International Immunopharmacology 76 (2019) 105871

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

PM2.5 inhibits SOD1 expression by up-regulating microRNA-206 and promotes ROS accumulation and disease progression in asthmatic mice

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ARTICLE INFO

Keywords:
Asthma
MicroRNA-206
SOD1
ROS

ABSTRACT

Bronchial asthma is the most common chronic respiratory disease. Chronic airway inflammation, airflow restriction and airway hyper-responsiveness are its main manifestations. In recent decades, the prevalence and mortality of asthma have been increasing all over the world, which seriously threatens public health. Research suggests that air pollution is associated with the increased incidence of asthma. PM2.5 is one of the most complex pollutants in the atmospheric environment and harmful to human health. It is related to the incidence of asthma. However, the molecular mechanism of PM2.5 in the development of asthma is still unclear. In this study, we established a mouse model of asthma using CRE to observe the effect of PM2.5 on the symptoms of asthmatic mice and its possible molecular mechanism. The results showed that PM2.5 could significantly increase airway resistance and pulmonary inflammation, increase the number of inflammatory cells, eosinophils, macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid in asthmatic mice. Moreover, PM2.5 could reduce the contents of antioxidant enzymes such as CAT, GSH, GSH-Px and T-SOD in lung tissue of mice, and increase the ROS level. PM2.5 can promote the expression of microRNA-206 in lung tissue of mice. miR-206 can target the 3'-UTR of SOD1 to inhibit SOD1 expression, which leads to the increase of ROS level and aggravates pulmonary inflammatory response and asthma symptoms in asthmatic mice. This study found the possible molecular mechanism of PM2.5 aggravating asthma, and miR-206 may be a potential target for asthma treatment.

1. Introduction

Bronchial asthma is the most common chronic respiratory disease in children, which seriously affects children's physical and mental health [1]. In recent years, the prevalence and mortality of asthma have been increasing all over the world. At present, there are 300 million asthmatic patients, asthma has become a chronic respiratory disease which seriously threatens public health in the world [2].

Particular matter < 2.5 μm (PM2.5) refers to particulate matter with aerodynamic equivalent diameter < 2.5 μm, which attracts more and more attention because of its impact on public health. Previous studies indicated that PM2.5 was related to the occurrence and development of many respiratory diseases such as COPD, asthma, lung cancer, etc. [3,4]. The concentration of PM2.5 with the most obvious symptoms during acute exacerbation of asthma ranged from 4.00 to 7.06 μg/ m^3, suggesting that the correlation between PM2.5 and acute exacerbation of asthma may not be constant in different concentrations of PM2.5 [5]. Many studies have confirmed that PM2.5 could increase ROS production. ROS increased in peripheral blood supernatants, lung and heart tissues in mice exposed to PM2.5 [6–9]. Excessive ROS could cause a series of damage to airway epithelial cells and alveolar cells, including structural damage, activation of inflammatory-related transcription factors, and the release of inflammatory mediators [10–12]. Frossi et al. found that oxidative stress increased Th2-mediated inflammatory response, the severity of asthma, the bronchial hyperresponsiveness, and promoted airway remodeling [13].

miRNA is an endogenous small molecule RNA with a length of about 22 nt. It does not contain an open reading frame (ORF) and has no ability to encode proteins. The conventional way of its action is to regulate the expression of target genes by binding to the 3′-untranslational region (3'-UTR) of target genes [14]. PM2.5 can affect gene and microRNA expression. Study on zebrafish showed that embryo exposure to PM2.5 could cause significant changes in gene and microRNA expression patterns in zebrafish. The most influential gene functional

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https://doi.org/10.1016/j.intimp.2019.105871
Received 7 May 2019; Received in revised form 14 August 2019; Accepted 30 August 2019
Available online 11 September 2019

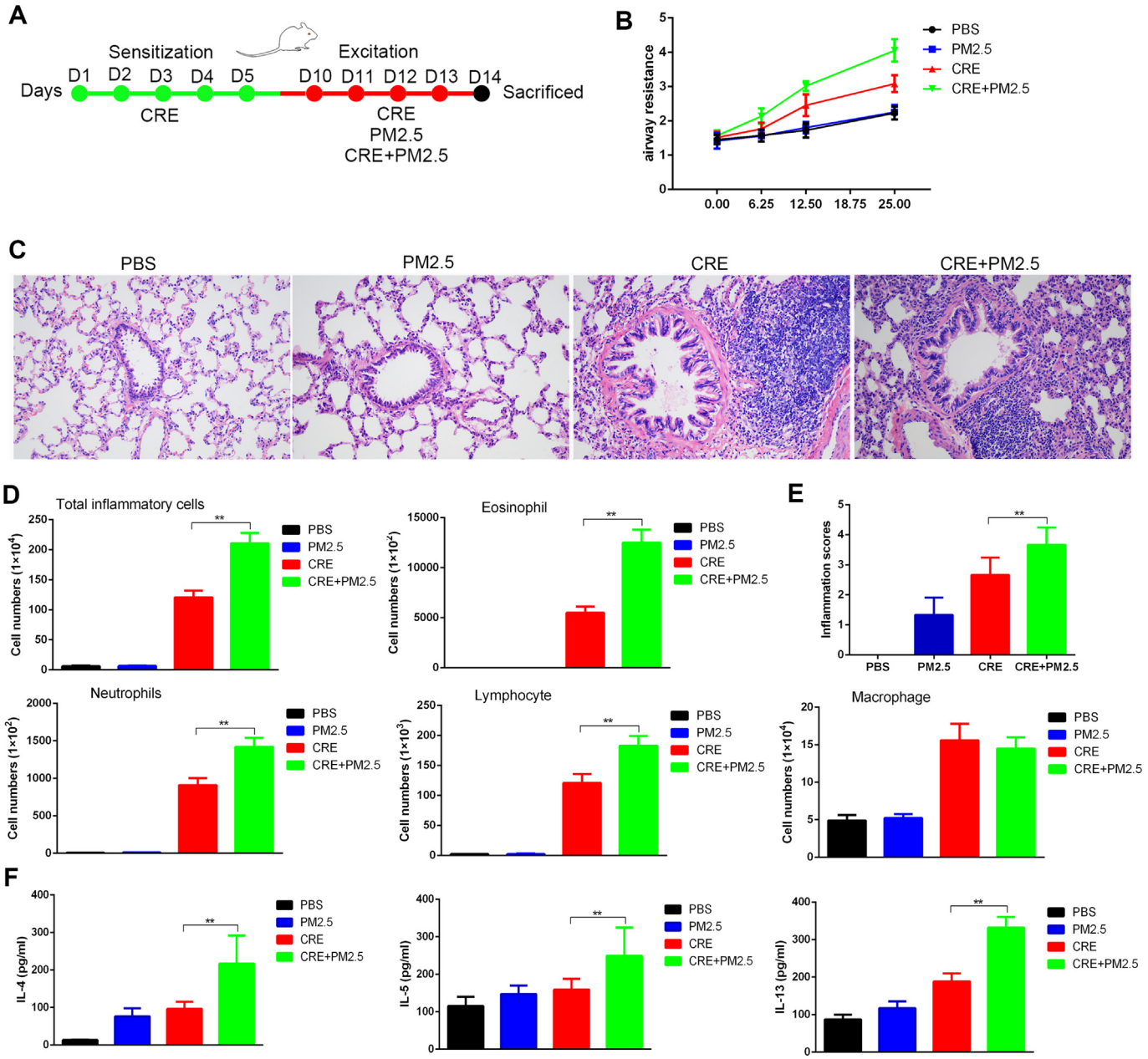

Fig. 1. PM2.5 could aggravate CRE-induced asthma symptoms, inflammation and pathological damage in mice.
A: A sketch map for establishing animal models; B: Detection of airway hyperresponsiveness in mice; C: H&E staining of lung and bronchus in mice; D: Classification and counting of inflammatory cells in alveolar lavage fluid of mice; E: Inflammation scores; F: IL-4, IL-5 and IL-13 levels. $^{**}P < 0.01$.

categories are oxidative stress and metabolic processes [15].

After investigating the baseline circulating small RNAs in 738 children with asthma before ICS treatment, 12 microRNAs were found to be significantly correlated with the severity of asthma in the following year. Each doubling of the expression of these small RNAs increases the risk by 25–67%, of which the most significant is the expression of microRNA-206 [16]. Many studies have reported that PM2.5, miR-206 and oxidative stress played important roles in the pathogenesis of asthma, it is not clear whether there is a correlation between them. PM2.5 could activate NF-κB, activation of NF-κB usually resulted in the up-regulation of some microRNAs [17]. Therefore, we speculate that PM2.5 may affect the role of oxidative stress in the progression of asthma by altering the expression of miR-206.

In this study, we established a mouse asthma model and exposed mice to PM2.5 to analyze the expression level of miR-206 in lung tissue of mice. The possible targets related to oxidative stress in microRNA-206 was predicted and was confirmed by the luciferase reporter gene. We also explored whether PM2.5 promotes oxidative stress by regulating the expression of miR-206 and participates in the aggravation of asthma.

2. Materials and methods

2.1. Experimental animals

A total of 42 SPF healthy female C57BL/6 mice weight 18–20 g (6–8 weeks old) were purchased from Shanghai Slac Laboratory Animal Co., Ltd. They were fed at 25 °C and 70% humidity with national standard solid mixed feed without pathogen, and they were free to eat and drink. The mice were placed in a comfortable and quiet room for one week before the initiation of the experimental procedure. We only use female mice in this study because our current experimental

Table 1
Primers used in this study.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-206	ACACTCCAGCTGGGTGGAATGTAAGGAAGT	Kit available
U6	GCTTCGGCAGCACATATACTAA	Kit available
SOD1	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
NRF2	TCCAGTCAGAAACCAGTGGAT	GAATGTCTGCGCCAAAAGCTG
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

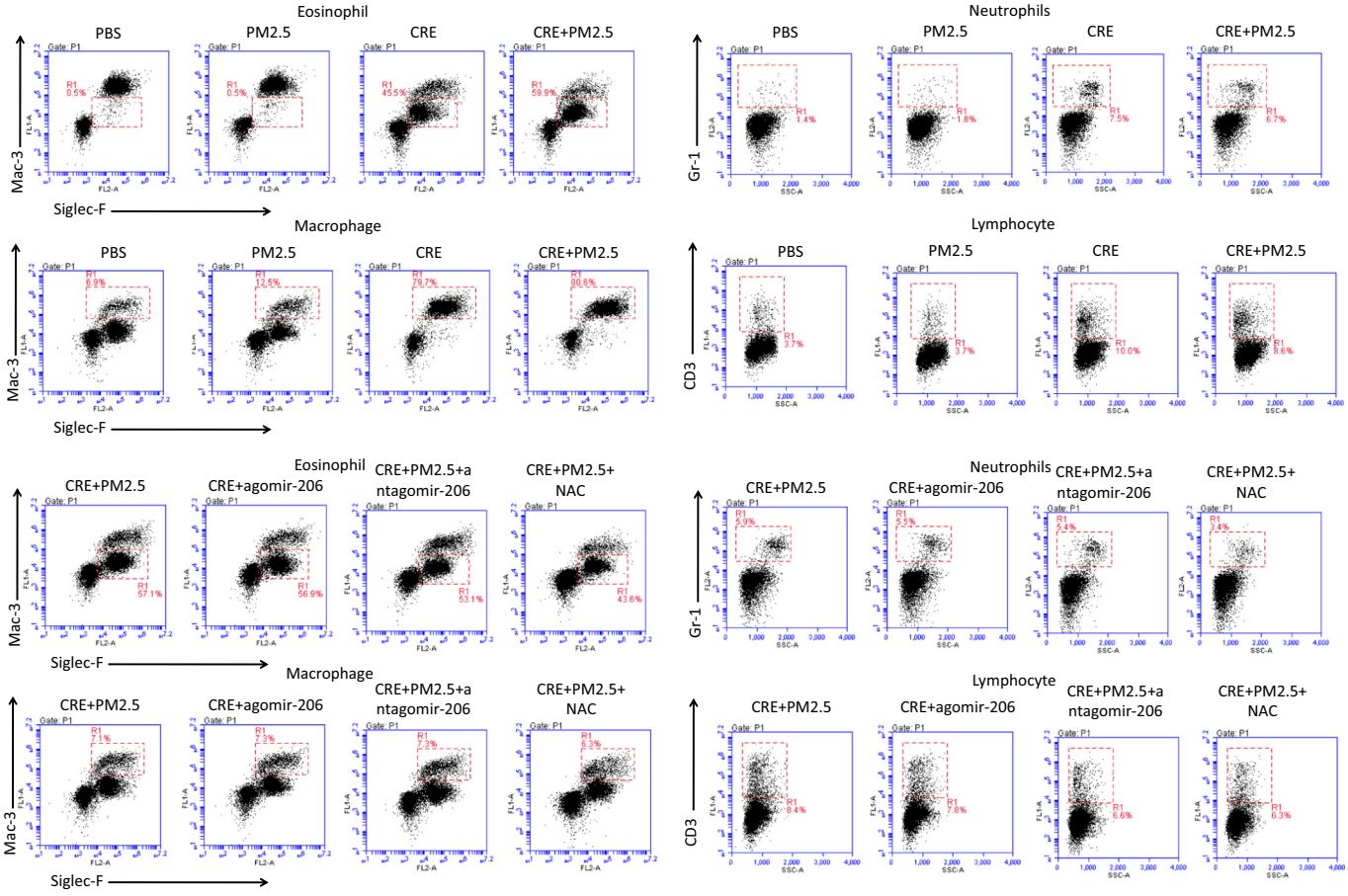

Fig. 2. Representative FACS plots.

conditions are limited, we will add male mice in the future.

This study was approved by Animal Ethics Committee of Shanxi medical university. All experimental procedure and animal care were carried out under the guidance of the Ethics Committee in order to minimize the suffering of animals.

2.2. Isolation of primary mouse tracheal epithelial cells

According to the reference [17], the mice were executed using carbon dioxide anesthesia. The mice were immersed in 75% alcohol for 15 min to sterilize, the intrapulmonary bronchus and extrapulmonary trachea were isolated aseptically and placed in a pre-cooled PBS. They were cut into $1mm^3$ size after the connective tissue was removed, 0.1% type I collagenase (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) diluted with DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium was added to digest overnight at 4 °C. They filtered through 200 mesh filters and centrifuged at 500 ×*g* and the supernatant was discarded at the next day. They were resuspended in DMEM/F12 medium containing 10% FBS. The cells were inoculated into the cell culture flask and subcultured for three times. Indirect immunofluorescence assay was performed using pan cytokeratin (Abcam, Cambridge, UK; 1:500) and Vimentin (Abcam, Cambridge, UK; 1:500) monoclonal antibodies to detect them.

2.3. Cell culture and siRNA transfection

The primary mouse tracheal epithelial cells were cultured with DMEM/F12 medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C with 5% CO2. The cells were treated with 1.6, 0.8, 0.4 and 0.2 mg/mLPM2.5 or 200/100/50/25 μg/mL CRE respectively for 48 h. The siRNA transfection was performed using Lipofectamine 2000 kit (Life Technologies, Carlsbad, CA, USA) according to the manual protocol.

2.4. PM2.5 acquisition

Ambient air particulate matter sampler (Model ZR-3930, Qingdao Zhongrui Intelligent Instrument Company) was put on the top floor of our hospital, PM2.5 was collected continuously from October to December 2017. The quartz film with PM2.5 was cut to the size of

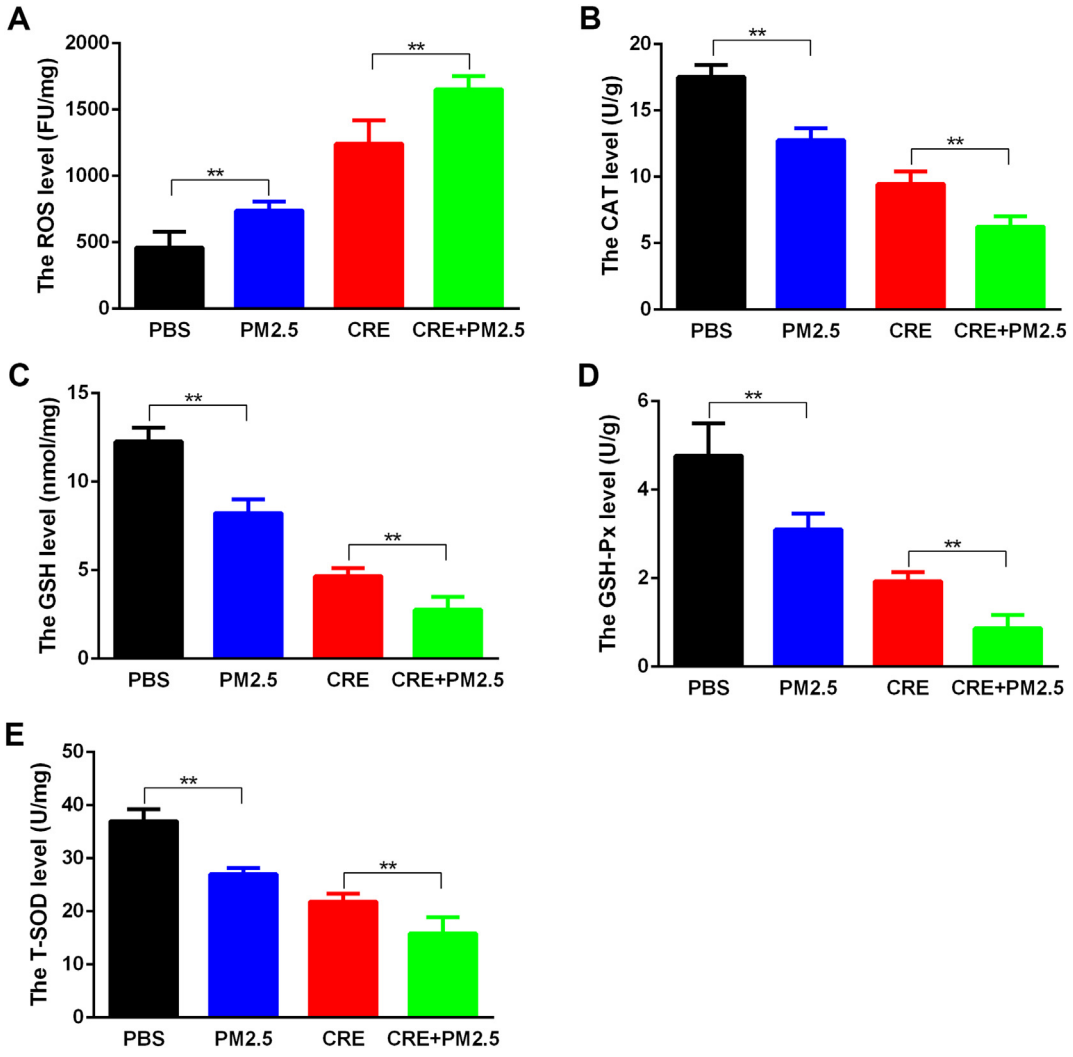

Fig. 3. PM2.5 could inhibit the expression of antioxidant enzymes in lung tissue of asthmatic mice and promotes ROS accumulation.
A: ROS levels in lung tissue of mice in different groups; B: CAT levels in lung tissue of mice in different groups; C: GSH levels in lung tissue of mice in different groups; D: GSH-Px levels in lung tissue of mice in different groups; E: T-SOD levels in lung tissue of mice in different groups.
$^{**}P < 0.01$.

1 cm × 3 cm and immersed in three-distilled water with ultrasound oscillation for 1 h. The oscillating solution was filtered with 70 μm filter and the filtrate was freeze-vacuum dried and sterilized.

2.5. Establishment of asthma mice model

According to the reference [18], the 20 μg cockroach extract (CRE) (GREER Laboratories, USA) was injected into D0-D4 trachea for 5 times respectively (sensitization stage), and stimulated into D10–13 trachea for 4 times (excitation stage). Airway hyperresponsiveness was detected in D14 trachea. The mice were executed to collect alveolar lavage fluid and lung tissue (Fig. 1). The effect of PM2.5 on asthma attack in mice was detected by intratracheal drip at the stimulation stage while allergen stimulation was given to mice [19]. The mice were anesthetized with isoflurane. The sterile cannula hose connected with 1 ml syringe was inserted into the trachea through the mouth for intratracheal drip. In the preliminary experiment, the mice were treated with different doses of PM2.5 (25, 50, 100 μg). It was found that PM2.5 could significantly enhance the CRE-induced allergic reaction and 100 μg PM2.5 had the strongest effect among them, so 100 μg PM2.5 was used to treat the mice in vivo. The mice were divided into seven groups randomly. PBS control group: The mice were treated with 50 μL PBS in both sensitization and excitation stages. CRE group: The mice were treated with 50 μL CRE (20 μg) in both sensitization and excitation stages. PM2.5 group: The mice were treated with 50 μL PBS in sensitization stage and 50 μL PM2.5 (100 μg) in excitation stage. PM2.5 + CRE group: The mice were treated with 50 μL CRE (20 μg) in sensitization stage and 50 μL PM2.5 (100 μg) + CRE (20 μg) mixed solution in excitation stage. PM2.5 + CRE + miR-206 Antagomir group: The mice were treated with 50 μL CRE (20 μg) in sensitization stage and 50 μL PM2.5 (100 μg) + CRE (20 μg) mixed solution in excitation stage, at the same time, miR-206 Antagomir (200 nmol/day) was injected into the tail vein of each mouse. miR-206 + CRE group: The mice were treated with 50 μL CRE (20 μg) in both sensitization and excitation stages, at the same time, miR-206 agomir (200 nmol/day) was injected into the tail vein of each mouse. PM2.5 + CRE + NAC (*n*-acetyl-L-cysteine, BSZH Scientific Inc.) group: NAC was administered to mice at 3 mmol/kg body weight every morning from the week before stimulation. The mice were treated with 50 μL CRE (20 μg) in sensitization stage and 50 μL PM2.5 (100 μg) + CRE (20 μg) mixed solution in excitation stage. There were 3 mice in each group.

2.6. Measurement of airway hyperresponsiveness in mice

After 24 h of last stimulation, mice were anesthetized with pentobarbital and tracheal intubation was performed, the ventilator and the

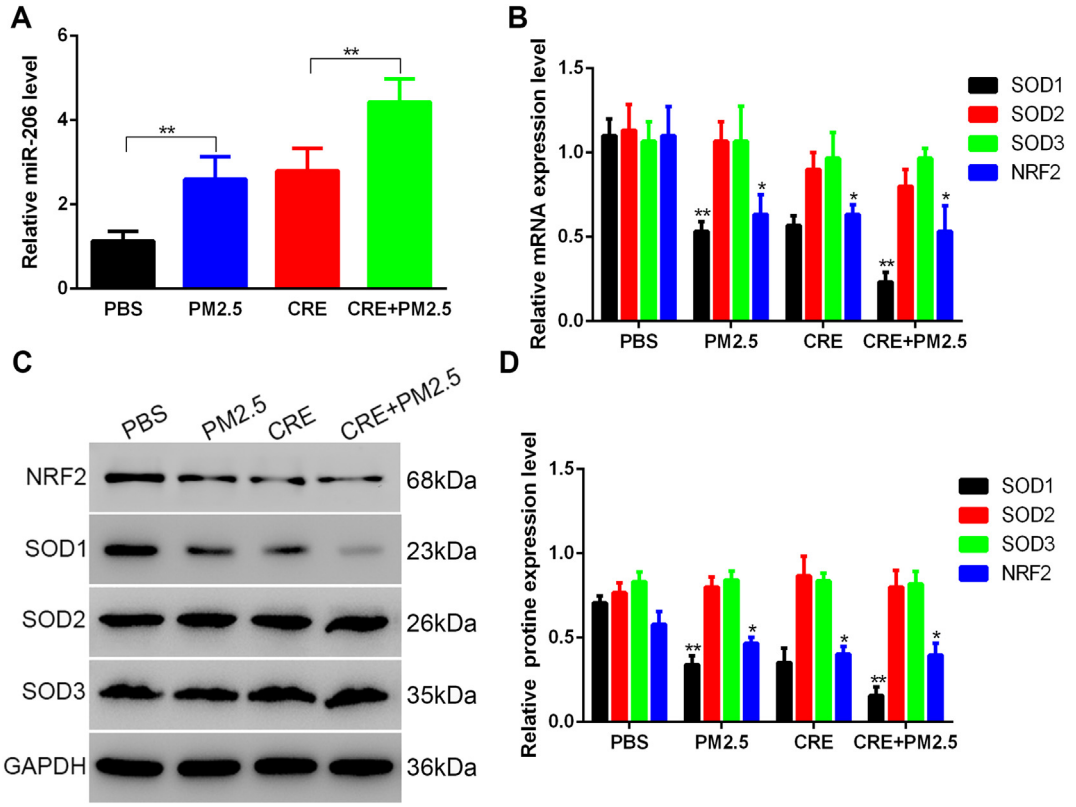

Fig. 4. PM2.5 could promote the expression of miR-206 and inhibit the expression of SOD1 in lung tissues of mice.
A: RT-PCR results showed that PM2.5 up-regulated the expression of miR-206 (U6 as internal reference); B: PM2.5 inhibited the expression of SOD1 and NRF2 but had no effects on SOD2 and SOD3 expression (GAPDH as internal reference); C, D: Western blotting results showed that PM2.5 inhibited the SOD1 and NRF2 protein expression, but had no effects on SOD2 and SOD3 expression.
$^{**}P < 0.01$.

atomizer were connected, the airway responsiveness of mice in different groups was measured by invasive mouse pulmonary function instrument (Buxco Company, USA). Methacholine (Mch) was used as a stimulant to detect the changes of airway resistance and lung compliance in mice induced by Mch at 0, 6.25, 12.5 and 25 mg·mL^{-1} concentrations respectively, which was an index for evaluating airway resistance in mice.

2.7. Classification and counting of inflammatory cells

Flow cytometry (Becton Dickinson FACS Canto II) was used to detect the classification and counting of inflammatory cells in alveolar lavage fluid of mice in different groups [17]. Eosinophil phenotype is side scatter(SSC)high Siglec-F$^+$ Mac-3$^-$ cell, Alveolar macrophages are defined as SSC high Siglec-F$^+$Mac-3$^+$cell, Granulocytes are defined as SSC high Gr-1$^+$cell, Lymphocyte is forward scatter (FSC)low/SSC low CD3$^+$cell. Fluorescent labelled antibodies were purchased from e-Bioscience Inc.

2.8. Pathological analysis of pulmonary inflammation

Following BAL, the lung tissues were infused with 10% neutral buffered formalin solution (1 ml). The fixed tissues were embedded in paraffin cut into slices (4 mm). They were stained with hematoxylin and eosin. A random number was assigned to each hematoxylin and eosin-stained slices from the groups. A pathologist blinded to the random numbers evaluated the inflammation degree of the slices using a Zeiss photomicroscope (Zeiss, Oberkochen, Germany). The degree of peribronchial inflammation was evaluated on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked and severe inflammation respectively, with an increment of 0.5 if the inflammation fell between two integers [21]. The total lung inflammation was defined as the sum of peribronchial inflammation scores.

2.9. ROS generation assay

Lung tissues were taken and cut into1mm^3 size using eye scissors, they were washed in pre-cooling PBS and blood stains and cell debris were removed. The tissues were ground on a 300 mesh filter and washed with pre-cooled PBS to obtain cell suspension. After centrifugation for 10 min, the supernatant was removed and washed twice with pre-cooled PBS to obtain lung tissue cell suspension. The cells ($> 10^6$ per well) were added to 96-well plate, and the DCF-DA (final concentration 10 μM) was added to them. They were incubated at 37 °C for 30 min. The fluorescence intensity of DCF was detected at 488 nm excitation wavelength by multifunctional microplate reader. Protein content of the cells was detected by BAC protein quantitative kit (Pierce, Rockford, IL, USA), and ROS level was expressed by fluorescence intensity (FU)/protein content (mg).

2.10. Detection of catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GSH-Px) and total superoxide dismutase (T-SOD) content

Proper amount of lung tissues were taken and weighed, equal amount of PBS was added and homogenate was prepared in ice bath. The supernatant was collected after centrifugation. The CAT, GSH, GSH-Px and T-SOD contents were detected using CAT, GSH, GSH-Px and T-SOD detection kits (Nanjing Jiancheng Bioengineering Institute) respectively according to the manufacturer's instructions.

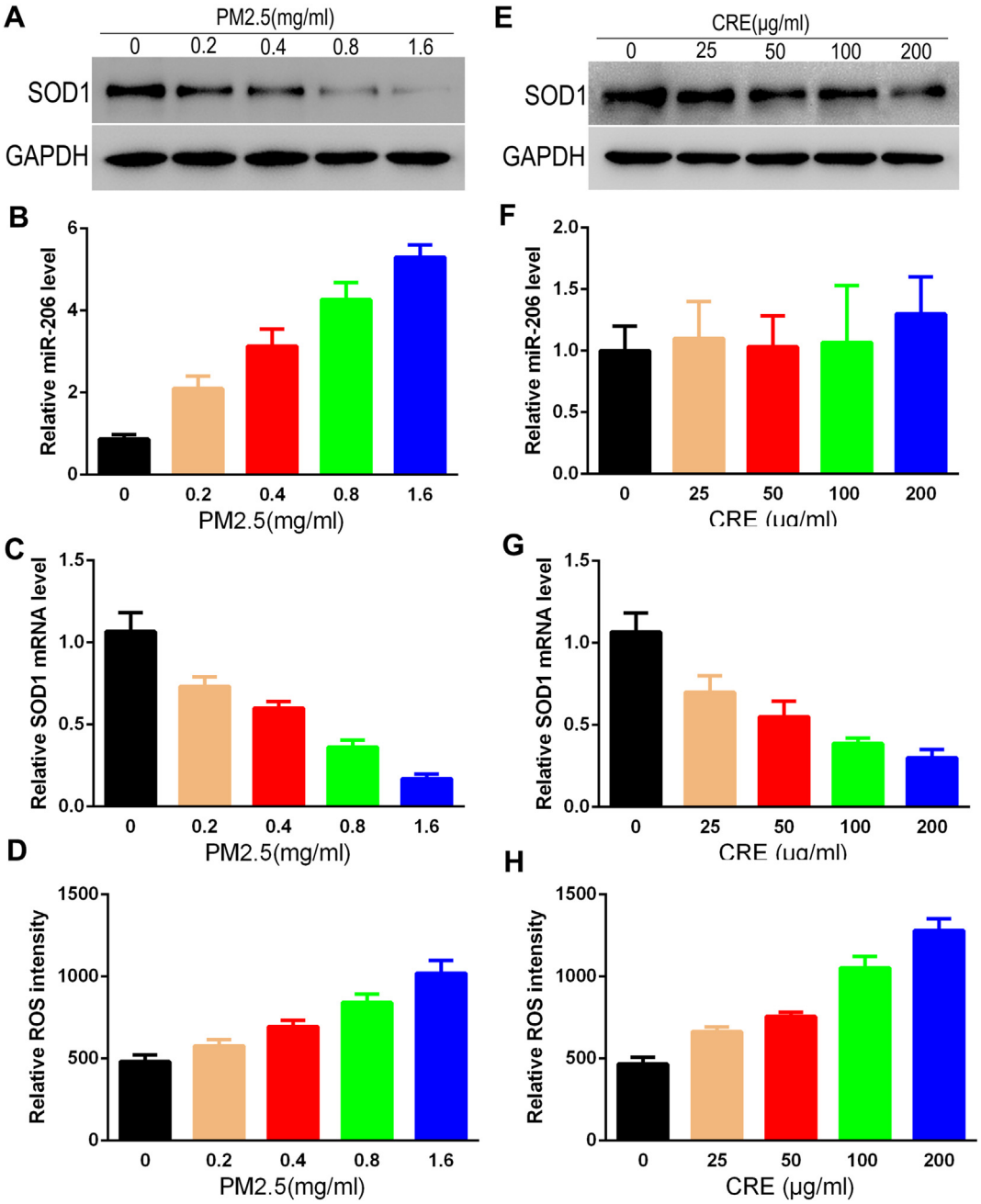

Fig. 5. PM2.5/miR-206/SOD1/ROS signal axis existed in primary mouse tracheal epithelial cells.
A: The SOD1 protein expression in primary mouse tracheal epithelial cells decreased with the increase of PM2.5 concentration; B: The expression of miR-206 in primary mouse tracheal epithelial cells increased with the increase of PM2.5 concentration; C: The SOD1 mRNA expression in primary mouse tracheal epithelial cells decreased with the increase of PM2.5 concentration; D: The ROS levels in primary mouse tracheal epithelial cells increased with the increase of PM2.5 concentration; E: The SOD1 protein expression in primary mouse tracheal epithelial cells decreased with the increase of CER concentration; F: There was no significant change in the expression of miR-206 in primary mouse tracheal epithelial cells with the increase of CER concentration; G: The SOD1 mRNA expression in primary mouse tracheal epithelial cells decreased with the increase of CER concentration; H: The ROS levels in primary mouse tracheal epithelial cells increased with the increase of CER concentration.
$^{**}P < 0.01$.

2.11. RNA extraction and quantitative real-time PCR

Total RNAs were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manual. The absorbance of 260 and 280 nm was measured by One drop ultramicro spectrophotometer and the purity and content of RNA were analyzed. For SOD1 mRNA, the purified RNA (1 μg) was reverse transcribed using Prime Script Kit (Takara Bio Inc., Otsu, Japan). The resulting cDNA samples were amplified by Real-time PCR with SYBR Premix Ex Taq™ (Takara Bio Inc., Otsu, Shiga, Japan) and ABI StepOne Plus System (Applied Biosystems, USA) according to the manufacturer's instructions. For miR-206, it was reverse transcribed using specific miRNA reverse transcription kit (D350, Takara, Tokyo, Japan), the cDNA samples were amplified by Real-time PCR with Power SYBR Green (DRR081A, Takara, Tokyo, Japan) and ABI StepOne Plus System (Applied Biosystems, USA) according to the manufacturer's instructions. The thermocycler parameters were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The levels of mRNA expression were

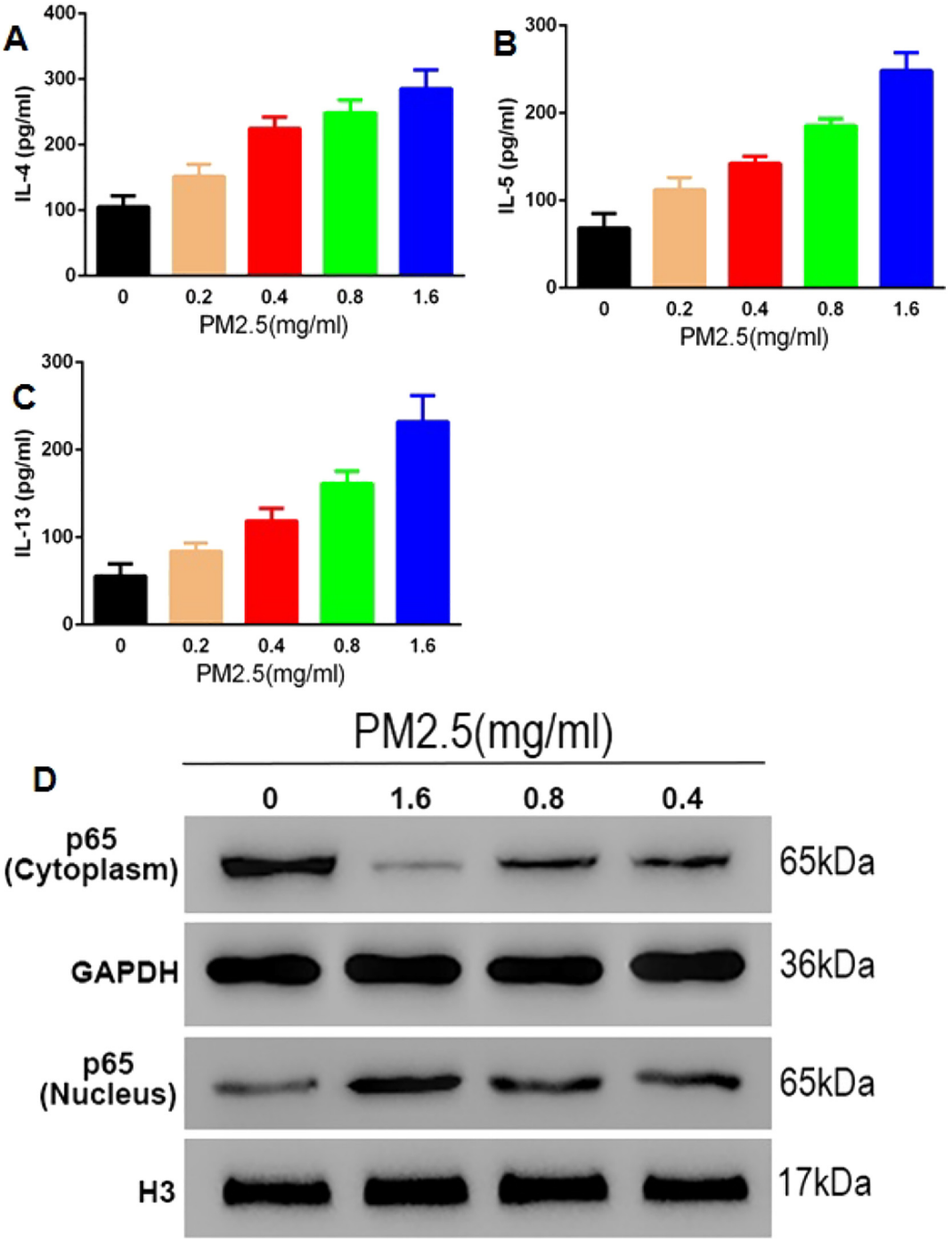

Fig. 6. The effects of PM2.5 on NF-κB activation and Th2 cytokine release in cell culture supernatants.
A: The effects of different concentrations of PM2.5 on IL-4 levels; B: The effects of different concentrations of PM2.5 on IL-5 levels; C: The effects of different concentrations of PM2.5 on IL-13 levels; D: The effects of PM2.5 on NF-κB p65 activation.

calculated using the $2^{-\Delta\Delta Ct}$ method, U6 and GAPDH was used as internal reference. PCR primers' sequences are shown in Table 1.

2.12. Western blotting test

The cells from different groups of mice were harvested and lysed with Cell Lysis Solution (Sigma-Aldrich, St. Louis, MO, USA), then they were centrifuged with 1000 rpm at 4 °C for 5 min. The extract supernatants were added protein electrophoresis buffer and boiled for 5 min, and then they were loaded on 12% SDS-polyacrylamide gels (40 μg per lane) and transferred onto polyvinylidene fluoride membranes (PVDF, Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked for 2 h at 37 °C with 5% non-fat milk, and were incubated with primary antibodies (1:1500 NRF2, 1:1000 SOD-1, 1:5000 SOD-2, 1:1000 SOD-3 and 1:500 GAPDH, Abcam, Cambridge, UK) respectively at 4 °C overnight. Following incubation with HRP-conjugated secondary antibody (1:50,000, Abcam, Cambridge, UK) for 1 h at 37 °C. The membrane was coated with ECL luminescence (Perkin-Elmer Inc.) after washing for 3 times, they were observed using Imagequant LAS4000 (GE Healthcare, Japan). GAPDH was used as normalization.

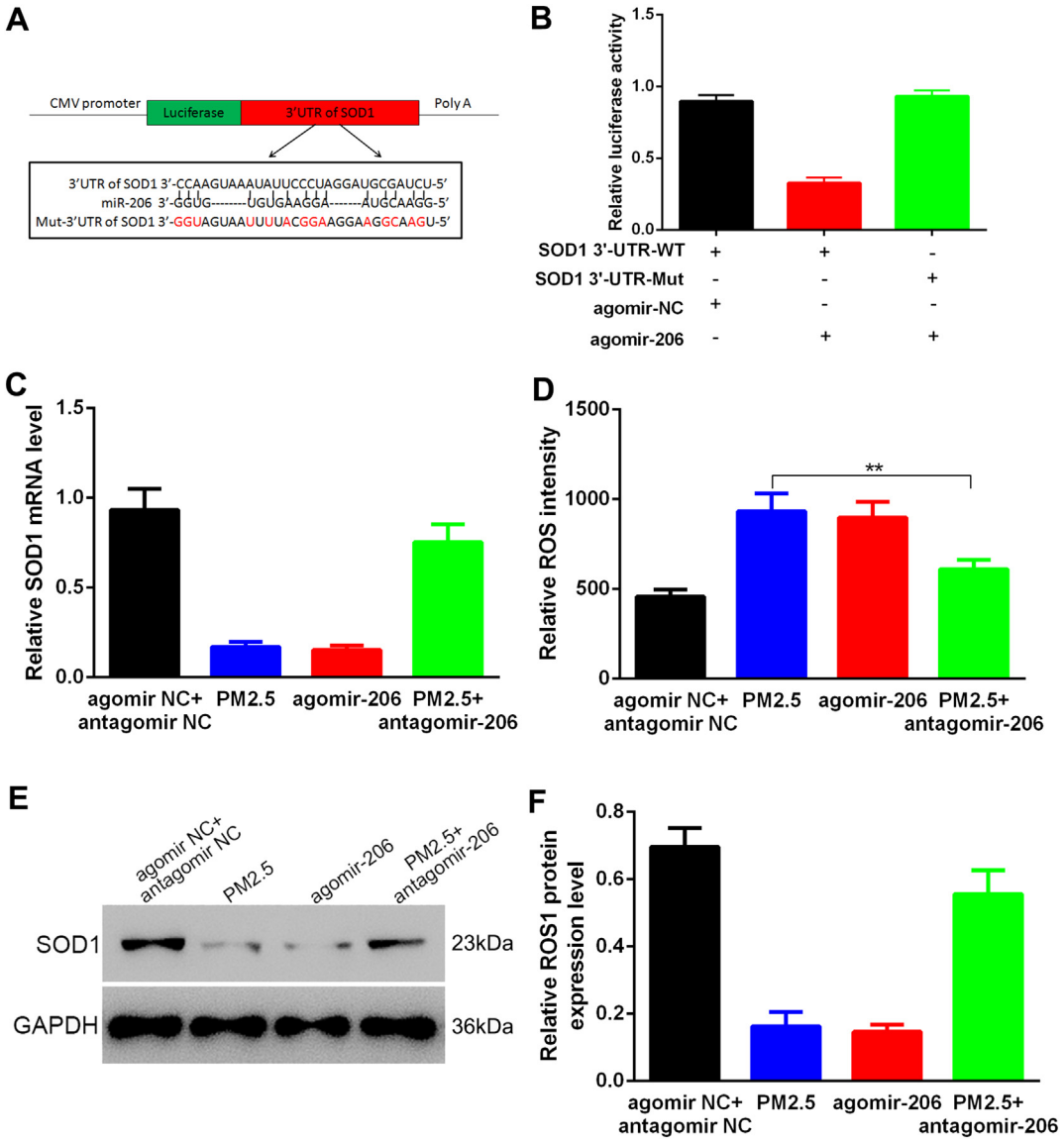

Fig. 7. miR-206 could target the 3'-UTR of SOD1 to inhibit its expression.
A: Construction of pGL3-SOD1-UTR-WT and pGL3-SOD1-UTR-Mut; B: Luciferase reporter gene test results showed that miR-206 could target the 3'UTR of SOD1; C: RT-PCR results showed that both miR-206 and PM2.5 treatments could inhibit SOD1 expression in primary mouse tracheal epithelial cells, and transfection of antagomir-206 into PM2.5-treated cells could up-regulate SOD1 expression; D: Both transfection of miR-206 and PM2.5 could promote ROS accumulation in primary mouse tracheal epithelial cells, and transfection of antagomir-206 into PM2.5-treated cells could inhibit ROS accumulation; E, F: Both transfection of miR-206 and PM2.5 could inhibit SOD1 protein expression in primary mouse tracheal epithelial cells, and transfection of antagomir-206 into PM2.5-treated cells could up regulate SOD1 protein expression.
$^{**}P < 0.01$.

2.13. Dual-luciferase reporter gene assays

The luciferase reporter gene plasmid WT SOD-1 3'-UTR (pGL3-SOD1-UTR-WT) and point mutation of luciferase reporter gene plasmid Mut SOD-1 3'-UTR (pGL3-SOD1-UTR-Mut) were constructed to analyze the interaction between 3'-UTR of SOD-1 and Mi-206. The 293 T cells were inoculated into 24 well plates and cultured overnight, luciferase reporter plasmid, Renilla luciferase and agomiR-206 mimic or control were transfected into 293 T cells simultaneously. The cells were split using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the instructions after culture for 48 h. The results were detected using Panomics Luminometer (Affymetrix, Santa Clara, CA, USA) after the luminescence was added. The sea renin fluorescence was used as internal reference.

2.14. Detection of IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid and cell culture supernatant

The contents of IL-4, IL-5 and IL-13 in the bronchoalveolar lavage fluid and cell culture supernatant were detected by ELISA kit (Abcam, Cambridge, UK) according to the manual.

2.15. Statistical analysis

The data were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). All results are presented as the mean ± standard deviation (SD). Student's *t*-test, the Wilcoxon signed-rank test and Pearson's chi-square test were used to evaluate the differences among groups. The correlation between the expression levels of miR-206 and SOD-1 was analyzed by Pearson's correlation analysis. $P < 0.05$ was considered to

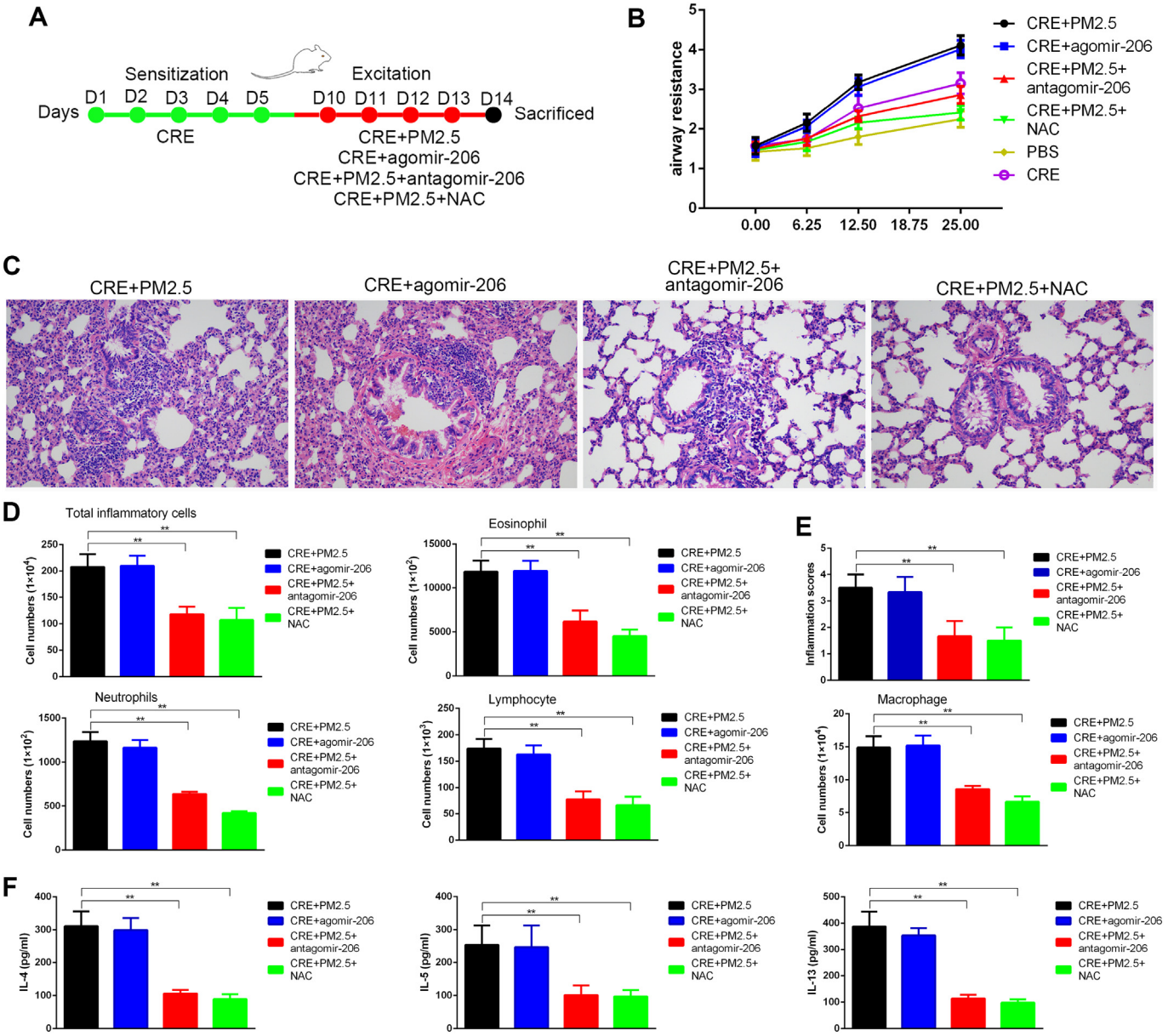

Fig. 8. Anagomir-206 or ROS inhibitors could alleviate the asthma symptoms, pathological damage and inflammation induced by PM2.5 and CRE in mice.
A: A sketch map for establishing animal models; B: Detection of airway hyperresponsiveness in mice; C, E: H&E staining of lung and bronchus in mice and inflammation scores; D: Classification and counting of inflammatory cells in alveolar lavage fluid of mice. $^{**}P < 0.01$.

indicate a statistically significant difference.

3. Results

3.1. PM2.5 could aggravate CRE-induced asthma symptoms in mice

The airway hyperresponsiveness test showed that the airway resistance increased significantly in CRE group compared with that of PBS group when the concentration of Mch was 12.5 mg/ml [(2.457 ± 0.314)vs(1.726 ± 0.211) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.002$] and 25 mg/ml [(3.085 ± 0.247)vs(2.231 ± 0.187) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.003$], which indicating that the asthma model was successfully established. The airway resistance increased in CRE + PM2.5 group compared with that of CRE group [Mch12.5 $mg \cdot mL^{-1}$: (3.107 ± 0.145) vs (2.457 ± 0.314) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.040\ 9$; Mch 25 $mg \cdot mL^{-1}$: (4.058 ± 0.326) vs (3.085 ± 0.247) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.015\ 0$]. However, there was no significant difference between PM2.5 group and PBS group (Fig. 1A). Pulmonary inflammation increased in CRE group compared with that of PBS group, which showed a large number of inflammatory cells infiltrating around bronchus. The total number of inflammatory cells, eosinophils, macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid increased. The pulmonary inflammation of mice was significantly increased in CRE + PM2.5 group compared with that of the CRE group, the total number of cells, eosinophils, neutrophils and lymphocytes and the IL-4, IL-5, IL-13 levels in bronchoalveolar lavage fluid increased significantly, while the change of macrophages was not obvious. There were no significant changes in pathology, cytokine levels and inflammatory cell numbers between the PBS group and PM2.5 group (Fig. 1B–F). Representative FACS plots showing the gating strategy utilized were shown in Fig. 2. These results suggested that PM2.5 could aggravate the symptoms of CRE-induced asthma and promote inflammation in mice.

3.2. PM2.5 could inhibit the expression of antioxidant enzymes in lung tissue of asthmatic mice and increase ROS level

Compared with PBS group, the contents of CAT, GSH, GSH-Px and T-SOD in CRE group decreased significantly, while the level of ROS increased. PM2.5 could further promote the contents of CAT, GSH, GSH-Px and T-SOD in lung tissue of mice to decrease significantly, while the level of ROS increased (Fig. 3).

3.3. PM2.5 could promote the expression of miR-206 and inhibit the expression of SOD1 and NRF2 in lung tissues of mice

RT-PCR results showed that the expression of miR-206 in lung tissues of mice in CRE + PM2.5 group was significantly increased, while the expression of SOD1 was significantly decreased compared with that of CRE group, there was no obvious difference in the expression of SOD2 and SOD3. Compared with PBS group, PM2.5 also significantly increased the expression of miR-206 and decreased the expression of SOD1 and NRF2 in lung tissues of mice. Western blotting results also showed that PM2.5 could inhibit SOD1 and NRF2 protein expression (Fig. 4).

3.4. PM2.5 promoted the expression of miR-206 and up-regulated ROS by inhibiting SOD1 expression in primary mouse tracheal epithelial cells

Indirect immunofluorescence assay showed that the primary mouse tracheal epithelial cells could express pan cytokeratin, but not Vimentin (Data not shown). PM2.5 treatment of primary mouse tracheal epithelial cells could promote the expression of miR-206, inhibit the expression of SOD1, and increase the ROS level. Moreover, the effect of PM2.5 was dose-dependent. The primary tracheal epithelial cells treated with CRE had no significant effect on the expression of miR-206, but could also inhibit the expression of SOD1 and increase the ROS level (Fig. 5). PM2.5 promoted nuclear transfer of NF-κB and Th2 cytokine release (Fig. 6). These results indicated that PM2.5/miR-206/SOD1/ROS signal axis existed in primary mouse tracheal epithelial cells.

3.5. miR-206 could target the 3'-UTR of SOD1 to inhibit its expression

Bioinformatics (TargetScan and RNAhybrid) predictions show that there are binding sites between miR-206 and the 3'-UTR of SOD1, which indicated that SOD1 may be the target gene of miR-206. Double luciferase reporter gene analysis showed that miR-206 could target the 3'UTR of SOD1 to inhibit luciferase expression. When the 3'UTR domain of SOD1 was mutated, the inhibitory effect of miR-206 disappeared. Transfection of agomir-206 into primary mouse tracheal epithelial cells could also inhibit SOD1 expression and increase ROS level. Transfection of antagomir-206 into PM2.5-treated primary mouse tracheal epithelial cells could up-regulate SOD1 expression and decrease ROS level (Fig. 7).

3.6. Inhibiting the expression of miR-206 or using ROS inhibitors in PM2.5-treated asthmatic mice could alleviate their symptoms

Airway hyperresponsiveness test showed that agomir-206 could aggravate asthma symptoms in CRE mice, the airway resistance increased significantly when the concentration of Mch was 12.5 mg/ml [(3.067 ± 0.217) vs (3.185 ± 0.183) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.002$] and 25 mg/ml [(4.018 ± 0.221) vs (4.116 ± 0.242) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.003$], which was similar to CRE + PM2.5. Treatment of antagomir-206 or NAC in CRE + PM2.5 mice could alleviate their asthma symptoms. Compared with that of CRE + PM2.5 group, airway resistance reduced in CRE + PM2.5 + antagomir-206 group [Mch12.5 $mg \cdot mL^{-1}$: (2.318 ± 0.138) vs (3.185 ± 0.183)$cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.0409$; Mch 25 $mg \cdot mL^{-1}$: (2.867 ± 0.215) vs (4.116 ± 0.242) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.0150$], the airway resistance also reduced in CRE + PM2.5 + NAC group [Mch12.5 $mg \cdot mL^{-1}$: (2.156 ± 0.144) vs (3.185 ± 0.183) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.0409$; Mch 25 $mg \cdot mL^{-1}$: (2.415 ± 0.189) vs (4.116 ± 0.242) $cmH_2O \cdot mL^{-1} \cdot s^{-1}$, $P = 0.0150$]. The intravenous injection of agomir-206 in CRE mice aggravated the pulmonary inflammation, the peribronchial inflammatory cell infiltration increased, the expression levels of IL-4, IL-5 and IL-13 and the number of inflammatory cells, eosinophils, macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid increased. Treatment of antagomir-206 or NAC in CRE + PM2.5 mice could alleviate the pulmonary inflammation. The total number of cells, eosinophils, neutrophils and lymphocytes and the expression levels of IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid decreased significantly, but there was no obvious change in macrophages. These results suggested that PM2.5 could increase the asthmatic symptoms induced by CRE through up-regulating the levels of miR-206 and ROS, and promote the inflammatory response in mice (Fig. 8).

4. Discussion

Asthma is one of the most common chronic respiratory diseases, it is associated with inflammatory cells, chronic airway inflammation, airflow restriction and airway hyperresponsiveness are the main manifestations [20]. The prevalence of asthma in children is on the rise in China. In 1990, the prevalence of asthma among urban children aged 0–14 years was 0.91%, it became 3.02% in 2010. In the past 20 years, the prevalence of asthma among children in China increased by 2.3 times [3,4,21,22]. It is no longer possible to explain the rapidly rising incidence of asthma by genetic changes, so many studies have focused on air pollutants.

PM2.5 is considered to be one of the most complex pollutants in the atmospheric environment and most harmful to human health because of its large surface area, more harmful substances adsorbed on the surface and long time in the air [23,24]. Epidemiological data suggested that atmospheric pollutants, especially particulate matter, are associated with increased mortality [25], it could also increase the incidence of cardiopulmonary diseases, such as asthma, chronic obstructive pulmonary disease, myocardial infarction and coronary heart disease [26–28]. Gary et al. [29] found that the emergency visiting rate of children with asthma increased with the increase of PM2.5 concentration. For every 11 μg /m3 increase of PM2.5 concentration, the OR value of children with asthma in the same period was 1.15 (95% CI: 1.08–1.23). Lee et al. [30] found that PM2.5 was significantly correlated with the admission rate of asthma. PM2.5 increased by 20.6 μg/m3 and the admission rate increased by 3.24% (95% CI: 0.93–5.60). Lokman et al. [31] found that PM2.5 was positively correlated with admission rate of children with asthma. During Beijing Olympic Games in the 2008, the pollutants were effectively treated. The results showed that the number of asthma patients in hospital outpatient clinics during the Olympic Games was significantly lower than before [32,33]. These suggested that the change of PM2.5 concentration may be related to the occurrence of asthma.

In this study, we found that PM2.5 could significantly increase airway resistance and pulmonary inflammatory response in asthmatic mice, and increase the total number of inflammatory cells, eosinophils, macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid. Moreover, PM2.5 could further promote the contents of antioxidant enzymes such as CAT, GSH, GSH-Px and T-SOD in lung tissue of mice to decrease, while the level of ROS increased. PM2.5 could promote the expression of miR-206 and inhibit the expression of SOD1 in lung tissue of mice. PM2.5 could also promote the expression of miR-206, inhibit SOD1 expression and up-regulate ROS level in primary mouse tracheal epithelial cells. The results of luciferase reporter gene analysis showed that miR-206 could target the 3'-UTR of SOD1 to inhibit its expression. miR-206 Antagomir or NAC, an ROS inhibitor, could inhibit ROS levels in lung tissue, reduce airway resistance and

pulmonary inflammation in PM2.5-treated asthmatic mice. These results suggested that PM2.5 at least partially aggravated pulmonary inflammation and asthmatic symptoms in asthmatic mice by promoting the expression of miR-206, inhibiting SOD1 expression and increasing ROS levels.

Under normal circumstances, the immune response is strictly regulated and balanced through complex activation and inhibition pathways. The occurrence of asthma may interfere with this balance, which leading to airway inflammation dominated by eosinophils and CD4 + T lymphocytes. They secret a large number of Th2 cytokines such as IL-4, IL-5 and IL-13, promoting asthma by enhancing the growth, differentiation and recruitment of eosinophils, basophils, mast cells and IgE-producing B cells, and directly inducing airway hyperresponsiveness [34,35]. This change in microenvironment can lead to changes in gene expression levels. It has been found that the expression level of multiple loci and > 100 genes has changed in asthma, and of course, the expression level of miRNAs can also change [36]. Study showed that the expression of miR-206 in peripheral blood of asthmatic patients significantly increased, miR-206 may be a molecular marker of asthma exacerbation [37]. In the course of asthma, inflammatory reaction activates pulmonary epithelial cells, pulmonary macrophages and so on, resulting in the accumulation of ROS, which may further promote the activity of redox-sensitive transcription factors such as NF-κB and AP-1, and induce the expression of pro-inflammatory genes [38]. Superoxide dismutase family (SOD) includes SOD1, SOD2 and SOD3, which are the first antioxidant enzymes to play a role in ROS scavenging process [39]. SOD1 plays a key role in cell scavenging reactive oxygen species (H_2O_2、O^{2-} and –OH) [40]. This study showed that PM2.5 could promote the miR-206 expression in lung tissue of asthmatic mice. miR-206 could target the 3'-UTR of SOD1 to inhibit its expression, thus leading to ROS accumulation.

5. Conclusions

In a word, in this study we found that PM2.5 could promote the occurrence and development of asthma, its molecular mechanism may be to promote the expression of miR-206 in lung tissue of asthmatic mice. miR-206 could target the 3'-UTR of SOD1 to inhibit its expression, thus leading to ROS accumulation, which further promoted inflammatory response and aggravated asthma symptoms. miR-206 may be an important molecular marker and therapeutic target for asthma. The limitation of animal number and sex in this study may lead to deviations in results, we will further verify it in the future.

Authors' contributions

Conceived and designed the experiments: Hailong Hao; Execution of experiments: Lei Wang and Jianying Xu; Data analysis: Lei Wang, Hu Liu and Jianying Xu; Discussion of results: Lei Wang, Jie Li and Hailong Hao; Wrote and or critical reading of manuscript: Lei Wang and Hailong Hao.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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