

# PM<sub>2.5</sub> and water-soluble components induce airway fibrosis through TGF- $\beta$ 1/Smad3 signaling pathway in asthmatic rats

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## ABSTRACT

Epidemiological studies have suggested that fine particulate matter (PM<sub>2.5</sub>) and asthma have been independently associated with pulmonary fibrosis but rarely studied together. Furthermore, it is unknown whether airway fibrosis in asthma is more attributable to water-soluble ions of PM<sub>2.5</sub>. Our current study was to explore the potential mechanism of PM<sub>2.5</sub> and water-soluble components on airway fibrosis in ovalbumin (OVA)-sensitized asthmatic rats. Rats were intratracheally instilled with PM<sub>2.5</sub> and water-soluble components every 3 days for 4 times or 8 times. Histopathological examination demonstrated that lung inflammatory and airway fibrosis were induced after PM<sub>2.5</sub> and water-soluble components exposure. Meanwhile, PM<sub>2.5</sub>, in particular water-soluble extracts, increased expression of collagen 1 (COL-1), connective tissue growth factor (CTGF), interleukin-6 (IL-6), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), Smad family member 3 (Smad3), and p-Smad3, whereas decreased secretion of heme oxygenase-1 (HO-1). However, pretreating asthmatic rats with SB432542, the inhibitor of TGF- $\beta$ 1, and SIS3 HCl, the antagonist of Smad3, both reversed the activation of airway fibrosis induced by water-soluble extracts. Therefore, TGF- $\beta$ 1/Smad3 signaling pathway may be responsible for the pathological process of airway fibrosis in asthmatic rats following PM<sub>2.5</sub> and water-soluble components exposure.

## 1. Introduction

Asthma is a chronic disorder of the airways characterized by inflammation infiltration, mucus hypersecretion, airway hyperresponsiveness, and airway remodeling (Licari et al., 2019). The prevalence of asthma has continued to increase, which affects millions of people in the world (Asher et al., 2020). The reasons for this rise in asthma have still not been fully answered; one factor may be exposure to fine particulate matter (PM<sub>2.5</sub>, aerodynamic diameter  $\leq 2.5 \mu\text{m}$ ) (Mirabelli et al., 2016). Epidemiological studies had reported the relationship between PM<sub>2.5</sub> exposure and the increased risk of asthma (Gleason et al., 2014). PM<sub>2.5</sub> is a complex mixture and its water-soluble constituents have attracted much attention in recent years (Chowdhury et al., 2018; Zhao et al., 2020). For example, prolonged exposure to the

water-soluble PM<sub>2.5</sub> extracts can exacerbate lung damage (Zhao et al., 2019). Hydrogen ions and water-soluble organic compounds of PM<sub>2.5</sub> are positively associated with asthma attacks (Yamazaki et al., 2019). Although it has largely proved that water-soluble components of PM<sub>2.5</sub> could cause adverse effects on asthma, the disease progress underlying the association between water-soluble components of PM<sub>2.5</sub> and asthma remains uncertain.

Airway fibrosis is a critical late-life event for asthma development (Hur and Broide, 2019). It is characterized by excessive infiltration of inflammatory cells, proliferation of fibroblasts, and deposition of extracellular matrix (ECM), being considered important in the airway (Ogger et al., 2020). Current report indicated that airway fibrosis is an important health problem that imposes a significant cost on patients and their quality of life (Zaghloul et al., 2019). However, the question of why

**Abbreviations:** PM<sub>2.5</sub>, Fine particulate matter; OVA, Ovalbumin; COL-1, Collagen 1; CTGF, Connective tissue growth factor; IL-6, Interleukin-6; TGF- $\beta$ 1, Transforming growth factor- $\beta$ 1; Smad3, Smad family member 3; HO-1, Heme oxygenase-1; ICP-MS, Inductively coupled plasma mass spectrometer; IC, Ion chromatography; WSL, PM<sub>2.5</sub> water-soluble components of low level 3.6 mg/kg-bw; WSH, PM<sub>2.5</sub> water-soluble components of high level 14.4 mg/kg-bw; SB, SB432542; SIS3 HCl; PCR, Polymerase chain reaction; kg, Kilogram; bw, Body weight; TWC, Total white cells; MON, Monocytes; NEU, Neutrophils; EOS, Eosinophils; BASO, Basophils; LYM, Lymphocyte.

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asthmatics develop airway fibrosis has not been fully answered. Recently, a vivo and vitro study has confirmed that PM<sub>2.5</sub> exposure could induce pulmonary fibrosis (Zheng et al., 2018), but whether airway fibrosis in asthma is attributable to PM<sub>2.5</sub> has been inconclusive. Meanwhile, the role of the water-soluble components of PM<sub>2.5</sub> requires further investigation.

There is growing evidence indicating that TGF- $\beta$ 1 but not TGF- $\beta$ 2 or TGF- $\beta$ 3 participated in the pathological changes of fibroblast proliferation, myofibroblast differentiation, and collagen synthesis (Li et al., 2020). Thus, TGF- $\beta$ 1 is considered as a predominant profibrotic cytokine (Pan et al., 2020). TGF- $\beta$ 1 promotes the activation of intracellular Smad family member 3 (Smad3) via binding to its receptor and thus exerting the downstream effect of TGF- $\beta$ 1-mediated pulmonary fibrosis (Liu et al., 2017; Xu et al., 2019). Previous studies have shown that TGF- $\beta$ 1 and Smad3 were both up-regulated in pulmonary fibrosis (Bacharier and Guilbert, 2019; Guo et al., 2019). Although the profibrotic role of TGF- $\beta$ 1/Smad3 signaling pathway has been deeply studied, activation of airway fibrosis in asthma through above mechanism has not been reported. Therefore, it is necessary to clarify whether airway fibrosis in asthma is triggered by water-soluble components of PM<sub>2.5</sub> through TGF- $\beta$ 1/Smad3 signaling pathway.

In the present study, we observed the inflammatory response and fibrosis effect of PM<sub>2.5</sub> and water-soluble components in asthmatic rats sensitized by repeated OVA challenge. Importantly, the TGF- $\beta$ 1/Smad3 signaling pathway was further explored to reveal the potential pathogenesis of airway fibrosis in asthmatic rats.

## 2. Materials and methods

### 2.1. Reagents

Ovalbumin (OVA) was obtained from Sigma (St Louis MO, USA). SB431542 and SIS3 HCl were purchased from Selleck (USA). Heme oxygenase-1 (HO-1) and Interleukin-6 (IL-6) ELISA kits were bought from Xitang Biotech (Shanghai, China). HO-1, TGF- $\beta$ 1, Smad3, CTGF, collagen 1, and  $\beta$ -actin antibodies were purchased from Santa Cruz Biotech (Santa Cruz, USA). TGF- $\beta$ 1 and IL-6 antibodies were obtained from Abcam (Cambridge Science Park, UK). Oligonucleotides for polymerase chain reaction (PCR) were obtained from Sangon Biotech (Shanghai, China). TRIZOL reagent and the perfect real-time quantitative PCR kits were purchased from Takara Bio (Japan).

### 2.2. PM<sub>2.5</sub> collection and preparation

The atmospheric PM<sub>2.5</sub> samples were collected from November to December in 2018 at a crossroad of Shanxi Medical University in Taiyuan, where the main source of ambient PM<sub>2.5</sub> is traffic exhausts. The PM<sub>2.5</sub> samples were collected on glass fiber filter membrane (Taishan, China) by high-volume sampler (TH1000H, Wuhan Tianhong, China) for 12 h (7:00–19:00) at the height of breathing zone. Subsequently, the filter membranes were cut into pieces (1 cm  $\times$  1 cm), immersed in distilled water, and sonicated 20 min for 3 times. After filtering with 6 layers of sterile gauze, one half of the solution was freeze-dried to extract PM<sub>2.5</sub>. The other half of the suspension was centrifuged at 12,000 rpm for 20 min, and then the supernatant was lyophilized to extract PM<sub>2.5</sub> water-soluble components. Weighted PM<sub>2.5</sub> and water-soluble components were suspended in saline for the establishment of rat model. Meanwhile, the concentration and chemical elements of metal elements and water-soluble ions in PM<sub>2.5</sub> were analyzed by inductively coupled plasma mass spectrometer (ICP-MS, Thermo Fisher, USA) and ion chromatography (IC, Dionex, USA), respectively. The specific results were shown in Table 1.

According to the physiological indices of respiratory volume, breathing rate of adult rats, and 75  $\mu\text{g}/\text{m}^3$  daily mean concentration of PM<sub>2.5</sub> recommended by China, the dose of PM<sub>2.5</sub> was set as 6 mg/kg-bw. Considering water-soluble components accounted for about 60 % of

**Table 1**

The primers used for reverse transcription and quantitative PCR reactions.

| Gene name      | Primer (5'-3')             | Primer Length (bp) |
|----------------|----------------------------|--------------------|
| TGF- $\beta$ 1 | F:ATGGTGGACCGCAACAACGC     | 20                 |
|                | R:CTGGCACTGCTTCCCGAATGTC   | 22                 |
| Smad3          | F:GTGCCAGCCATGTCGTCCATC    | 21                 |
|                | R:ACTTCTCCTCTGCCCGTTCTG    | 22                 |
| CTGF           | F:CACCGCACAGAACCCACAC      | 21                 |
|                | R:ATGGCAGGCACAGGCTTGTATG   | 22                 |
| COL I          | F:GGTGAGACAGGCGAACAAGGTG   | 22                 |
|                | R:GCCAGGAGAGCCAGGAGGAC     | 20                 |
| $\beta$ -actin | F:GGAGATTACTGCCCTGGCTCCTA  | 23                 |
|                | R:GACTCATCGTACTCTGCTTGTCTG | 24                 |

PM<sub>2.5</sub> mass, we selected 3.6 mg/kg-bw as the low exposure dose of water-soluble components. During the period of our study, the highest daily concentration of PM<sub>2.5</sub> was 295  $\mu\text{g}/\text{m}^3$ , nearly four times of Chinese guidelines (<http://www.cnemc.cn/>). So we selected 14.4 mg/kg-bw which was four times of 3.6 mg/kg-bw as the high exposure dose of water-soluble components.

### 2.3. Animals and treatments

Animal experiments have been approved by the animal and ethics review committee of the laboratory animal center at Shanxi Medical University (No. SCXK (jin) 2015–0001, Shanxi, China). 4-week-old male SD rats with body weight of 180–220 g were purchased from the Animal Center of Shanxi Medical University. All animals were maintained under standard laboratory conditions (temperature: 23  $\pm$  2  $^{\circ}\text{C}$ ; relative humidity: 60  $\pm$  10 %) and adaptively fed for 1 week with ad libitum access to food and water.

70 rats were randomly divided into seven groups (n = 10 per group) and treated with normal saline (NS); OVA; OVA + PM<sub>2.5</sub> 6 mg/kg-bw (OVA + PM<sub>2.5</sub>); OVA + PM<sub>2.5</sub> water-soluble components of low level 3.6 mg/kg-bw (OVA + WSL); OVA + PM<sub>2.5</sub> water-soluble components of high level 14.4 mg/kg-bw (OVA + WSH); OVA + PM<sub>2.5</sub> water-soluble components of high level 14.4 mg/kg-bw + SB431542 (OVA + WSH + SB); OVA + PM<sub>2.5</sub> water-soluble components of high level 14.4 mg/kg-bw + SIS3 HCl group (OVA + WSH + SI), respectively. OVA sensitization and PM<sub>2.5</sub> treatment were performed as follows. Briefly, on day 1, rats were intraperitoneally injected with 1 mL saline containing 10 % ovalbumin and Al(OH)<sub>3</sub>. On days 8 and 15, rats underwent booster injection with 1% ovalbumin. During days 22–43, the rats were given aerosol inhalation exposure with 3% OVA for 30 min daily. The rats in normal saline control group were treated with saline during the whole procedure. Following OVA sensitization, from day 22, rats in control group and asthma model group were intratracheally instilled with normal saline, and rats in other groups were instilled with PM<sub>2.5</sub> (6 mg/kg) or water-soluble components (3.6 and 14.4 mg/kg) at 3-days intervals. The inhibitor solution SB431542 (5 mg/kg-bw) and SIS3 HCl (1.25 mg/kg-bw) were intraperitoneally injected 15 min before the instillation of PM<sub>2.5</sub> water-soluble components (14.4 mg/kg-bw) following the manufacturer's instructions. Half of rats were sacrificed for sample collection within 24 h of the above treatment on day 31, the rest of 35 rats were sacrificed for sample collection on day 43.

### 2.4. Animal tissue specimens and broncho-alveolar lavage fluid (BALF) collection

The rats were anesthetized before drawing blood from abdominal aorta. Serum was collected by centrifugation at 3000 rpm for 10 min. The inferior lobe of left lungs was fixed in 4% polychlorinated formaldehyde for histopathologic and immunohistochemical examination. All the samples obtained from rats were stored at -80  $^{\circ}\text{C}$  for biochemical analysis.

The right lungs were lavaged three times with 1  $\times$  phosphate

buffered saline (5 mL) to obtain bronchoalveolar lavage fluid (BALF). After centrifugation at 1500 rpm for 10 min at 4 °C, the precipitated cell pellets were re-suspended with PBS. Total BALF cells were fixed on glass slides and stained with Giemsa (Beijing solar bioscience & technology co., ltd) to count the differential cells under light microscopy (the specific results were shown in Fig. 1).

## 2.5. Histological scoring of peribronchial inflammation and airway fibrosis

The fixed tissues were embedded in paraffin. The 5 µm sections were stained with hematoxylin-eosin (H&E) and masson's trichrome staining to evaluate the degree of peribronchial inflammation and airway fibrosis. The severity of peribronchial inflammation was graded semi-quantitatively as previously described (Myou et al., 2003). The lung fibrosis was scored by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) as previously described (Ding et al., 2019).

## 2.6. Western blot analysis for TGF-β1, Smad3, HO-1 and IL-6 expression

The expression levels of TGF-β1, Smad3, HO-1, and IL-6 in lung tissues were determined by western blot. Total protein was extracted using RIPA lysis buffer, quantified with bicinchoninic acid (BCA) protein assay kit (Beyotime Biological Technology Co. Ltd), separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, PVDF membranes were incubated with rabbit anti-rat TGF-β1 (1:2000 dilution, Abcam), or mouse anti-rat Smad3 (1:200 dilution, Santa Cruz), or HO-1 (1:2000 dilution, Santa Cruz), or IL-6 (1:5000 dilution, Abcam) primary antibodies. After washing 3 times by PBST, the membranes were incubated with goat anti-rabbit/mice secondary antibodies (1:5000 dilution, Boster). Finally, immunoreactive bands were visualized by ECL reagent, quantified by densitometry using Image-J software (National Institutes of Health, America) with β-actin as an internal reference.

## 2.7. Quantitative real-time PCR (qRT-PCR) analysis for TGF-β1, Smad3, CTGF, and collagen 1 expression

Total RNA from lung tissues of rats were extracted using RNAiso Plus reagent (TaKaRa, Japan), reverse transcribed with PrimeScript<sup>TM</sup> RT reagent Kit with gDNA Eraser (TaKaRa, Japan), and amplified by TB Green<sup>®</sup> Premix Ex Taq<sup>TM</sup> II kit (TaKaRa, Japan) for quantifying of TGF-β1, Smad3, CTGF, and collagen 1 mRNA at following conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 30 s, 60 °C for 20 s, and 10 s at 72 °C. β-actin was used for normalization. Relative RNA expression was determined using the 2<sup>-ΔΔCt</sup> method. The sequences of primers were listed in Table 1.

## 2.8. ELISA analysis for HO-1 and IL-6 expression

The expression levels of HO-1 and IL-6 in plasma were detected by ELISA kit (Xitang Biotechnologies Co. Ltd, Shanghai, China) according to the manufacturer's instructions.

## 2.9. Immunohistochemical analysis for TGF-β1, Smad3, p-Smad3, CTGF, and collagen 1 expression

The expression levels of TGF-β1, Smad3, p-Smad3, CTGF, and collagen 1 were measured by immunohistochemical staining as previously described (Yang et al., 2019). The paraffin-embedded rat lung tissues were sectioned (5 µm) and incubated overnight at 4 °C with primary antibodies, including rabbit anti-rat TGF-β1 (1:200 dilution, Santa Cruz), or mouse anti-rat Smad3 (1:200 dilution, Santa Cruz), or rabbit anti-rat p-Smad3 (1:100 dilution, Abclonal) or CTGF (1:200 dilution, Santa Cruz) or collagen 1 (1:500 dilution, Santa Cruz). After staining with secondary antibodies for 25 min, the slides were washed 3 times by PBS and visualized with diaminobenzidine (DAB). Following counterstaining with hematoxylin, the protein expression levels were detected by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) and expressed as an average optical density (AOD) value.

## 2.10. Statistical analysis

The data were analyzed with SPSS 22.0 software program. Data were presented as the mean ± standard deviation. One-way ANOVA followed by post hoc analysis (Dunnett, post-test) was performed to compare the difference between groups. *P* < 0.05 was considered statistically significant.

## 3. Results

### 3.1. PM<sub>2.5</sub> and water-soluble components induced pulmonary inflammation and fibrosis in OVA-sensitized rats

H&E staining was performed to examine the pathological changes of pulmonary tissues. As shown in Fig. 2A, mild inflammatory cell infiltration was found in asthma model group. PM<sub>2.5</sub>, in particular water-soluble extracts at 14.4 mg/kg-bw, obviously induced inflammatory changes featured with considerable inflammatory cell infiltration into the bronchioli and alveoli areas in OVA-sensitized asthmatic rat lung tissues. In contrast, the inhibitors of TGF-β1 and Smad3 suppressed the bronchioli and alveoli inflammation induced by PM<sub>2.5</sub> water-soluble components. More importantly, the degree of inflammatory injury in rats consecutively exposed to PM<sub>2.5</sub> and water-soluble components for 8 times seemed worse than that of rats exposed for 4 times (Fig. 2B, D). Additionally, the results of semi-quantitative HE staining supported the pro-inflammatory effect of PM<sub>2.5</sub> and water-soluble components

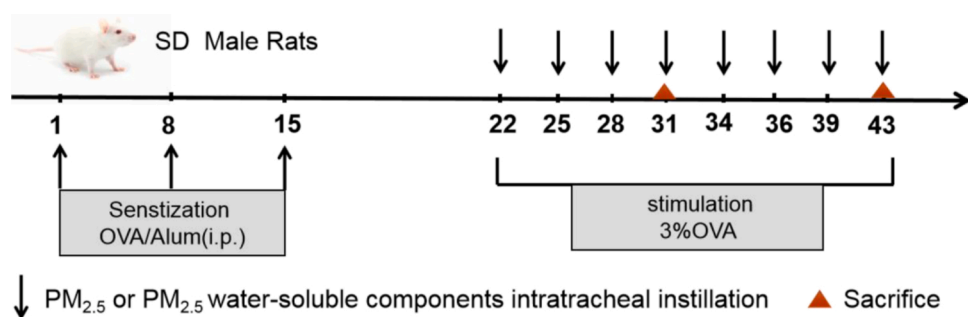


**Fig. 1.** Experimental protocol for the induction of asthma model and exposure to PM<sub>2.5</sub> and water-soluble components. The asthmatic rats were induced by intraperitoneal injection and inhalation aerosol of OVA. After asthma modeling, the rats were intratracheally instilled with PM<sub>2.5</sub> at 6 mg/kg-bw and water-soluble components at 3.6 or 14.4 mg/kg-bw for 4 times or 8 times at 3-days intervals. The inhibitor solution SB431542 (5 mg/kg-bw) and SIS3 HCl (1.25 mg/kg-bw) were intraperitoneally injected 15 min before the instillation of PM<sub>2.5</sub> water-soluble components (14.4 mg/kg-bw) following the manufacturer's instructions. Half of rats were sacrificed within

24 h of the above treatment on day 31, the rest of 35 rats were sacrificed on day 43.

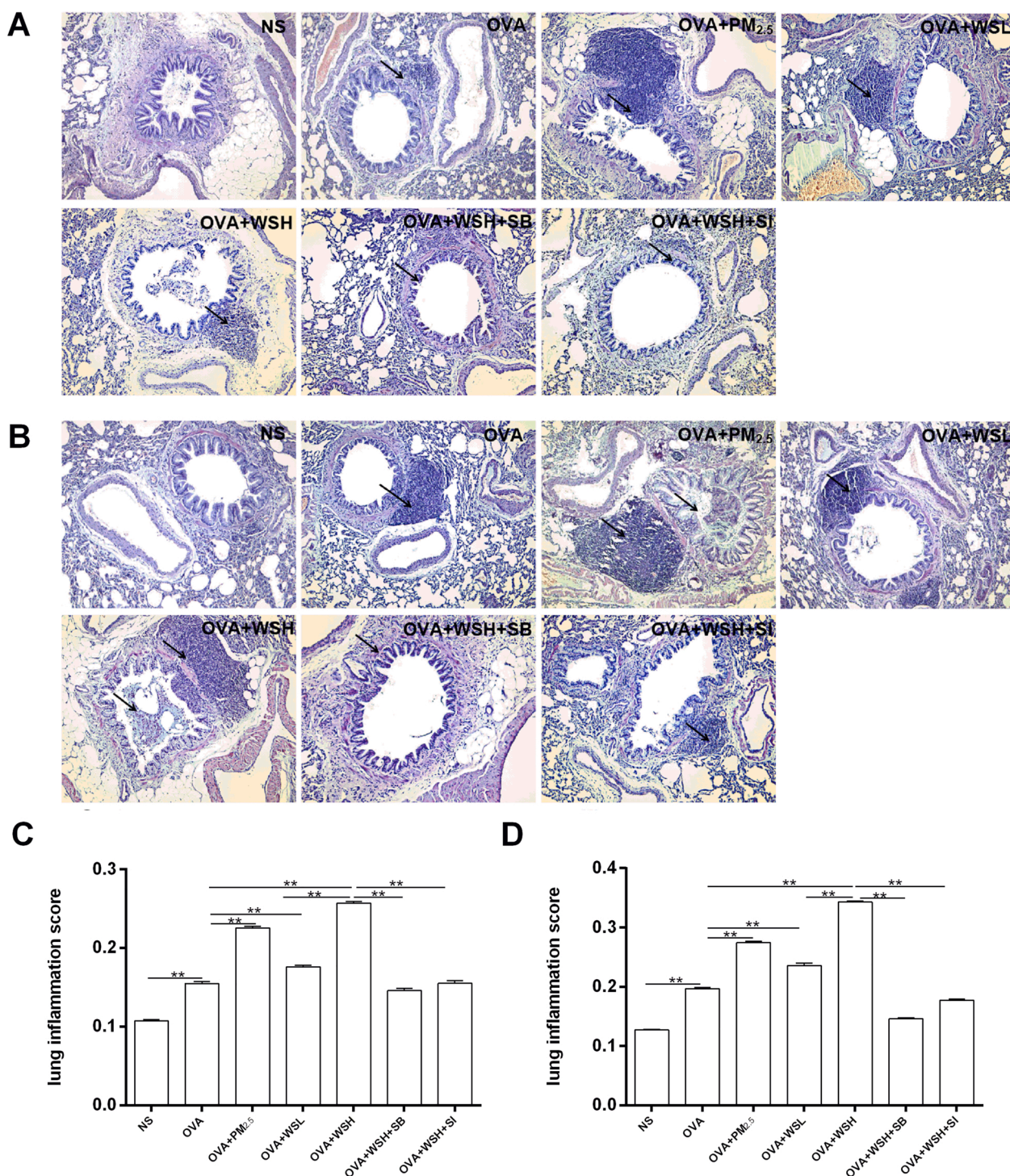


**Fig. 2.** Pathological alteration in OVA-induced asthmatic rats exposed to PM<sub>2.5</sub> and water-soluble components. Lung tissue specimens in asthmatic rats exposed to PM<sub>2.5</sub> and water-soluble components for 4 times (A) or 8 times (B) were stained with hematoxylin and eosin (H&E) at 100× magnification. The severity of peribronchial inflammation was analyzed semiquantitatively (C, D). The average of the inflammation scores is represented as the mean ± SD (n = 3 in each group), \*\**P* < 0.01. Black arrows indicate inflammatory cell infiltration.

(Fig. 2C, D).

The profibrotic effect of PM<sub>2.5</sub> and water-soluble components was further determined in asthmatic rats (Fig. 3). Trichrome-stained lung tissue sections showed that after exposure to PM<sub>2.5</sub> and water-soluble components, large collagen existed around the small airways. Interestingly, with the increase of water-soluble components doses, the degree of collagen deposition significantly elevated, showing that water-soluble components can potentially cause airway fibrosis in asthmatic rats

(Fig. 3A). The effective inhibitors of TGF-β1 and Smad3 were used to pre-treat asthmatic rats, followed by exposure to water-soluble components at a dose of 14.4 mg/kg-bw. Fig. 3A showed that the fibrosis levels in the presence of SB431542 and SIS3 HCl were obviously lower than those in the absence of these inhibitors, indicating that SB431542 and SIS3 HCl both could inhibit airway fibrosis. Similarly, as shown in Fig. 3B, collagen accumulation in the lungs was similar to the former features when exposure times double increased. This further proved the

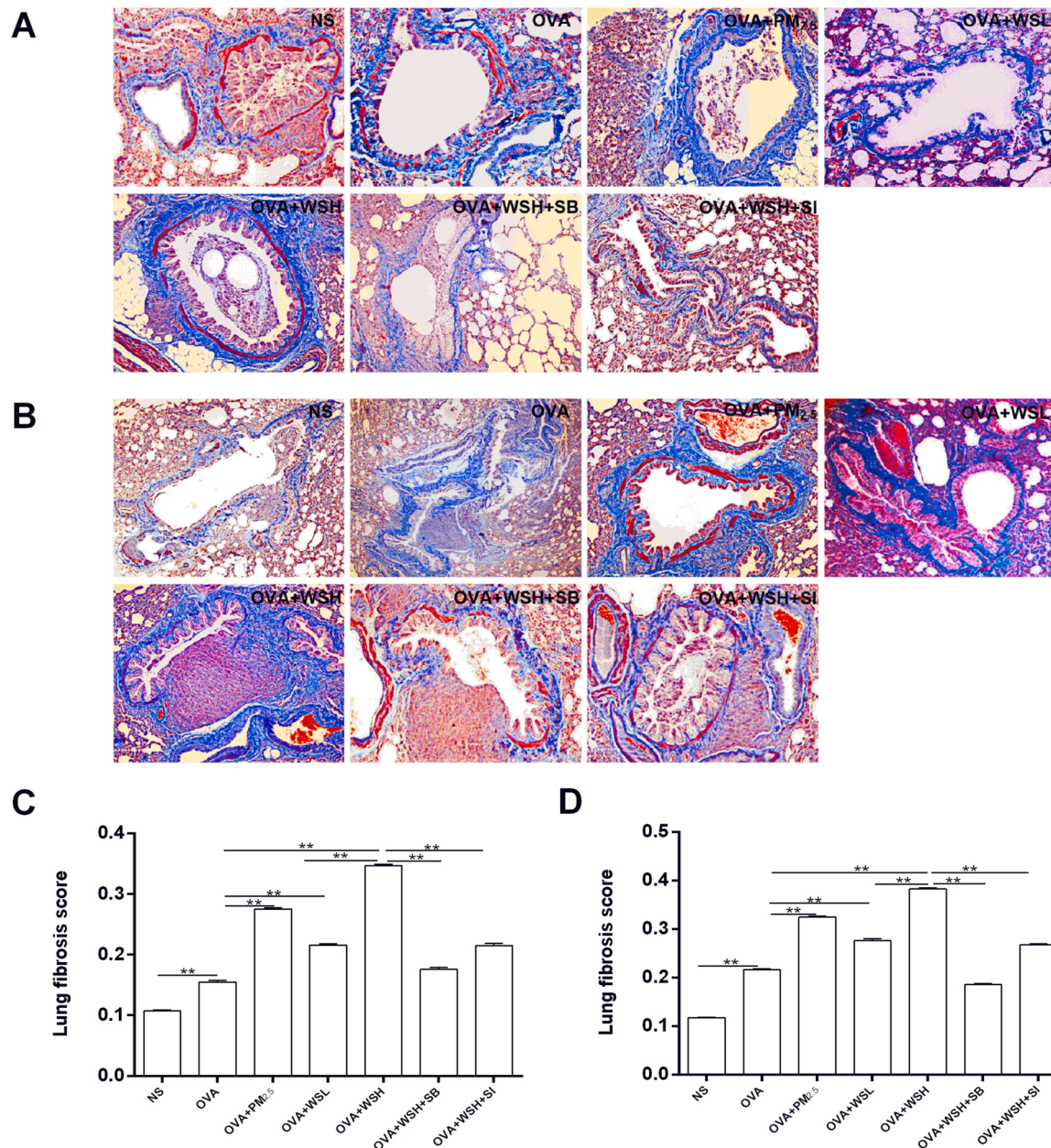


**Fig. 3.** Fibrotic changes in OVA-induced asthmatic rats exposed to PM<sub>2.5</sub> and water-soluble components. Masson's trichrome staining of lung tissues in asthmatic rats exposed to PM<sub>2.5</sub> and water-soluble components for 4 times (A) or 8 times (B) ( $\times 200$ ). Fibrosis degree of lung (C, D). Data are expressed as the mean  $\pm$  SD ( $n = 3$  in each group).  $**P < 0.01$ . The blue staining areas indicate the deposition of collagen fibrosis in lungs.

profibrotic effect of PM<sub>2.5</sub> and water-soluble components in asthmatic rats.

### 3.2. PM<sub>2.5</sub> and water-soluble components induced the expression of fibrosis-related factors

CTGF and collagen 1 are critical factors indicating the activation of pulmonary fibrosis. The gene and protein expression of CTGF and collagen 1 in the lung of asthmatic rats were determined by immunohistochemical and PCR analyses. After exposure to 6 mg/kg-bw PM<sub>2.5</sub>, 3.6 and 14.4 mg/kg-bw PM<sub>2.5</sub> water-soluble components for 4 or 8 times, the gene and protein expression levels of CTGF and collagen 1 in the lung markedly enhanced compared with that of OVA-sensitized asthmatic rats (Fig. 4). Meanwhile, the gene and protein expression levels of CTGF and collagen 1 significantly enhanced as the concentration of PM<sub>2.5</sub> water-soluble components increased, showing a dose-

dependent behavior (Fig. 4). This finding suggested that PM<sub>2.5</sub> and water-soluble components can potentially promote airway fibrosis in asthmatic rats through activation of collagen 1 and CTGF. Elevated gene and protein expression of collagen 1 and CTGF in OVA-sensitized rats in response to water-soluble components of PM<sub>2.5</sub> can be inhibited by the inhibitor of TGF- $\beta$ 1 and Smad3, which indicated that TGF- $\beta$ 1/Smad3 signaling pathway was involved in the activation of collagen 1 and CTGF, then inducing airway fibrosis.

### 3.3. PM<sub>2.5</sub> and water-soluble components increased the expression of inflammatory factors in plasma and lung tissues

Airway fibrosis occurrence is ascribed to the dysregulation of cytokines of HO-1 and IL-6. We then evaluated inflammation-related molecules by ELISA to further investigate the role of PM<sub>2.5</sub> and water-soluble components in airway fibrosis in asthmatic rats. As shown in Fig. 5A, the

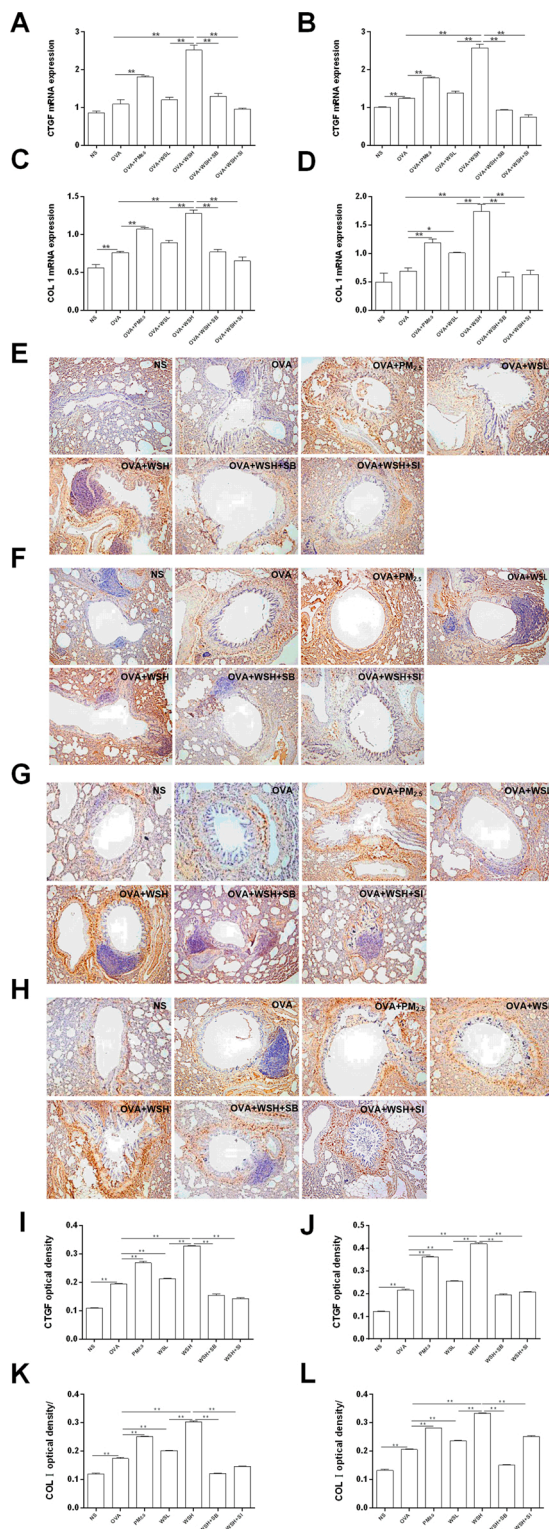


**Fig. 4.** Effect of PM<sub>2.5</sub> and water-soluble components on the fibrosis-related factors CTGF and COL 1. The gene expression of CTGF in the lung tissues of asthmatic rats intratracheally instilled with PM<sub>2.5</sub> and water-soluble components for 4 times or 8 times (A, B). The gene expression of COL 1 in rat lungs after instillation 4 times or 8 times (C, D). IHC analysis of the protein expression of CTGF in the lung tissue of asthmatic rats intratracheally instilled with PM<sub>2.5</sub> and water-soluble components for 4 times or 8 times (E, F) ( $\times 200$ ). IHC analysis of the protein expression of COL 1 in rat lungs after instillation 4 times or 8 times (G, H) ( $\times 200$ ). CTGF (I, J) and COL 1 (K, L) staining density analysis. Data are expressed as the mean  $\pm$  SD ( $n = 3$  in each group). \*\* $P < 0.01$ .

levels of IL-6 in OVA-sensitized rats treated with 6 mg/kg-bw PM<sub>2.5</sub> and 14.4 mg/kg-bw PM<sub>2.5</sub> water-soluble components were significantly higher than that in the untreated rats. More importantly, after double increase of exposure times, the IL-6 level in the group exposed to PM<sub>2.5</sub> water-soluble components at 14.4 mg/kg-bw was higher than that in the 3.6 mg/kg-bw group (Fig. 5B). In contrast, SB431542 treatment significantly decreased the level of IL-6 (Fig. 5A, B). As for anti-oxidative stress cytokine HO-1, the OVA-sensitized rats presented a lower level of HO-1 in the plasma compared to the saline control group (Fig. 5C, D). After treating the OVA-sensitized rats with PM<sub>2.5</sub> and water-soluble components, significant reduction of HO-1 was observed in the plasma (Fig. 5D). However, pretreatment with SB431542 and SIS3 HCl could remarkably reverse reduced HO-1 secretion (Fig. 5D).

Consistent with the ELISA, western blot results showed that IL-6 was up-regulated, whereas HO-1 was less expressed following exposure to PM<sub>2.5</sub> and water-soluble components (Fig. 5E, F). Moreover, TGF- $\beta$ 1 and Smad3 inhibitors both inhibited increased IL-6 and activated decreased HO-1 in lung tissues. The results suggested that PM<sub>2.5</sub> and water-soluble components can activate IL-6 and inhibit HO-1, which could be reversed by TGF- $\beta$ 1 and Smad3 inhibitors. Further research needs to confirm whether TGF- $\beta$ 1/Smad3 signaling pathway is involved in the occurrence of oxidative stress and inflammation response.

#### 3.4. PM<sub>2.5</sub> and water-soluble components up-regulated TGF- $\beta$ 1/Smad3 signaling pathway in OVA-sensitized rats

Since TGF- $\beta$ 1/Smad3 signaling pathway plays a vital role in the development of tissue fibrosis, whether PM<sub>2.5</sub> and water-soluble components affected the activation of TGF- $\beta$ 1, Smad3, and p-Smad3 in asthmatic rats were determined as well. We observed that PM<sub>2.5</sub> and water-soluble components significantly induced the gene and protein expression of TGF- $\beta$ 1 and Smad3, and the protein expression of p-Smad3 in lung tissue of asthmatic rats, which was most pronounced under the condition of high concentration of PM<sub>2.5</sub> water-soluble components (14.4 mg/kg) (Fig. 6A, C). Contrarily, the elevated expression of TGF- $\beta$ 1, and Smad3 was suppressed by SB431542 and SIS3 HCl (Fig. 6A, C). In addition, increased protein levels further validated the activation of TGF- $\beta$ 1/Smad3 signaling pathway in airway fibrosis in asthmatic rats (Fig. 6E, I, K, Q). Meanwhile, after double increase of the exposure times, the gene and protein expression of TGF- $\beta$ 1, Smad3, and the protein expression of p-Smad3 are in keeping with the above results (Fig. 6B, D, F, J, L, R). These results demonstrated that PM<sub>2.5</sub> and water-soluble components could induce airway fibrosis in OVA-sensitized asthma model via TGF- $\beta$ 1/Smad3 signaling pathway.

#### 4. Discussion

Water-soluble components are the main toxic substances of PM<sub>2.5</sub>, whether it could contribute to airway fibrosis in asthmatic and its underlying mechanism were rarely studied. In the present study, we not only found that PM<sub>2.5</sub> and water-soluble components could induce lung inflammation and then trigger collagen deposition in OVA-sensitized rats lung tissues, but also identified the crucial role of TGF- $\beta$ 1/Smad3 signaling pathway in occurrence and development of airway fibrosis in asthmatic rats.

Airway fibrosis is a progressive pulmonary disease featured with varying degrees of thickening and folding of luminal wall (Jeganathan et al., 2020). Airway fibrosis formation, one of the exacerbated outcomes of asthma, has been extensively studied, which was involved in the following two steps: the early stage of airway fibrosis was characterized by considerable inflammatory cell infiltration and oxidative stress damage; in the later stage, massive augmentation of extracellular matrixes (ECM) with abundant accumulation of collagen and fibroblasts occurred in the lung, implying that progressive airway remodeling has developed (Milara et al., 2020). In the current study, OVA-sensitized rats showed mild inflammatory cell infiltration and collagen fiber

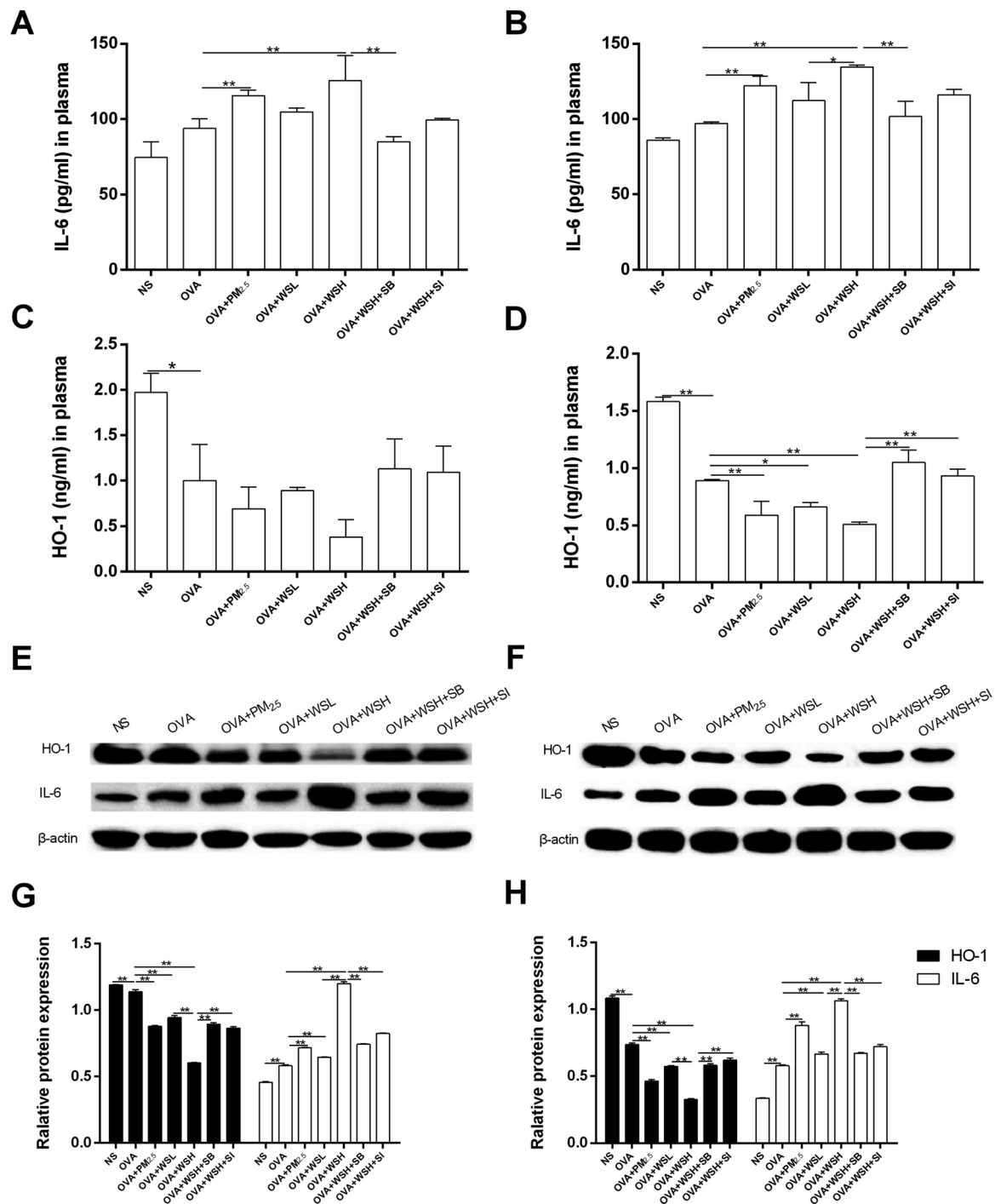


**Fig. 5.** Effect of PM<sub>2.5</sub> and water-soluble components on inflammatory cytokine and oxidative stress factor levels. IL-6 expression in plasma after instillation of PM<sub>2.5</sub> and water-soluble components for 4 times or 8 times (A, B), HO-1 expression in plasma after instillation 4 times or 8 times (C, D). Western blot analysis of the relative protein expression of IL-6 and HO-1 in rat lungs after instillation of PM<sub>2.5</sub> and water-soluble components for 4 times or 8 times (E, F). The protein gray analysis of IL-6 and HO-1 after instillation 4 times or 8 times, respectively (G, H). Data are expressed as the mean  $\pm$  SD ( $n = 3$  in each group). \* $P < 0.05$ . \*\* $P < 0.01$ .

deposition, suggesting we have well established the asthma model in rats. In addition, destroyed alveolar structure with a great inflammatory cell infiltration and collagen deposition was observed in PM<sub>2.5</sub> and water-soluble components stimulated lungs of asthmatic rats. It means that the profibrotic effects of PM<sub>2.5</sub> and water-soluble components in the airway of asthmatic should be paid more attention.

As a crucial profibrotic factor in the downstream of TGF- $\beta$ 1, CTGF was regulated by a functional Smad3-binding site in the CTGF promoter (Ziller et al., 2020). After exposure to PM<sub>2.5</sub>, CTGF coordinated with TGF- $\beta$ 1 to induce airway fibrosis through activation of extracellular

matrix proteins, such as collagen 1 (Guo et al., 2019). A vitro study has identified that human lung fibroblasts recruited more CTGF and collagen 1 factors (Guan and Zhou, 2017). The finding was verified in our OVA-sensitized rats model again. PM<sub>2.5</sub> and water-soluble components effectively increased the expression of CTGF and collagen 1 in the lungs of asthmatic rats (Fig. 4). The result underlines the profibrotic effects of PM<sub>2.5</sub> and water-soluble components in asthmatic rats.

Excessive release of proinflammatory cytokines such as IL-6 following PM<sub>2.5</sub> exposure can trigger the aggravation of inflammation and oxidant load. (Huang et al., 2019). It was reported that IL-6 triggers

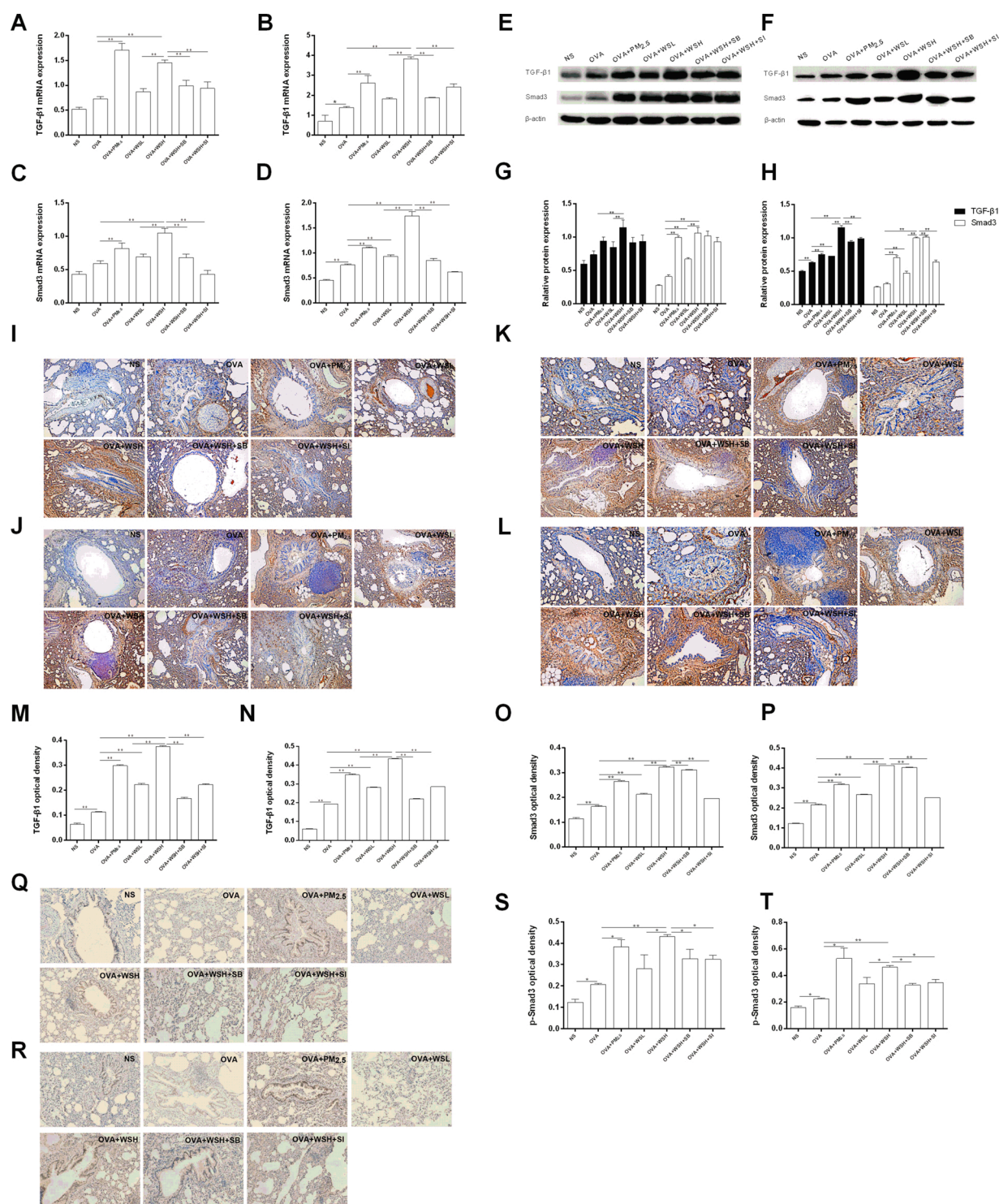


**Fig. 6.** Effect of PM<sub>2.5</sub> and water-soluble components on the TGF-β1/Smad3 signaling pathway. The gene expression of TGF-β1 in rat lungs after instillation of PM<sub>2.5</sub> and water-soluble components for 4 times or 8 times (A, B). The gene expression of Smad3 in rat lungs after instillation 4 times or 8 times (C, D). Western blot detection of the protein expression of TGF-β1 and Smad3 in rat lungs after instillation of PM<sub>2.5</sub> and water-soluble components for 4 times or 8 times (E, F). The relative protein expression levels of TGF-β1 and Smad3 in rat lungs after instillation 4 times or 8 times, respectively (G, H). IHC detection of the protein expression of TGF-β1, Smad3, and p-Smad3 in rat lungs after instillation 4 times (I, K, Q) or 8 times (J, L, R) (× 200). TGF-β1 staining density analysis after instillation 4 times or 8 times (M, N). Smad3 staining density analysis after instillation 4 times or 8 times (O, P). p-Smad3 staining density analysis after instillation 4 times or 8 times (S, T). Data are expressed as the mean ± SD (n = 3 in each group). \* *P* < 0.05, \*\**P* < 0.01.

the accumulation of collagen and enhances the TGF-β signaling pathway, which eventually results in pulmonary fibrosis (Dong et al., 2020; Tan et al., 2020). Meanwhile, the relative levels of IL-6 increased significantly in the TGF-β1 treatment group in HPMCs (Shentu et al.,

2021). These results suggested that the interaction between TGF-β1 and IL-6 is associated with pulmonary fibrosis. As an important redox regulator, HO-1 plays a critical role in anti-oxidant and pro-survival in response to cellular stressors, which could maintain the redox

homeostasis and thus ameliorate fibrosis response (Hauptmann et al., 2020). Up-regulation of HO-1 could suppress TGF- $\beta$ 1 dependent ROS production, which is useful in the treatment of kidney fibrosis (Clark et al., 2016). HO-1 protects hepatocellular carcinoma cells against TGF- $\beta$  growth by increasing Smad3 phosphorylation (Park et al., 2018). Furthermore, TGF- $\beta$ 1 also could stimulate HO-1 expression in pulmonary epithelial cells (Ning et al., 2002). These studies showed that HO-1 and TGF- $\beta$ 1/Smad3 signaling pathway reciprocally regulate each other. In the present study, after exposure to PM<sub>2.5</sub> and water-soluble components, IL-6 was up-regulated, whereas HO-1 was less expressed. The results were consistent with increased pulmonary histopathology scores (Fig. 3), eosinophils and neutrophils accumulation (Fig. 1), and the paralleled increased airway fibrosis levels (Fig. 4), suggesting that aberrant oxidative stress exaggerated airway fibrosis in asthmatic rats following PM<sub>2.5</sub> and water-soluble components exposure.

In the pathogenesis of pulmonary fibrosis, TGF- $\beta$ 1 is an attractant for inducing differentiation of fibroblasts into myofibroblasts and ECM synthesis (Qian et al., 2018). Activated TGF- $\beta$ 1 can stimulate Smad3 to be phosphorylated in cytoplasm, and then migrate into nucleus to mediate transcriptions of various fibrogenic cytokines including collagen 1 and CTGF (Pan et al., 2020). In this work, after PM<sub>2.5</sub> and water-soluble extracts exposure, the expression of TGF- $\beta$ 1, Smad3 and p-Smad3 was obviously increased in the lung tissues of asthmatic rats, which was reversed after treatment with SB431542 and SIS3 HCl. Similarly, decreased collagen 1, CTGF, IL-6, and increased HO-1 were also observed paralleled with alleviated airway fibrosis when pretreatment with SB431542 and SIS3 HCl. The data indicated that PM<sub>2.5</sub> and water-soluble components could stimulate airway fibrosis in asthma, potentially by impacting TGF- $\beta$ 1/Smad3 signaling pathway.

The present study revealed that subacute exposure to PM<sub>2.5</sub> and water-soluble components could induce sustained pulmonary fibrosis development in OVA-sensitized asthmatic rats, which was confirmed by a significant increase in fibrosis scoring. However, there was no significant difference among the different exposure times, implicating that subacute exposure to PM<sub>2.5</sub> and water-soluble components still did not inflict maximum pulmonary damage. Actually, it must be acknowledged that our study provides a new idea to explore the long-term effect of PM<sub>2.5</sub> and water-soluble components in the future. Furthermore, water-soluble components played the most significant role in inducing airway fibrosis in our present study, suggesting that the subacute toxicity of water-soluble components still should be paid close attention even if it accounts for a portion of PM<sub>2.5</sub>. More importantly, although all the data acquired from asthmatic rats cannot be fully extrapolated to the human being, we must acknowledge that pathogenetic mechanism and therapeutic intervention in the fibrosis outcome of asthmatic patients are worthy of further exploration.

## 5. Conclusions

In summary, this study provides scientific evidence to support that PM<sub>2.5</sub> and water-soluble components could induce lung inflammation and ensue airway fibrosis in asthmatic rats through activation of TGF- $\beta$ 1/Smad3 signaling pathway. It may provide a novel therapeutic target for preventing ambient pollutants-induced fibrosis in asthmatic patients.

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## CRediT authorship contribution statement

**Hongyan Wu:** Conceptualization, Methodology, Writing - original

draft. **Dan Wang:** Writing - review & editing, Formal analysis. **Hao Shi:** Visualization. **Nannan Liu:** Investigation. **Caihong Wang:** Formal analysis, Resources. **Jiayu Tian:** Formal analysis, Investigation. **Xin Wang:** Formal analysis. **Zhihong Zhang:** Methodology, Conceptualization, Supervision.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2021.06.005>.

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# Supplementary Material

## S1. Chemical components of PM<sub>2.5</sub>

The chemical components of metal elements and water-soluble ions were determined by ICP-MS and IC separately in our study. The concentrations and elemental compositions of PM<sub>2.5</sub> were shown in STable 1. 12 metal elements (Zn, Fe, Mn, Pb, Cr, Cu, Ni, Ti, As, Se, V, and Cd) accounted for 2.74% of total PM<sub>2.5</sub> mass, and Fe and Zn were the major two metals. Total water-soluble ions in PM<sub>2.5</sub> accounted for 56.14% of total PM<sub>2.5</sub> mass. The major water-soluble ions (NO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, NH<sub>4</sub><sup>+</sup> and F<sup>-</sup>) accounted for 30.00% of total PM<sub>2.5</sub> mass.

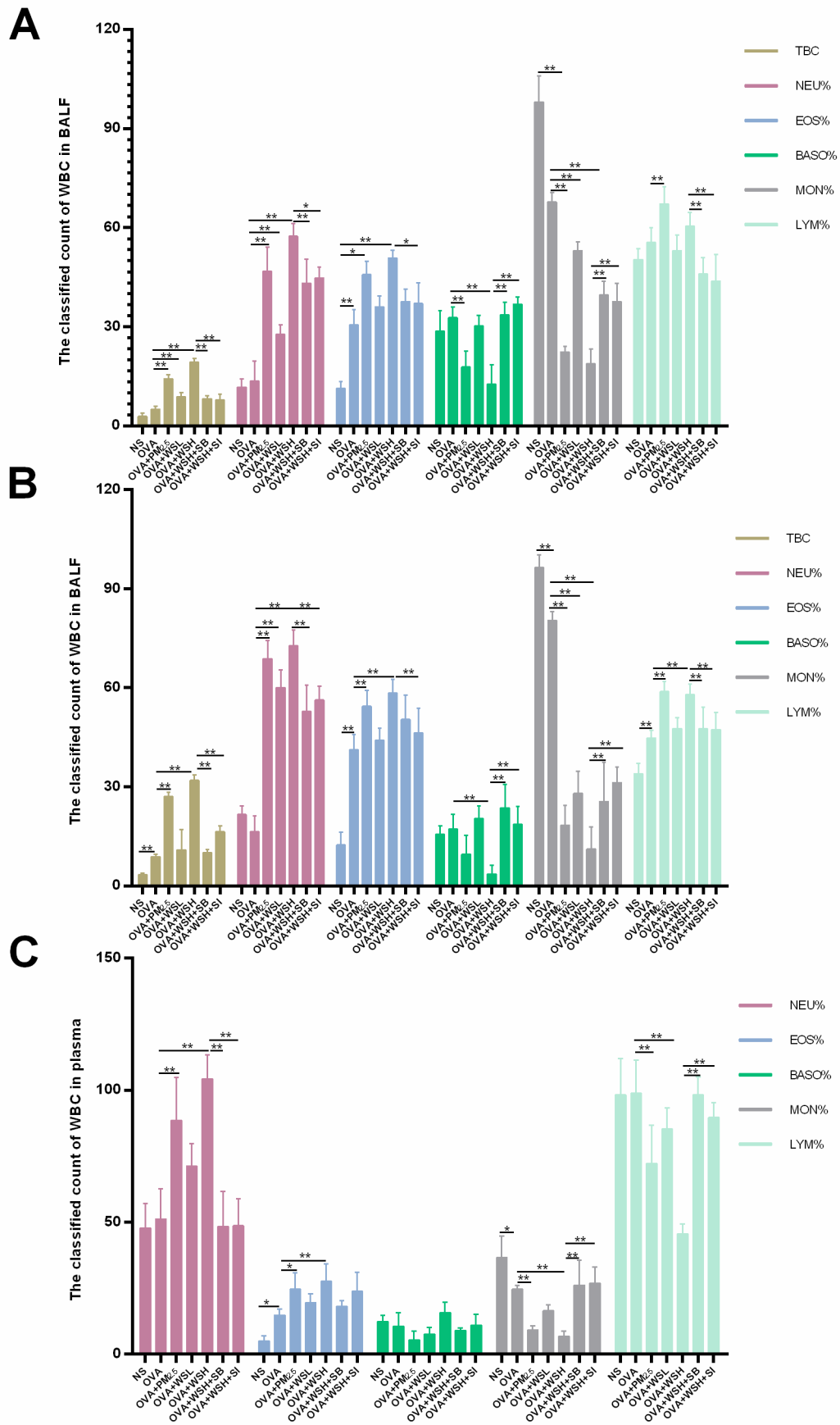
**STable 1.** 12 metals and 5 soluble anions concentrations in PM<sub>2.5</sub>

| elements   | Concentration (ng/m <sup>3</sup> ) | water-soluble ions            | Concentration (µg/m <sup>3</sup> ) |
|------------|------------------------------------|-------------------------------|------------------------------------|
| Zn         | 126.16 ± 51.36                     | NO <sub>3</sub> <sup>-</sup>  | 16.24 ± 6.14                       |
| Fe         | 2100.10 ± 259.69                   | Cl <sup>-</sup>               | 0.75 ± 0.12                        |
| Mn         | 64.01 ± 11.75                      | SO <sub>4</sub> <sup>2-</sup> | 9.98 ± 2.86                        |
| Pb         | 71.48 ± 35.07                      | NH <sub>4</sub> <sup>+</sup>  | 8.24 ± 2.68                        |
| Cr         | 22.71 ± 4.96                       | F <sup>-</sup>                | 0.01 ± 0.00                        |
| Cu         | 20.69 ± 3.44                       | total                         | 35.22 ± 11.64                      |
| Ni         | 8.62 ± 1.86                        | percentage                    | 30.00%                             |
| Ti         | 28.67 ± 3.83                       |                               |                                    |
| As         | 5.78 ± 3.32                        |                               |                                    |
| Se         | 6.76 ± 2.32                        |                               |                                    |
| V          | 2.40 ± 0.57                        |                               |                                    |
| Cd         | 0.79 ± 0.34                        |                               |                                    |
| total      | 2458.16 ± 186.90                   |                               |                                    |
| percentage | 2.74%                              |                               |                                    |

## S2. Total number of white cells and differential cell counts in BALF and plasma

The total white cells (TWC) and differential cell counts in BALF were detected. As shown in SFig. 1A, 1B, OVA obviously increased the percentage of eosinophils (EOS), whereas decreased the ratio of monocytes (MON) compared with saline control group ( $P < 0.01$ ). After exposure to PM<sub>2.5</sub> and water-soluble components, the total white cells and the percentages of neutrophils (NEU), eosinophils (EOS), and lymphocyte (LYM) were significantly elevated, while basophils (BASO) and monocytes (MON) were reduced compared with OVA group ( $P < 0.05$ ), especially in the high concentration of water-soluble components (14.4 mg/kg·bw) group. Pretreatment with TGF- $\beta$ 1 and Smad3 inhibitors can restore the total white cells and the percentages of differential cell counts in the BALF. The data suggested that PM<sub>2.5</sub> and water-soluble components could aggravate pulmonary inflammatory response in asthmatic rats.

As shown in SFig. 1C, leukocyte differential counts by blood smear image indicated that the mild-increased percentages of NEU and EOS, while decreased proportions of MON and LYM in OVA-induced asthmatic rats were further strengthened in PM<sub>2.5</sub> and water-soluble components challenged groups. In contrast, the inhibitors of TGF- $\beta$ 1 and Smad3 obviously attenuated the degree of inflammatory cell infiltration, which was largely consistent with the BALF result. The data indicated that the systematic inflammatory response was obviously aggravated in asthmatic rats exposed to PM<sub>2.5</sub> and water-soluble components.



26 **SFig. 1 Effect of PM<sub>2.5</sub> and water-soluble components on inflammatory cell infiltration in bronchoalveolar**  
27 **lavage fluid (BALF) and plasma of asthmatic rats.** The classified count of WBC in BALF of asthmatic rats  
28 exposed to PM<sub>2.5</sub> and water-soluble components for 4 times (A) and 8 times (B). The classified count of WBC in  
29 plasma of asthmatic rats exposed to PM<sub>2.5</sub> and water-soluble components for 8 times (C). Data are expressed as  
30 the mean  $\pm$  SD (n = 3 in each group). \*  $P < 0.05$ , \*\* $P < 0.01$ .