therapy.

Conclusions

In the COPD mouse model, IL-33 aggravates COPD lung injury by increasing the inflammatory response and oxidative stress. IL-33 provides the experimental basis for the study of molecular targets for prediction and treatment of COPD.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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