

RESEARCH ARTICLE

Indoor PM_{2.5} from coal combustion aggravates ovalbumin-induced asthma-like airway inflammation in BALB/c mice

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Yu J, Li K, Xu J. Indoor PM_{2.5} from coal combustion aggravates ovalbumin-induced asthma-like airway inflammation in BALB/c mice. *Am J Physiol Lung Cell Mol Physiol* 317: L29–L38, 2019. First published April 10, 2019; doi:10.1152/ajplung.00012.2019.—We hypothesized that indoor PM_{2.5} exposure from coal combustion exacerbates airway inflammation in the lung tissue of asthmatic mice induced with ovalbumin (OVA). Forty BALB/c mice, randomly divided into four groups ($n = 10$ per group), were intratracheally instilled with normal saline alone, PM_{2.5} (2.5 mg/ml PM_{2.5} alone), OVA (15 μ g/ml OVA alone), and PM_{2.5}+OVA (2.5 mg/ml PM_{2.5} and 15 μ g/ml OVA), respectively, four times at 2-wk intervals. Daily mean concentration of PM_{2.5} from indoor coal combustion was 156.95 μ g/m³. The highest metal composition in PM_{2.5} was Zn (34.81 ± 1.8 μ g/m³). Exposure to PM_{2.5}+OVA significantly elevated IL-4 and decreased IFN- γ production in mice compared with the control ($P < 0.05$). Exposure to PM_{2.5}+OVA showed a significant increase in the protein levels of granulocyte-macrophage colony-stimulating factor and IL-8 and a decrease in the protein level of transforming growth factor- β 1 in bronchoalveolar lavage fluid of mice compared with the control ($P < 0.05$). The expression of IL-4 mRNA was significantly increased, whereas the expression of IFN- γ mRNA was decreased in lung tissue of the PM_{2.5}+OVA group ($P < 0.05$). The expression level of Foxp3 mRNA in the PM_{2.5}+OVA group was significantly lower than that in the control group in lung tissue ($P < 0.05$). Treatment with PM_{2.5}+OVA promoted a prominent neutrophil sequestration into the lung parenchyma, goblet cell proliferation, and severe inflammatory cell infiltration in the airways. Exposure to PM_{2.5} from indoor coal combustion might induce airway inflammatory immune responses and exacerbate peribronchiolar inflammation due to infiltration of inflammatory cells into the airway submucosa and airway structural pathological changes.

airway inflammation; asthma; coal combustion; indoor; lung tissue; PM_{2.5}

INTRODUCTION

Asthma and asthma-related symptoms are a common chronic condition, and there is evidence that its prevalence is increasing worldwide. Our cross-sectional epidemiologic studies examined whether indoor air pollution could account for a high prevalence rate of asthma and asthma-related symptoms and deterioration of pulmonary function among adults of an acid rain-plagued city in southwest China (18a, 18b). The city of Zunyi with vast coal reserves is renown as the city most seriously polluted by acid rain. The sulfur content in coal used

in Zunyi households was ~3% (16). Taking into account the chilly climate in winter in Zunyi, there is a great need for coal, which is used as the main source of energy for heating, cooking, and baking in households. The city of Zunyi has been strongly impacted by particulate matter (PM) and other air pollutants from domestic (coal combustion) sources and industrial sources (e.g., power plant). The overall prevalence of adult asthma and asthma-related symptoms was reported to be 13% in Zunyi in 2012 (17). Indoor factors most consistently associated with asthma and asthma-related symptoms and decreased pulmonary function in adults included fuel combustion, mold growth, and environmental tobacco smoke (18). These results from our previous studies suggested that exposure to indoor air pollutants, including PM_{2.5} (PM <2.5 μ m aerodynamic diameter), could promote sensitization to common air contaminants and further development or exacerbation of asthma morbidity. However, the adverse effect of PM_{2.5} from indoor coal combustion on airway inflammation in asthma has never been elaborated.

In the present study, our objective was to determine whether the results observed in these epidemiologic studies could be replicated in an animal asthma model and identify indoor PM_{2.5} exposure from coal combustion as a causative factor in the exacerbation of airway inflammation in the lung tissue of asthmatic mice induced with ovalbumin.

MATERIALS AND METHODS

Ethics Statement

All the experimental procedures were conducted at Zunyi Medical University and adhered to the guidelines and regulations set out by the Zunyi Medical University Ethics Committee, with full approval from its Animal Care and Use Committee.

Sample Collections of Coal-Fired PM_{2.5}

Raw coal was purchased from a coal field in Zunyi in southwest China. In a 60 m³ clean laboratory in the School of Public Health of Zunyi Medical University, coal was processed to the size of 3–5 cm and ignited with coke, followed by sampling after obtaining stable coal flame. Particle size distribution and concentration were measured using a precalibrated sampler (TH150-A, Wuhan Tianhong INST Group). PM_{2.5} samples were collected on glass fiber filters (90 mm in diameter, Wuhan Tianhong INST Group) by the sampler with PM_{2.5} cyclone at a flow rate of 100 l/min, and the filter was changed every 4 h for the analysis of PM_{2.5} concentration. The samplers were placed 1.5 m above the ground and 1 m away from the stove horizontally within the room. The ambient temperature and humidity were recorded before and after sampling. Before and after collection, the glass fiber membrane was weighted after being equilibrated in a drying tower for 24 h. Indoor PM_{2.5} concentration was calculated

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based on the formula: $C_{PM_{2.5}} = \frac{m_0 - m_1}{Q}$, where C is concentration, m_0 is the weight of filter membrane after sampling (mg), m_1 is the weight of filter membrane before sampling (mg), and Q is the volume under the standard condition (V).

Coal-Fired PM_{2.5} Sample Extraction

The PM_{2.5} filters were extracted with ultra-pure water in an ultrasonic bath. The filter containing particles was cut into 1 × 3 cm sections, submerged in deionized distilled water, and subjected to ultrasonic oscillation three times for 20 min. The suspensions were filtered through 12 layers of cotton gauze, and filtrates were centrifuged at 12,000 rpm for 30 min at 4°C. The resulting supernatant liquids were collected and freeze-dried under vacuum. Before use, the dried samples were diluted with sterilized 0.9% physiological saline and mixed for 15 min. After ultrasonic elution and freeze-drying, the samples were prepared and stored at -20°C until used (2).

Analysis of Metal Elements in PM_{2.5}

The filter for indoor PM_{2.5} collection of burning coal was cut into pieces and put into the TFM (modified polytetrafluoroethylene) digestion tank, with 40 ml of concentrated HNO₃-HCl (vol/vol = 1:3) added, capped, and then placed on the turntable for 15 min of digestion at 200°C. After cooling to room temperature, we opened the can and rinsed the filter with 40 ml triple-distilled water. The solution was allowed to sit for half an hour before extraction. It was drained into a 200-ml volumetric flask, washed with triple-distilled water 5 times, and the washing water drained into a volumetric flask. Triple-distilled water was added to a volume of 200 ml. The obtained solution was subjected to instrumental detection after filtration by 0.45-μm filter. The spiked recovery of this method is between 94% and 105% (9).

Animals

A total of 40 male BALB/c mice (weighing 190 ± 10 g) aged 6–8 wk were purchased from the Animal Center of the Third Military Medical University (Chongqing, China) and were housed in polypropylene cages (380 mm × 220 mm × 150 mm) with pinewood sawdust bedding. The cages, each housing up to 10 mice, were furnished with bedding materials, gnaw sticks, and cardboard tubes. The animals were maintained under controlled temperature (20 ± 1°C) and humidity (60 ± 5%), on a 12:12-h light-dark (light: 0900–2100; dark: 2100–0900) cycle. Food and water were freely available. The proposal of the study was reviewed and approved by the Zunyi Medical University. Animal experimental procedures were approved by Zunyi Medical University Ethics Committee. All methods were performed in accordance with guidelines and regulations of the Zunyi Medical University.

Study Design

The 40 mice were randomly divided into four groups ($n = 10$ per group), and each group was treated with a specific testing sample. The four testing samples prepared for the present study were control (containing normal saline alone), PM_{2.5} (2.5 mg/ml PM_{2.5} alone), ovalbumin (OVA; 15 μg/ml OVA alone), and PM_{2.5}+OVA (2.5 mg/ml PM_{2.5} and 15 μg/ml OVA). The tidal volume of mice is ~0.86 ml, with respiratory rate of ~85 breaths/min. PM_{2.5} concentration of the sampling environment was measured to be 156.95 μg/m³, with 2 wk of mice inhalation calculated to be $0.86 \times 85 \times 2 \times 7 \times 24 \times 60 \times 156.95 \div 106 = 231.3$ μg. PM_{2.5} of 500 μg per mouse was administered, which was ~2.2 times of the inhalation dose. Our preexperiment results indicated that the deposition rate of PM_{2.5} in the lungs of mice was ~20%. The deposition of PM_{2.5} in mice was ~46.26 μg, and the administered dosage was ~10.8 times the deposition amount. Additionally, OVA at 3 μg could stimulate asthma in mice.

OVA (Grade VII) was purchased from Sigma-Aldrich (Shanghai, China). OVA was dissolved in a sterile saline solution.

The instillation dose of PM_{2.5} was 500 μg/mouse, and the instillation dose of OVA was 3 μg per mouse. Mice were intratracheally instilled with a mixed or individual solution of normal saline alone, OVA, and PM_{2.5} through a polyethylene tube under 4% halothane anesthesia 4 times at 2-wk intervals (14). Therefore, the total instillation dose of PM_{2.5} was 0.8 mg/mouse. The control group was treated with a 80-μl sterile saline solution. One day after the last intratracheal administration, mice from all groups (aged = 13 wk) were euthanized by exsanguination under deep anesthesia by intraperitoneal injection of pentobarbital sodium. Serum was taken by centrifugal separation of blood. The serum samples were stored at -80°C until further analysis.

Quantitation of Cytokines and Chemokine Bronchoalveolar Lavage Fluid

The cytokine protein levels in the bronchoalveolar lavage fluid (BALF) were measured using an enzyme-linked immunosorbent assay (ELISA). Interleukin (IL)-4, IL-8, interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-β1 (TGF-β1) were measured by an ELISA kit (Beijing Suolaibao Technology, Beijing, China).

Serum Antigen-Specific IgE and IgG1 Antibodies

Serum OVA-specific IgE and IgG1 antibodies were measured by a Mouse OVA-IgE ELISA kit and a mouse OVA-IgG1 ELISA kit (Beijing Suolaibao Technology, Beijing, China). According to the manufacturer's protocol, 1 U of anti-OVA IgE was defined as 1.3 ng of antibody, and 1 U of anti-OVA IgG1 as 160 ng of antibody. The absorption at 450 nm (subwavelength, 620 nm) for OVA-specific IgE and IgG1 antibodies was measured using a microplate reader (Spectrafluor, Tecan, Salzburg, Austria).

Detection of IL-4 and IFN-γ mRNA in Lung Tissue by RT-PCR

Total RNA of whole lung tissue was extracted using TRIzol (Invitrogen, San Diego, CA, no. 1259673). Reverse transcriptase was obtained from Invitrogen (no. 696045), the thermal cycler was obtained from Biometra (Goettingen, Germany; model T1-Thermoblock), the electrophoresis apparatus was obtained from Bio-Rad (Hercules, CA; model: Power PAC 300), and the gel image analysis instrument was obtained from Bio-Rad (model: Gel Doc XR). Changes in the band brightness of IL-4 and IFN-γ were observed, and the optical density of each band was analyzed. The levels of IL-4 and IFN-γ are given relative to GAPDH levels as a control (23).

Pathological Evaluation

Pathological hematoxylin and eosin staining. The following procedures were followed: 1) for preparation, ~0.5 × 0.5 cm tissue was cut from the same position of the upper lung of mice, rinsed with normal saline, and quickly fixed in 10% paraformaldehyde for 24 h. 2) For dehydration, fixed tissue specimens were soaked in four concentrations of gradient ethanol in this order: 80%, 90%, 95% anhydrous ethanol; 2 h for each gradient. 3) For hyalinization, after the specimen was treated (step 2), it was then immersed in xylene solution for another 2 h. 4) For waxing, treated specimens were soaked in paraffin for 3 h. 5) For embedding, waxed specimens were immersed in melting paraffin, which turned into a paraffin block after condensation. 6) For slicing, paraffin blocks were sliced into slices of ~4- to 6-μm thickness. 7) For sectioning, the sliced specimen was completely paved and transferred to the middle of the slide, which was slightly angled to remove water, and placed into a temperature-controlled instrument for dewaxing. 8) For staining, hematoxylin and eosin staining was used, including dewaxing, extraction of benzene, rehydration, dyeing, dehydration, hyalinization, and sealing. 9) Microscopic examination (Olympus BX53).

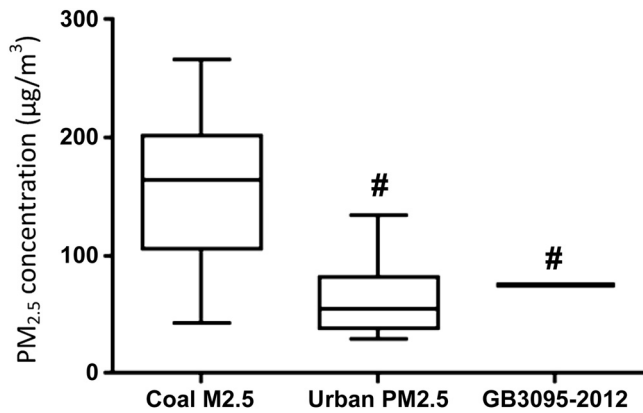


Fig. 1. A comparison of particulate matter ≤ 2.5 μm diameter (PM_{2.5}) concentrations among indoor and outdoor environments and China National Ambient Air Quality Standards. # $P < 0.05$ vs. coal PM_{2.5}.

Electron microscopic sections. The following procedures were followed: 1) For tissue treatment, 1 mm³ of lung tissue was obtained within 1 min and put into an Eppendorf tube (EP), which was fixed by 2.5% glutaraldehyde at 4°C for 2 h. 2) Fixation solution was removed from the EP tube and washed with phosphate buffer (0.01 M) for 5 min $\times$ 3 times. 3) Washing solution was removed and 1% osmium was added for 3-h infiltration at 4°C, followed by washing with ddH₂O for 10 min $\times$ 3 times. 4) For dehydration, at room temperature the specimens were infiltrated in acetone at different gradient concentrations (50%, 70%, 80%, 80%, and 100%) for 15 min, repeated twice for each gradient. 5) After infiltration in acetone and embedding solution (vol/vol = 1:1) for 3 h, the specimen was infiltrated in embedding solution at 37°C for 2 h. 6) The specimen was placed in the embedding plate and embedded in the embedding solution. The samples were naturally condensed overnight at 35°C, then placed at 45°C for 12 h, and then at 60°C for 48 h for polymerization, which was then dried for storage. 7) Leica extra-slim slicer was used for slicing the specimens into sections of 60-nm thickness. 8) The copper mesh was degreased with xylene, rinsed with alcohol, and covered in 1% Formar film. 9) The slices were placed onto copper mesh and stained with lead citrate, and then examined under 1,000-fold microscope. Electron microscopy was analyzed under a transmission electron microscope (TEM) (Philips CM10, 100 KV).

Statistical Analysis

The statistical analyses were performed with SPSS software, version 20.0 for Windows (SPSS, Chicago, IL). Values of all variables are presented as means $\pm$ SD. One-way ANOVA was used to determine the effects of different treatments, and Student-Newman-Keuls (SNK) test was used for paired comparison among multiple groups. A level of $P < 0.05$ was considered as statistically significant.

RESULTS

Concentration of PM_{2.5} from Indoor Coal Combustion

Daily mean indoor PM_{2.5} concentration was 156.95 $\mu\text{g}/\text{m}^3$, while indoor PM_{2.5} concentration varied from 42.14 $\mu\text{g}/\text{m}^3$ to 266.50 $\mu\text{g}/\text{m}^3$ at each time point. Whereas daily mean outdoor PM_{2.5} concentration in the urban area of Zunyi was 62.92 $\mu\text{g}/\text{m}^3$, outdoor PM_{2.5} concentration changed from 28.85 $\mu\text{g}/\text{m}^3$ to 134.72 $\mu\text{g}/\text{m}^3$ at each time point. Daily mean indoor PM_{2.5} concentration was significantly higher than that of outdoor PM_{2.5} concentration ($P < 0.05$). Daily mean indoor PM_{2.5} concentration exceeded the China National Ambient Air Quality Standards (GB3095-2012) for PM_{2.5} of 75 $\mu\text{g}/\text{m}^3$ (Fig. 1).

Effect of PM_{2.5} on the Distribution of Lung-Infiltrating Ovalbumin-Specific Inflammatory Cells

In the OVA-only, the number of total cells and of monocyte, lymphocytes, and eosinophils in BALF were increased over those in the control group ($P < 0.05$). In PM_{2.5}-only group, the number of total cells and of monocyte, lymphocytes, and neutrophil in BALF was significantly greater than that in the control group ($P < 0.05$). In the PM_{2.5}+OVA group, the number of total cells and number of monocytes, lymphocytes, neutrophils, and eosinophils in BALF was markedly and significantly increased when compared with control, OVA-only, and PM_{2.5}-only groups ($P < 0.05$) (Table 1).

Metal Composition in PM_{2.5} from Indoor Coal Combustion

Metals were measured in PM_{2.5} (Table 1). Among metals, the content of Zn (34.81 $\mu\text{g}/\text{m}^3$) was highest in PM_{2.5}, whereas the content of Mn (0.019 $\mu\text{g}/\text{m}^3$) was the lowest. Cu (3.29 $\mu\text{g}/\text{m}^3$) and Pb (2.76 $\mu\text{g}/\text{m}^3$) were more abundant in PM_{2.5}. PM_{2.5} contained the following concentrations of metals in descending order: Zn (34.81 $\pm$ 1.8 $\mu\text{g}/\text{m}^3$) > Cu (3.29 $\pm$ 1.9 $\mu\text{g}/\text{m}^3$) > Pb (2.76 $\pm$ 1.7 $\mu\text{g}/\text{m}^3$) > Fe (0.51 $\pm$ 0.08 $\mu\text{g}/\text{m}^3$) > Cd (0.075 $\pm$ 0.035 $\mu\text{g}/\text{m}^3$) > As (0.067 $\pm$ 0.076 $\mu\text{g}/\text{m}^3$) > Mn (0.019 $\pm$ 0.011 $\mu\text{g}/\text{m}^3$) (Table 2).

Effect of PM_{2.5} on OVA-Specific IgE and IgG1 in Serum

Coexposure to OVA and PM_{2.5} significantly increased OVA-specific IgE production in mice compared with the control, OVA-only, PM_{2.5}-only groups ($P < 0.05$). Difference in OVA-specific IgG1 production in mice was not statistically significant among the treatment groups ($P > 0.05$; Fig. 2).

Table 1. Effect of PM_{2.5} on the distribution of lung-infiltrating ovalbumin-specific inflammatory cells

Group	Total Cell	Eosinophil	Neutrophil	Lymphocyte	Monocyte
Control	6.61 $\pm$ 0.72	0	0.15 $\pm$ 0.04	1.34 $\pm$ 0.23	5.11 $\pm$ 0.59
OVA	41.78 $\pm$ 1.47*	2.47 $\pm$ 0.28*	3.22 $\pm$ 0.42	12.64 $\pm$ 0.95*	23.44 $\pm$ 1.19*
PM _{2.5}	37.75 $\pm$ 2.03*	0.30 $\pm$ 0.11	4.81 $\pm$ 1.77*	11.35 $\pm$ 0.66*	23.19 $\pm$ 1.44*
PM _{2.5} +OVA	103.54 $\pm$ 12.54*†‡	6.48 $\pm$ 1.51*†‡	12.47 $\pm$ 2.5*†‡	30.35 $\pm$ 3.91*†‡	54.90 $\pm$ 5.47*†‡

Values are means $\pm$ SD; $n = 10$. Four groups of mice were treated intratracheally with saline (control), particulate matter ≤ 2.5 μm diameter (PM_{2.5}; 2.5 mg/ml PM_{2.5} alone), ovalbumin (OVA; 15 $\mu\text{g}/\text{ml}$ OVA alone), and PM_{2.5}+OVA (2.5 mg/ml PM_{2.5} and 15 $\mu\text{g}/\text{ml}$ OVA) 4 times at 2-wk intervals. * $P < 0.05$ vs. control; † $P < 0.05$ vs. OVA; ‡ $P < 0.05$ vs. PM_{2.5}.

Table 2. Metal concentrations in PM_{2.5}

Metal	Concentration, $\mu\text{g}/\text{m}^3$
Zinc (Zn)	34.81 ± 1.8
Copper (Cu)	3.29 ± 1.9
Plumbum (Pb)	2.76 ± 1.7
Ferrum (Fe)	0.51 ± 0.08
Cadmium (Cd)	0.075 ± 0.035
Arsenic (As)	0.067 ± 0.076
Manganese (Mn)	0.019 ± 0.011

Values are means $\pm$ SD. PM_{2.5}, particulate matter $\leq 2.5 \mu\text{m}$ diameter.

Effect of PM_{2.5} on Cytokines and Chemokines Levels in BALF

OVA + PM_{2.5} significantly increased IL-4 in mice compared with the control, OVA-only, and PM_{2.5}-only groups ($P < 0.05$). OVA-only, PM_{2.5}-only, and PM_{2.5}+OVA significantly reduced the level of IFN- γ in mice compared with the control ($P < 0.05$; Fig. 3).

Effect of PM_{2.5} on GM-CSF, TGF- β 1, and Chemokine Protein Levels in BALF

PM_{2.5}+OVA increased the protein levels of GM-CSF and IL-8 in BALF of mice compared with the control, OVA-only, and PM_{2.5}-only groups ($P < 0.05$). Exposure to PM_{2.5}+OVA showed a significant decrease in the protein level of TGF- β 1 in BALF of mice compared with OVA-only and PM_{2.5}-only groups ($P < 0.05$). A significant difference in the protein levels of GM-CSF, TGF- β 1, and IL-8 in BALF were found in both PM_{2.5}-only, OVA-only groups compared with the control, respectively ($P < 0.05$; Fig. 4).

Effect of PM_{2.5} on Expressions of IL-4 and IFN- γ mRNA in Lung Tissue

The expression of IL-4 mRNA was obvious in lung tissue of the PM_{2.5}+OVA group, while it was significantly decreased ($P < 0.05$) in the control, OVA-only, and PM_{2.5}-only groups. In contrast, the expressions of IFN- γ mRNA were significantly increased ($P < 0.05$) in the control group compared with those in the OVA-only, PM_{2.5}-only, and PM_{2.5}+OVA groups (Fig. 5).

Effect of PM_{2.5} on Expressions of Foxp3 mRNA in Lung, Thymus, and Spleen Tissues

In the lung tissue, the expression level of Foxp3 mRNA in the control group was significantly higher than that in the PM_{2.5}+OVA group ($P < 0.05$). Similarly, in spleen tissue, the expression levels of Foxp3 mRNA in the OVA, PM_{2.5}, and PM_{2.5}+OVA groups were significantly higher than that in the control group ($P < 0.05$). In thymus tissue, the expression level of Foxp3 mRNA in the control group was higher than other treatment groups in a dose-effect manner ($P < 0.05$) (Fig. 6).

Effect of PM_{2.5} on Expression of Thymic Stromal Lymphopoietin mRNA in Thymus Tissue

The expression levels of thymic stromal lymphopoietin (TSLP) mRNA in the OVA and PM_{2.5}+OVA groups were significantly higher than that in the control group ($P < 0.05$). TSLP mRNA expression in the PM_{2.5}+OVA group was significantly higher than that in the PM_{2.5} group in the thymus tissue ($P < 0.05$). No significant difference was found between the control and PM_{2.5} group (Fig. 7).

Effect of PM_{2.5} on Histomorphology of Lung Tissue in Mice

Naked-eye observation of lung tissue. There was no obvious abnormal color observed in the lung tissue of control group, which was pink without edema or bleeding, and the contour was smooth (Fig. 8A). The lung tissue of OVA group was darker in color, with occasional edema, rough surface, and small pieces of dark gray lesions observed (Fig. 8B). Lung of the PM_{2.5} group was darker in color, with occasional edema, and the surface at bottom of the lung was rougher, while the right upper lobe appeared normal (Fig. 8C). Lung of the PM_{2.5} + OVA group showed abnormal color, which was slightly gray; with visible large white or translucent lesions, which was loose, soft, and flexible; significant edema; and a rough surface, with a pronounced convex and notched edge (Fig. 8D).

Histopathology. The control group showed a very small amount of inflammatory cell infiltration, without thickening of bronchial wall (Fig. 9A). A moderate amount of neutrophils and a small amount of eosinophil infiltration was seen at the bronchial wall and surrounding tissue of the OVA group, with

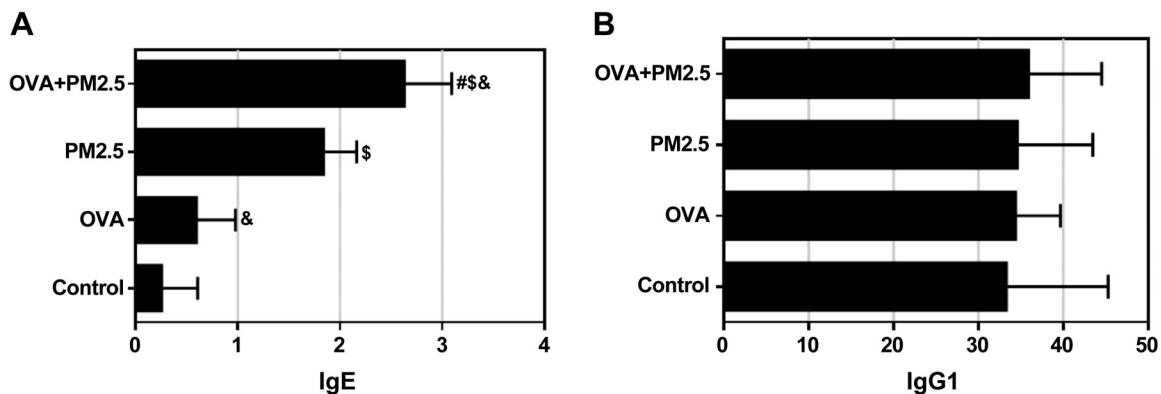


Fig. 2. Effect of particulate matter $\leq 2.5 \mu\text{m}$ diameter (PM_{2.5}) on ovalbumin (OVA)-specific IgE (A) and IgG1 (B) production in serum. Four groups of mice were treated intratracheally with saline (control), PM_{2.5} (2.5 mg/ml PM_{2.5} alone), OVA (15 $\mu\text{g}/\text{ml}$ OVA alone), and PM_{2.5}+OVA (2.5 mg/ml PM_{2.5}, and 15 $\mu\text{g}/\text{ml}$ OVA) 4 times at 2-wk intervals. All values are expressed as means $\pm$ SD; $n = 10$. & $P < 0.05$ vs. control; \$ $P < 0.05$ vs. OVA; # $P < 0.05$ vs. PM_{2.5}.

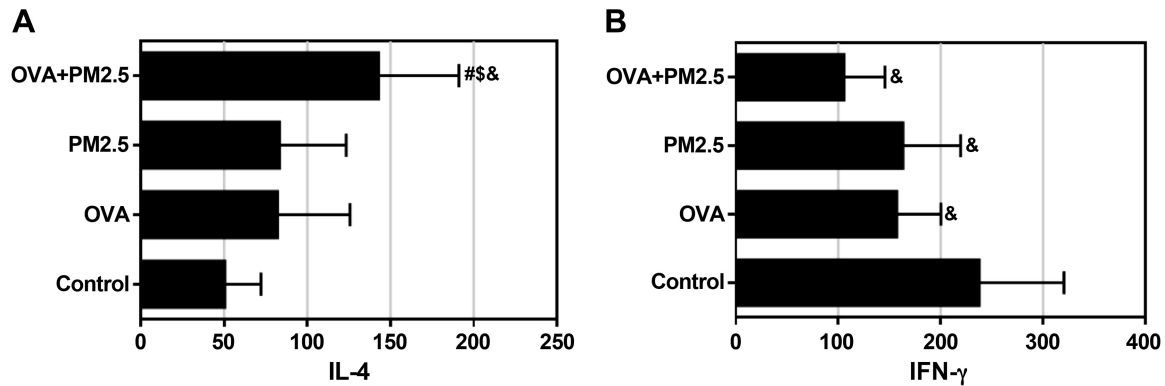


Fig. 3. Expression of cytokines (IL-4; *A* and IFN- γ ; *B*) in BALF. Four groups of mice were treated intratracheally with saline (control), particulate matter ≤ 2.5 μm diameter (PM_{2.5}; 2.5 mg/ml PM_{2.5} alone), ovalbumin (OVA; 15 $\mu\text{g/ml}$ OVA alone), and PM_{2.5}+OVA (2.5 mg/ml PM_{2.5}, and 15 $\mu\text{g/ml}$ OVA) 4 times at 2-wk intervals. All values are expressed as means $\pm$ SD; $n = 10$. & $P < 0.05$ vs. control; \$ $P < 0.05$ vs. OVA; # $P < 0.05$ vs. PM_{2.5}.

slightly thickened wall (Fig. 9*B*). A moderate amount of mononuclear cells lymphocytes and a small amount of neutrophil infiltration was seen at the bronchial wall and surrounding tissue of PM_{2.5} group, with a small amount of neutrophilic exudate observed at the bronchial wall, and thickening of the tube wall, but no eosinophils (Fig. 9*C*). A large number of mononuclear cells and lymphocytes and a small amount of eosinophil and neutrophil infiltration were observed at the bronchial wall of PM_{2.5}+OVA group, along with luminal inflammatory cell exudation, thickening of the wall, as well

as a small amount of fibrosis and goblet cell hyperplasia (Fig. 9*D*).

Electron microscopy. The control group showed clear alveolar, alveolar septum structure, and type I, II cell structure, with normal distribution, clear nuclear structure, continuous basement membrane without thickening, along with minimal alveolar septal connective tissue and fiber composition, as well as clear air-blood barrier structure (Fig. 10*A*). OVA group showed a large number of exfoliated cells (seen at the surface of cilia cells, which were slightly edematous, with deteriorating

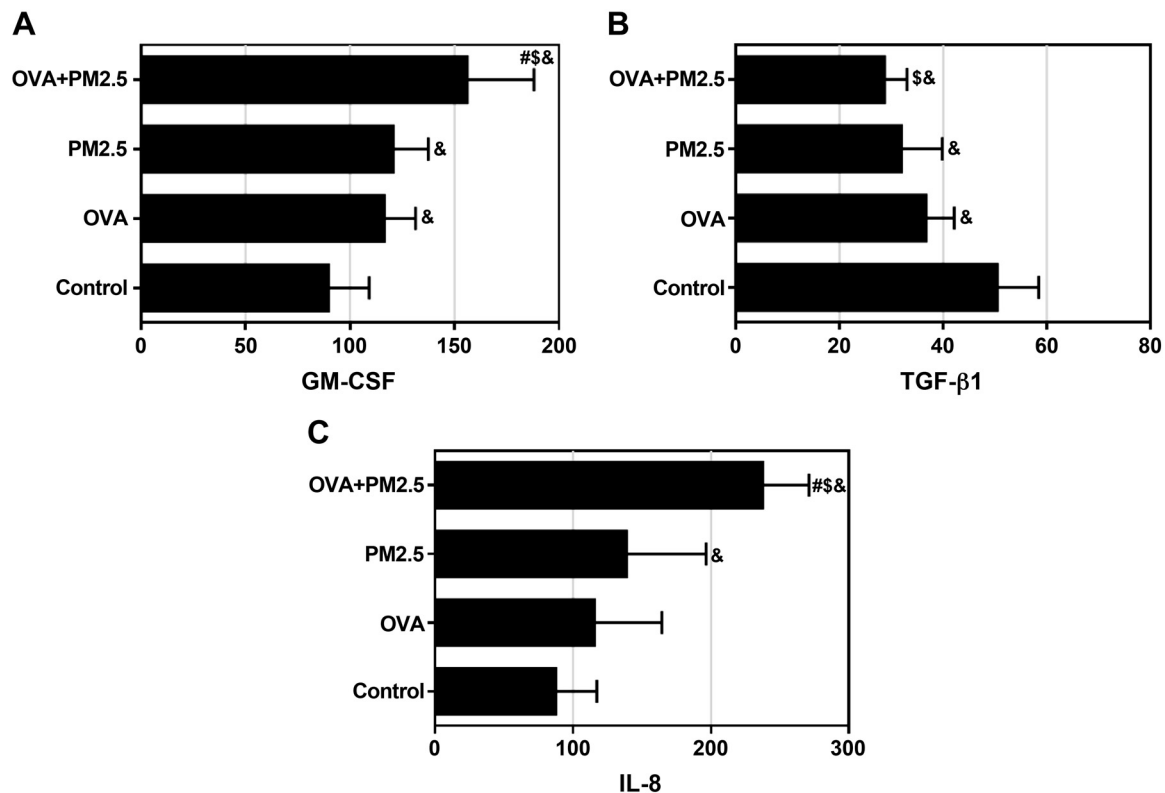
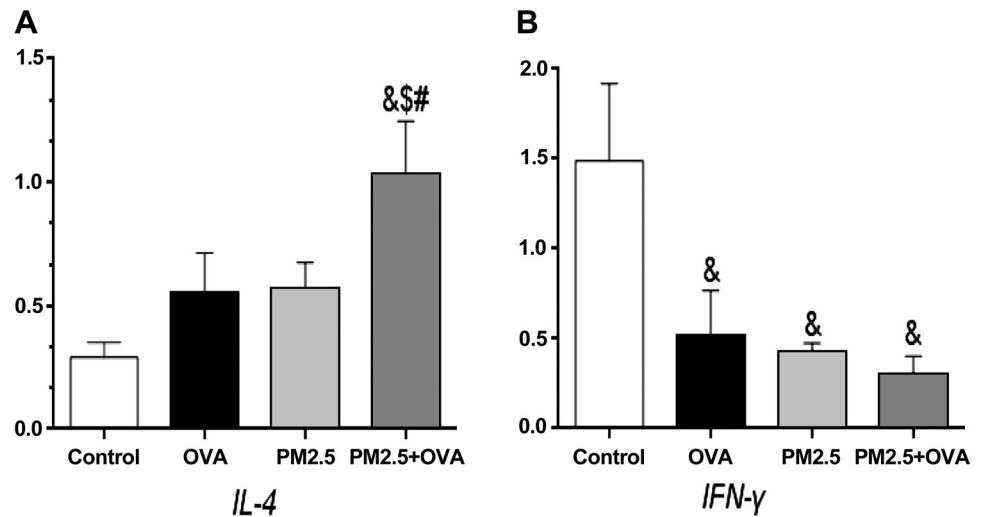


Fig. 4. Expression of protein levels of granulocyte-macrophage colony-stimulating factor (GM-CSF; *A*), transforming growth factor- $\beta 1$ (TGF- $\beta 1$ (*B*), and IL-8 (*C*) in bronchoalveolar lavage fluid (BALF). Four groups of mice were treated intratracheally with saline (control), particulate matter ≤ 2.5 μm diameter (PM_{2.5}; 2.5 mg/ml PM_{2.5} alone), OVA (15 $\mu\text{g/ml}$ OVA alone), and PM_{2.5}+OVA (2.5 mg/ml PM_{2.5}, and 15 $\mu\text{g/ml}$ OVA) 4 times at 2-wk intervals. All values are expressed as means $\pm$ SD; $n = 10$. & $P < 0.05$ vs. control; \$ $P < 0.05$ vs. OVA; # $P < 0.05$ vs. PM_{2.5}.

Fig. 5. Expression of IL-4 (A) and IFN- γ (B) mRNA in lung tissue. All values are expressed as means $\pm$ SD; $n = 10$. & $P < 0.05$ vs. control; \$ $P < 0.05$ vs. OVA; # $P < 0.05$ vs. PM_{2.5}.



structure observed), and type II cell damage was seen locally, with a less-lamellar particle structure, clear air-blood barrier, and occasional relatively high amount of alveolar septum fibers locally (Fig. 10B). A large number of exfoliated cells were observed in the bronchioles of PM_{2.5} group, with extensive edema of endothelial cells, and a large amount of fiber in the alveolar septum, along with occasional edema of type I cells, and decreased cytoplasmic electron density (Fig. 10C). PM_{2.5}+OVA group showed a large amount of local cell damage with organelle destruction, with locally observed nuclear condensation, and obvious edema of type II cells, infiltration of inflammatory cells, as well as unclear air-blood

barrier structure, and a basement membrane of uneven thickness (Fig. 10D).

DISCUSSION

In this study, we investigated indoor PM_{2.5} concentration, pathologic change, alterations in inflammatory cytokines, and chemokines in BALF, OVA-specific IgE and IgG1 antibodies in serum, and the expression of IL-4 and IFN- γ mRNA in lung tissue of mice, and we assessed the adverse effects of intratracheal exposure to PM_{2.5} from indoor coal combustion on allergic lung inflammation. To the best of our knowledge, this

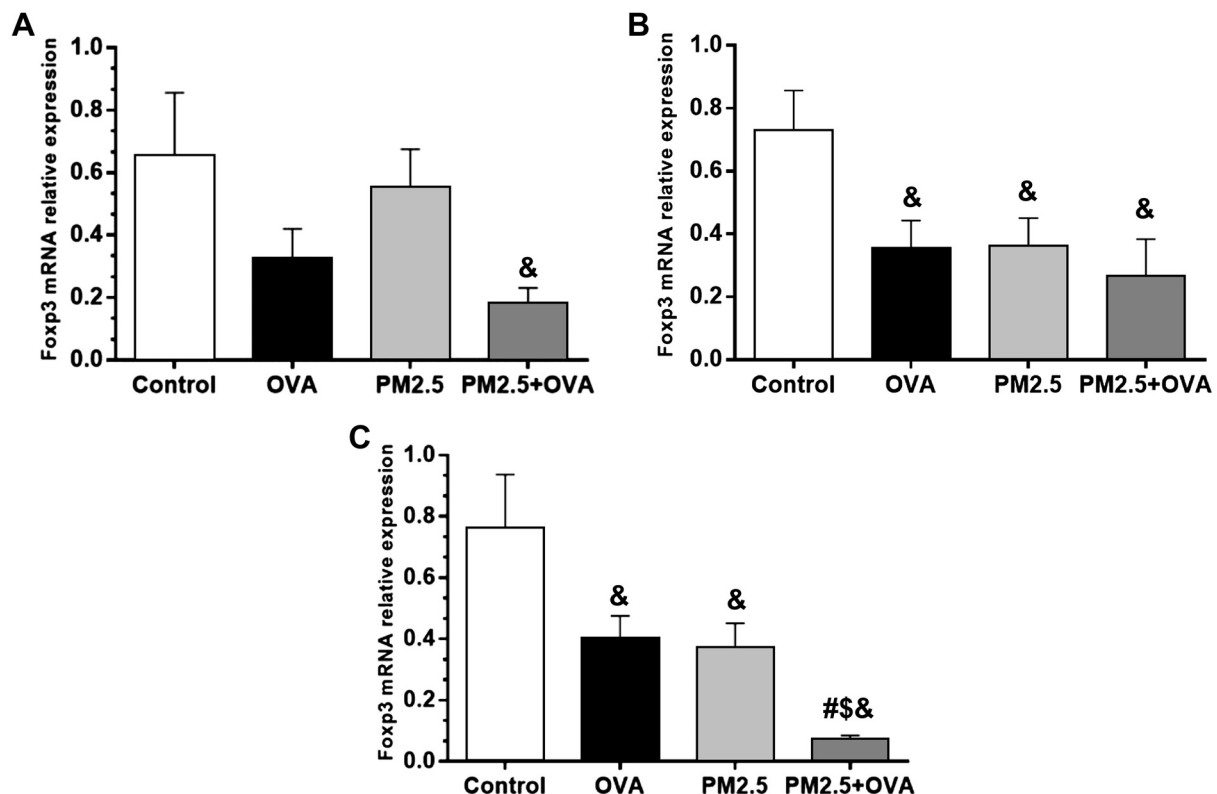


Fig. 6. Expression of Foxp3 mRNA in lung (A), spleen (B), and thymus (C) tissues. & $P < 0.05$ vs. control; \$ $P < 0.05$ vs. OVA; # $P < 0.05$ vs. PM_{2.5}.

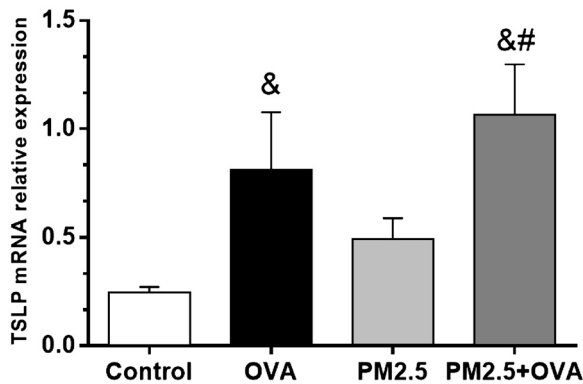


Fig. 7. Expression of thymic stromal lymphopoietin (TSLP) mRNA in thymus tissue. & $P < 0.05$ vs. control; # $P < 0.05$ vs. PM_{2.5}.

was the first study to evaluate the effects of PM_{2.5} from indoor coal combustion on the exacerbation of airway inflammation in the lung tissue of asthmatic animals induced with OVA. Our current study showed that PM_{2.5} from coal combustion exacerbated the degree of infiltration of inflammatory cells (neutrophils, eosinophils and lymphocytes) in the airway and airway remodeling in a mouse model of bronchial asthma.

Air pollution, including atmospheric particulate matter with diameter $\leq 2.5 \mu\text{m}$ (PM_{2.5}), has been shown in *in vitro* and experimental studies to affect the rate of lung function development, aggravates asthma, and causes other respiratory symptoms, such as cough and bronchitis in children and adults. Barría et al. (3) demonstrated that homes without heating showed a lower median PM_{2.5} of $58.6 \mu\text{g}/\text{m}^3$, while those using firewood, kerosene, and electricity ranged between $112.5 \mu\text{g}/\text{m}^3$ and $114.9 \mu\text{g}/\text{m}^3$, and coal users' homes reached 162.9

$\mu\text{g}/\text{m}^3$. The present study showed that daily average indoor PM_{2.5} concentration was $156.95 \mu\text{g}/\text{m}^3$, which exceeded the China National Ambient Air Quality Standards for the average 24-h PM_{2.5} concentration of $75 \mu\text{g}/\text{m}^3$, and which was 4.48 times that of the U.S. National Ambient Air Quality Standards ($35 \mu\text{g}/\text{m}^3$). Results from the China rural indoor air pollution study indicate that the highest PM_{2.5} was observed in the coal-use kitchens with a concentration of $222.54 \pm 41.12 \mu\text{g}/\text{m}^3$, followed by biomass-use kitchens at $130.48 \pm 26.53 \mu\text{g}/\text{m}^3$. PM_{2.5} in all scenarios exceeded the safe limit of $75 \mu\text{g}/\text{m}^3$ as compared with the Ambient Air Quality GB 3095-2012 (22). Some special intervention strategies should be developed among inner-city population, including decreasing household coal combustion, increasing the use of improved stoves with chimney or electric fan, and increasing kitchen and bedroom ventilation by increasing the number of windows.

Bronchial asthma is a chronic inflammatory lung disease, with an array of cells (e.g., eosinophils, neutrophils, bronchial epithelial cells, and others) implicated in the pathogenesis of the disease. The characteristic structural changes seen in the bronchial walls of asthma sufferers include epithelial denudation, reticular basement membrane fibrosis, smooth muscle hypertrophy and hyperplasia, goblet cell metaplasia/mucous gland hypertrophy, and others (1). Churg et al. (6) compared histologic sections from the lungs of women between Mexico City (a high PM locale) in Mexico and Vancouver in Canada (a low PM locale), and revealed that long-term exposure to high levels of ambient particulate pollutants was associated with small airway remodeling. Greatly thickened airway wall and the marked increase in fibrous tissue and muscle in the airway walls were found in the lungs of the Mexico City subjects. In another study, the pathological changes of lung

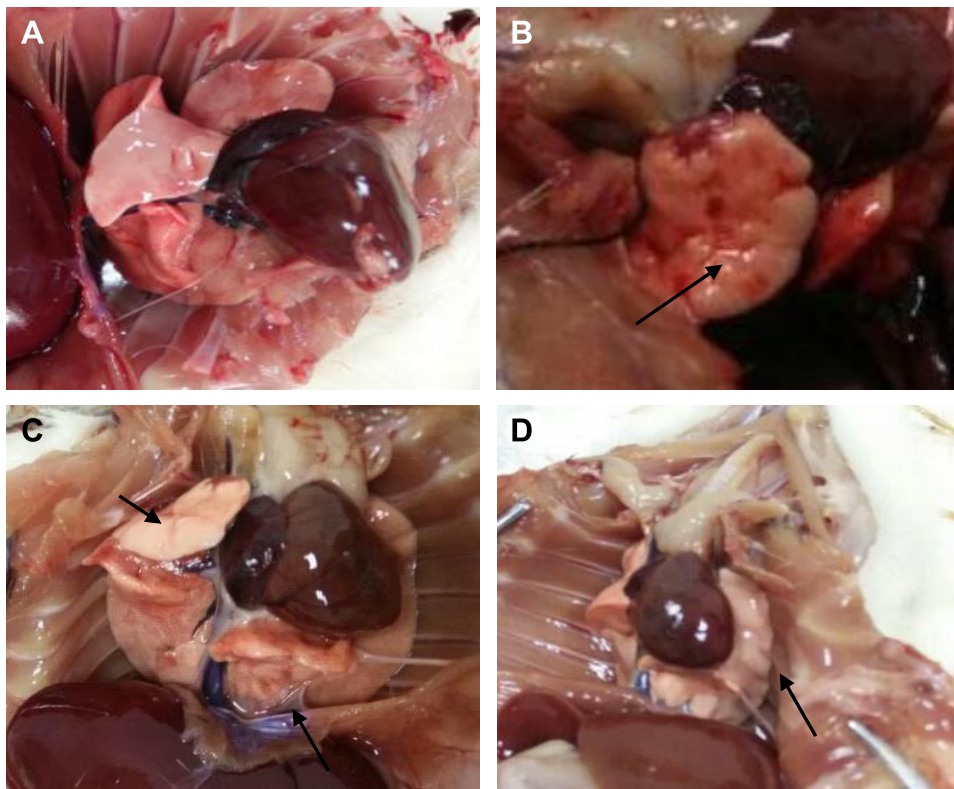
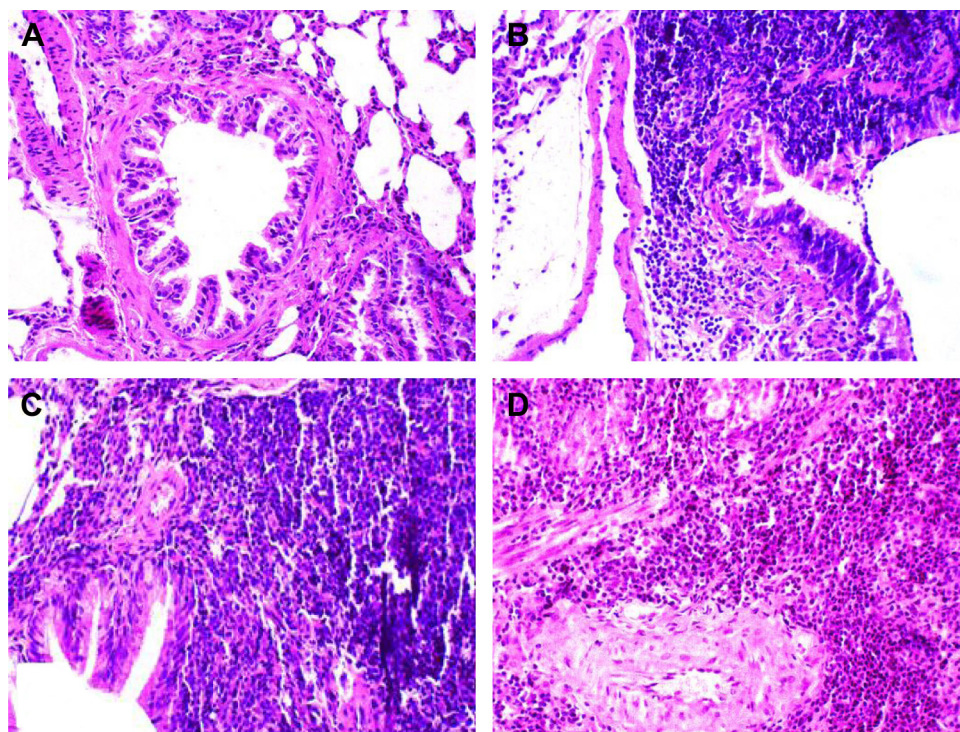


Fig. 8. Histomorphologic changes in lung tissue observed with the naked eye in mice. A: control group. B: ovalbumin (OVA) (15 $\mu\text{g}/\text{ml}$ OVA). C: particulate matter $\leq 2.5 \mu\text{m}$ diameter (PM_{2.5}) (2.5 mg/ml PM_{2.5}). D: PM_{2.5}+OVA (2.5 mg/ml PM_{2.5} and 15 $\mu\text{g}/\text{ml}$ OVA). Lung tissue is indicated by arrows.

Fig. 9. Histopathologic findings of the lung after intratracheal administration of testing samples (hematoxylin and eosin staining). *A*: control group. *B*: ovalbumin (OVA; 15 μ g/ml OVA) group; *C*: PM_{2.5} (2.5 mg/ml PM_{2.5}) group; *D*: PM_{2.5}+OVA (2.5 mg/ml PM_{2.5} and 15 μ g/ml OVA) group. Magnification, $\times 200$.



showed epithelial shedding, infiltration of inflammatory cells in peribronchial and fibrosis on the basement membrane in mice, which intratracheally exposed to diesel exhaust particles (5). In this study, PM_{2.5}+OVA caused serious peribronchiolar

inflammation, infiltration of inflammatory cells into the airway submucosa, and goblet cell proliferation in the airways. In comparison, PM_{2.5}-only and OVA-only caused comparatively slight peribronchiolar inflammation. Under TEM, PM_{2.5}+

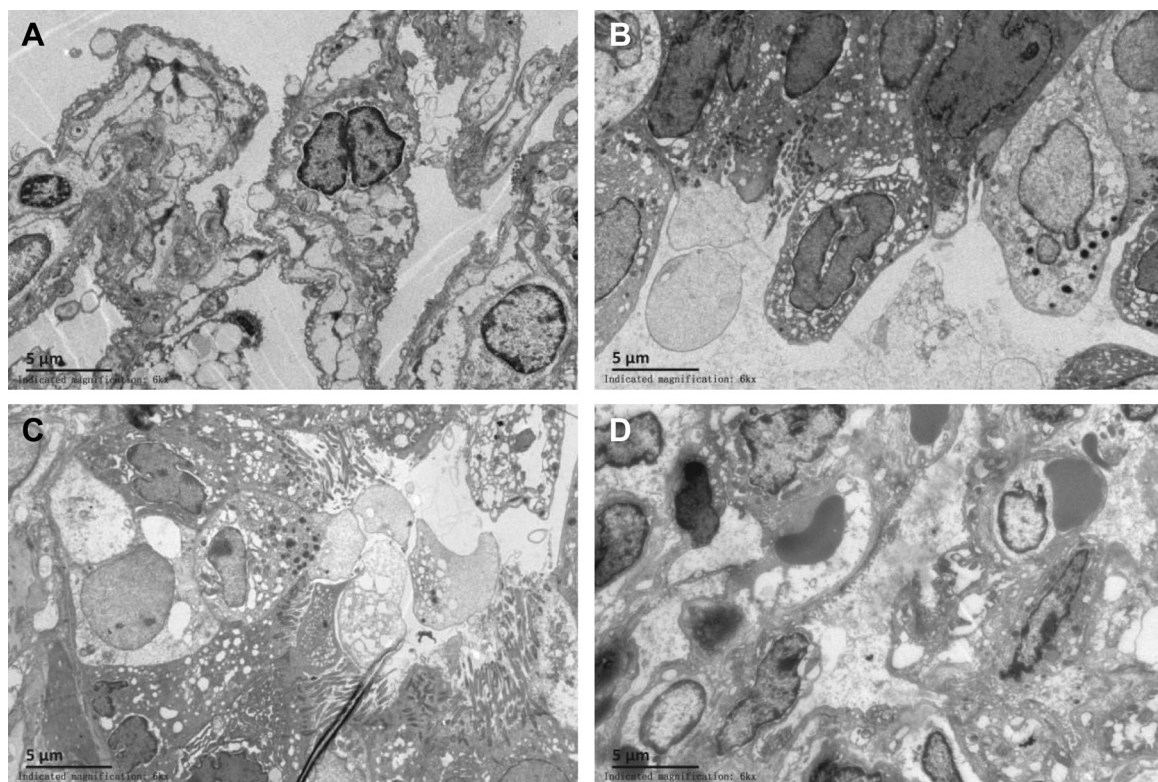


Fig. 10. Electron microscopic images of the lungs in mice. *A*: control group. *B*: OVA (15 μ g/ml OVA). *C*: PM_{2.5} (2.5 mg/ml PM_{2.5}). *D*: PM_{2.5}+OVA (2.5 mg/ml PM_{2.5} and 15 μ g/ml OVA).

OVA caused even more serious damage to cell organelles and nonuniform basement membrane width. Our results demonstrated that PM_{2.5} from coal combustion may exacerbate peribronchiolar inflammation due to infiltration of inflammatory cells into the airway submucosa and airway structural pathologic changes. We inferred that PM_{2.5} might enhance extracellular matrix (ECM) gene expression and induce fibroblast and other cells to excessively synthesize and secrete ECM; PM_{2.5} might further cause airway fibrosis and participate in airway remodeling. The airway remodeling caused by PM_{2.5} could be an interaction between airway inflammation and changes of mechanical property of the airway wall.

In the current study, coexposure to OVA and PM_{2.5} significantly increased OVA-IgE production rather than OVA-IgG1 production in mice compared with the control. He et al. (14) used mice administered intratracheally with urban PM (UPM) and/or ovalbumin (OVA) to observe the relationship between bronchial asthma and UPM. UPM induced adjuvant effects on specific IgE and IgG1 production by OVA. Both A/J and BALB/c mice produced ovalbumin-specific IgG1 antibodies. The C57BL/6 mice did not produce detectable levels of antibodies (4). In this study, the lack of an increase in OVA-IgG1 production induced by PM_{2.5}+OVA may be due to the difference in animal strain. The other reasons may be that specific IgE was associated with type I hypersensitivity reaction; specific IgG was also associated with type II and type III hypersensitivity reactions, and defended the body against the pathogens. In addition, the slight degree of eosinophilic lung infiltration was found in the OVA-only and PM_{2.5}+OVA groups, which illustrated that asthma model was successfully established. The slight eosinophilic lung infiltration may be the reason that the exposure doses of allergen-OVA were small, the exposure dose was just enough to induce the occurrence of asthma, but the intratracheally instilled dose of allergen-OVA needs further improvement.

Asthma is considered a chronic inflammation of the airway due to allergen-specific IgE production, as well as alterations in the balance between Th1 and Th2 responses and others. The IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. IFN- γ is a key cytokine which induces T cells to differentiate into Th1 cells (19). The imbalance of cytokine microenvironment is a key factor of the increase of serum IgE level in allergic diseases, including asthma. The house dust mite allergen was capable of promoting immune response by upsetting the balance between the polarizing cytokines IL-4 and IFN- γ (7). Han et al. (13) reported that outdoor PM_{2.5} exposure concentration was negatively correlated with serum IFN- γ level ($r = -0.536$, $P < 0.05$). Intratracheal administration of nano-SiO₂ could lead to the airway hyperresponsiveness and the airway remodeling. OVA-treated rats exhibit higher IL-4 and lower IFN- γ compared with the control rats (12). In our study, a negative significant correlation between IL-4 and IFN- γ was found, we proposed that PM_{2.5} may enhance Th2 polarization and reduce Th1 polarization, and induce IFN- γ /IL-4 imbalance in mice induced with OVA.

GM-CSF, a potent hematopoietic factor, was able to enhance the neural differentiation of bone marrow stromal cells (11). It stimulates the precursors of neutrophils, proliferation, and maturation of macrophages and eosinophils. IL-8 chemotactically accumulates inflammatory cells, extends the survival time

of neutrophils, aggravates lung injury, and promotes the release of leukotrienes and histamine-mediating inflammation, which is not dependent on IgE, causing late-onset asthma in synergy with IL-3. Transforming growth factor- β 1 (TGF- β 1) secreted by airway epithelium and eosinophils is thought to play an important role in airway remodeling in asthmatic subjects. PM_{2.5} exposure can reduce the viability of human keratinocyte cell line (HaCaT) cells (20) and human nasal epithelial cells (15), which induced the expression of inflammatory cytokines GM-CSF and IL-8. In OVA-sensitized rats, the levels of TGF- β 1 increased in the BALF (21), while PM_{2.5} exposure induced a decreased TGF- β 1 level in the lungs of asthmatic mice. In this study, PM_{2.5}+OVA increased the protein levels of GM-CSF and IL-8 in BALF of mice. We surmised that PM_{2.5} enhanced the expressions of GM-CSF and IL-8 in OVA-induced asthma, the increased GM-CSF and IL-8 levels promoted the differentiation and migration of inflammatory cells. We found that exposure to PM_{2.5}+OVA showed a significant decrease in the protein level of TGF- β 1, which was similar to the findings of Wang's study (24). The results obtained might be associated with TGF- β 1 signal transduction, which reduced the suppression of smad7 channel, resulting in extracellular matrix accumulation.

In conclusion, exposure to PM_{2.5} from indoor coal combustion might induce airway inflammatory immune responses and exacerbate peribronchiolar inflammation due to infiltration of inflammatory cells into the airway submucosa and airway structural, pathological changes. Further studies to understand the mechanisms underlying this potentiation are in order.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.Y. and J.X. conceived and designed research; K.L. performed experiments; J.Y., K.L., and J.X. analyzed data; K.L. prepared figures; J.Y. and K.L. interpreted results of experiments; J.Y. and K.L. drafted manuscript; J.Y. and J.X. edited and revised manuscript; J.Y. approved final version of manuscript.

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