Fine particulate matter aggravates allergic airway inflammation through thymic stromal lymphopoietin activation in mice

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Abstract. Fine particulate matter (PM2.5) has been linked to exacerbation of allergic airway inflammation in mice. However, the mechanism underlying exposure to PM2.5 and subsequent and adverse effects remains to be fully elucidated. Therefore, the present study aimed to investigate the effects of PM2.5 by different levels on airway inflammation in mouse models of in allergic and steroid-resistant asthma. BALB/c mice were nasally instilled with PBS (control) or 10, 31.6 or 100 μg PM2.5, and randomly assigned into nine groups. The acute asthma model was previously induced to investigate the change of inflammatory cells in bronchoalveolar lavage fluid (BALF). Histopathological changes of the lung were assessed, in addition to levels of interleukin (IL)-4 and IL-13 in BALF and immunoglobulin Ein serum. Thymic stromal lymphopoietin (TSLP) proteinexpression levels were assessed by western blotting. The present study demonstrated that medium- and high-dose PM2.5 is linked to acute exacerbation of allergic airway inflammation in mice. In conclusion, the pathological mechanisms of PM2.5 may be associated with allergic/steroid-resistant airway inflammation, T-cell helper (Th)1/Th2 cytokine production and upregulation of TSLP expression in a murine model of allergic and steroid-resistant asthma.

Introduction

Asthma is a respiratory disease that has been reported to represent a significant threat to human health, especially for children. In 2006, the Global Initiative for Asthma noted that asthma is a heterogeneous disease that is usually characterized by chronic airway inflammation (1). However, the complex mechanisms involved in the induction of a Th2-biased immunologic response remain to be fully elucidated. An important molecular mechanism involved in asthma is type 2 inflammation (2,3). Type 2 helper T cells release cytokines, including interleukin (IL)-4 and IL-13. They also activate B cells, and promote immunoglobulin E (IgE) expression, recruitment of mast cells and eosinophilia. Environmental factors have been implicated to serve important roles in the mechanism of asthma. Anumber of studies have demonstrated that exposure to ambient particulate matter is linked to acute exacerbations of asthma (4,5). To investigate the effects of fine particulate matter (PM2.5) at different dosages on airway inflammationin a mouse model of asthma, the present study generated a successful model of acute allergic asthma in mice by nasally instilling PM2.5 at different dosages (10, 31.6 or 100 μg). Glucocorticoids were intraperitoneally injected into the ovalbumin (OVA)-induced allergic mice that had been exposed to PM2.5 at three different dosages. To the best of our knowledge, the present study evaluated for the first time the effects of PM2.5 on various parameters of the allergic airway inflammation in these mouse models. Gavett *et al* (6) demonstrated that inhalation of three different concentrations (42, 134, or 425 μg/m^3) PM2.5 would produce human doses per tracheobronchial surface area equivalent to aspiration by mice of doses (10, 31.6 or 100 μg), respectively. Furthermore, the mouse-aspirated doses (10, 31.6 or 100 μg) in turn were equivalent to good, moderate and severe pollution, referring to a correlation chart consisting of PM2.5 standards, air quality index and air quality level. The chart was established by the US. Environmental Protection Agency in 2006 (7).

Materials and methods

Source of PM2.5. PM2.5 was collected with an Anderson-type high volume sampler (Wuhan Tianhong TH-150) at a moderate rate. This flow rate was aerodynamically calculated to obtain PM2.5 with a diameter of ≤2.5 μm. The fine particulate matter was stored in a freezer at -20°C until further required.

Animals. Female BALB/c mice (age, 8 weeks; n=70) were obtained from the Nanjing Medical University Experimental

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Animal Center (Nanjing, China). Animals (weight, 20-25 g) were held individually in ventilated cages, exposed to a 12-h light/dark cycle and were given free access to food and water under standard laboratory conditions of 22-28°C and relative humidity of 50-60%. The care and handling of the animals were in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the Nanjing Medical University, and the experimental protocol was approved by the institutional animal ethics group.

Experimental groups. The BALB/c mice were randomly divided into nine groups: Control (n=6), OVA (n=8), OVA/dexamethasone (DXM; n=8), OVA/PM2.5 (10 μg; n=8), OVA/PM2.5 (31.6 μg; n=8), OVA/PM2.5 (100 μg; n=8), OVA/DXM/PM2.5 (10 μg; n=8), OVA/DXM/PM2.5 (31.6 μg; n=8) and OVA/DXM/PM2.5 (100 μg; n=8).To establish the model of asthma, 64 mice were sensitizedby intraperitoneal injections of 20 μg OVA (Grade V; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) emulsified in 2.25 mg aluminum hydroxide in a total volume of 0.1 ml and challenged with aerosol inhalation of 1% OVA for 30 min daily for 5 consecutive days. In addition, mice were nasally instilled with PBS (control) or 10, 31.6 or 100 μg PM2.5, and DXM was intraperitoneally injected into the OVA induced allergic mice that were exposed to PM2.5 at the three different dosages.

Analysis of bronchoalveolar lavage fluid (BALF) and total IgE in serum. Each mouse was anaesthetised with an intraperitoneal injection of 1% sodium pentobarbital (200 μl/mouse), and a sample of blood (1 ml) was collected via retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged (2,000 x g for 10 min at 4°C), and the plasma was stored at -70°C. The BALF was centrifuged (1,500 x g for 10 min at 4°C) and washed twice with 0.5 ml saline. Differential cell counts were performed with Wright's staining (for 10 min at room temperature) following centrifugation, ≥200 cells were counted for each sample. The expression levels of IL-4 (cat. no. JEM-03; Shanghai Joyee Biotechnics Co. Ltd., Shanghai, China) and IL-13 (cat. no. BMS6015; eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in BALF and total IgE (cat. no. CSB-E07983m; Cusabio Biotech Co. Ltd., Wuhan China) in serum were measured by ELISA according to the manufacturer's protocol.

Histological experiments. The mice were sacrificed and the left lungs were removed (8), immersed in 4% formaldehyde overnight, and subsequently embedded in paraffin and sections. Sections (5-6 μm) were stained with haematoxylin-eosin (30 min at room temperature). An image-analysis system (Image-Pro Plus; Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the lung histopathology. The severity of the inflammatory cell infiltration was blindly assessed using a 5-point scoring system (9). The scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2-4 cells deep and 4, a ring of cells 4 cells deep.

Western blot analysis. The right lung tissues from the nine groups were added to 1 ml radioimmunoprecipitation assay lysis buffer (Wuhan Sevicebio Technology Co., Ltd., Wuhan, China) supplemented with 20 μl cocktail phosphatase inhibitors, 2 μl protease inhibitors and 10 μl 100 mM phenylmethanesulfonylfluoride, Tissue samples were lysed on ice and oscillated at 4°C for 30 min. Following lysis, the resulting supernatants were collected as total protein samples by centrifugation at 12,000 x g for 15 min at 4°C, and protein concentrations were determined using the Bicinchoninic Acid protein assay. Equal amounts of protein extracts (20 μg) were separated by 10% SDS-PAGE and blotted onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membrane was subsequently blocked in blocking solution (5% non-fat milk, 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) at room temperature for 1 h and incubated with the following primary antibodies: Anti-actin (1:3,000 dilution, cat. no. sc-1616r; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-TSLP (1:3,000 dilution, cat. no. ab188766; Abcam, Cambridge, MA, USA) polyclonal primary antibodies overnight at 4°C, Membranes were washed with 10 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20 (TBST) three times, for 5 min per wash. The membranes were incubated with the HRP-conjugated goat anti-rabbit IgG antibody (1:5,000 dilution, cat no. sc-2004; Santa Cruz Biotechnology, Inc.) at room temperature for 30 min. The membranes were then washed with TBST three times, for 5 min per wash (10). Densitometric analysis was performed using Image J software 1.47v (National Institutes of Health, Bethesda, MD, USA) after the scanning of the Kodak X-Omat LS X-ray films.

Statistical analysis. Data are expressed as the mean ± standard error, and were analysed by one-way analysis of variance followed by the least significant difference post hoc test. SPSS software version 20 (IBM Corp., Armonk, NY, USA) was used for data analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of PM2.5 on histopathological changes in the lungs. Compared with the lack of inflammation in the control group, a significant increase in the infiltration of inflammatory cells and bronchial wall thickening were observed in the OVA and OVA/PM2.5 (10, 31.6 and 100 μg) groups. The vast majority of the inflammatory cell infiltration resulted from a marked eosinophilia and a significant enhancement in the number of lymphocytes and neutrophils. In contrast, the inflammatory scores of the peribronchial and perivascular regions were significantly reduced in the OVA/DXM/PM2.5 (10, 31.6 and 100 μg) groups, compared with those in the OVA group (Fig. 1A). Furthermore, the statistical significance of the differences in the inflammatory scores in the OVA/DXM/PM2.5 (10, 31.6 and 100 μg) groups compared with those in the OVA group decreased with exposure levels (P=0.004, P=0.015 and P=0.04, respectively; Fig. 1B).

PM2.5 promotes asthmatic airway inflammation. In preliminary experiments, the control and glucocorticoid therapy group did not exhibit evidence of airway inflammation in terms of the total number of cells in BALF [the total cell counts in the control group, OVA/DXM group, OVA/DXM/PM2.5 (10 μg) group, OVA/DXM/PM2.5 (31.6 μg) group and OVA/DXM/PM2.5 (100 μg) group are 35.8±5.8x10^4, 56±15.1x10^4, 49.8±13.6x10^4,
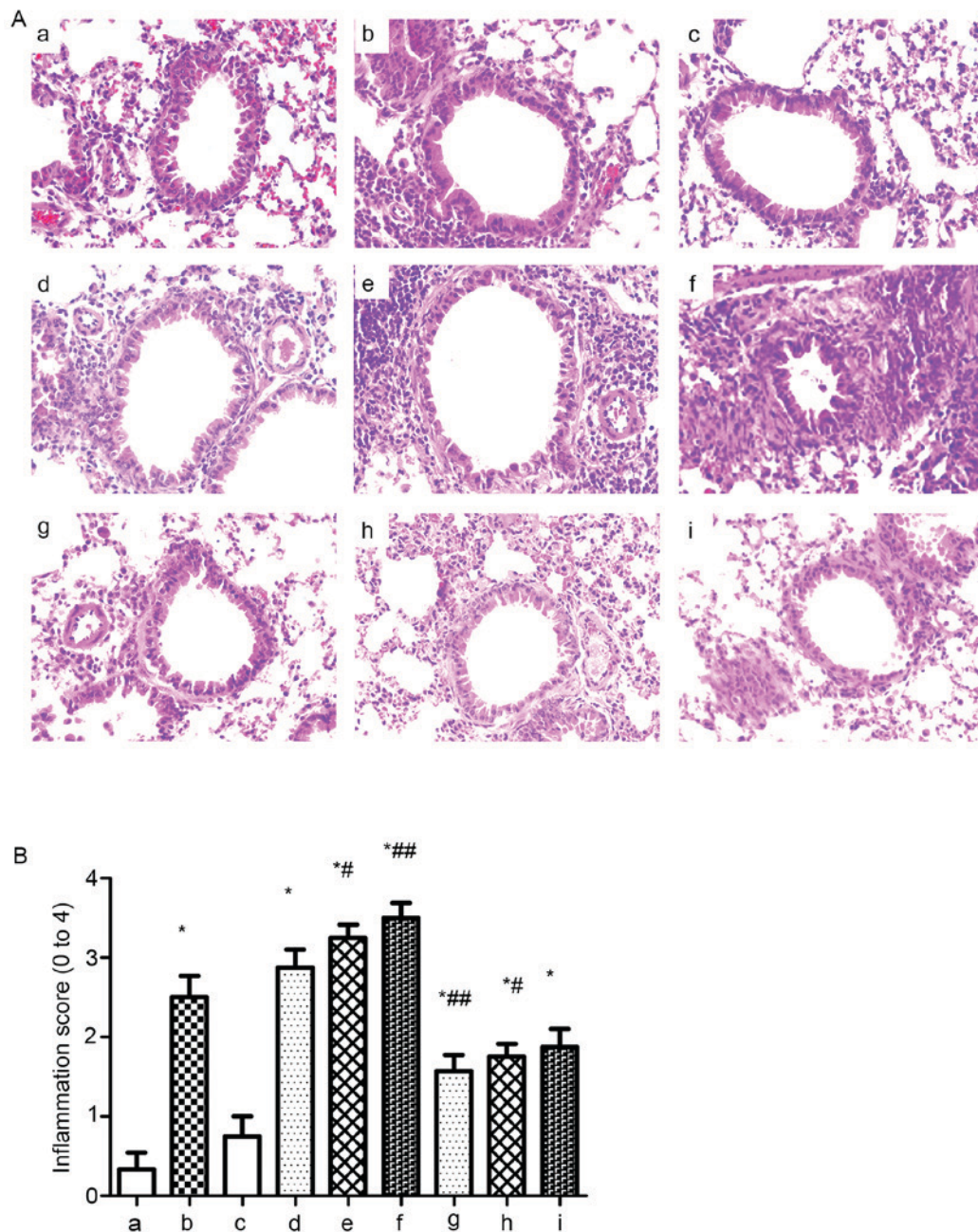
Figure 1. Effects of PM2.5 on the infiltration of inflammatory cells in allergic asthma and steroid-resistant asthma. (A) Hematoxylin and eosin staining of lung tissues from each group. (magnification, x400). (B) Lung inflammation scores. Data are expressed as the mean ± standard error (n=6-8/group). *P<0.05 vs. control group; #P<0.05 and ##P<0.01 vs. OVA group. a, control group; b, OVA group; c, OVA/DXM group; d, OVA/PM2.5 (10 µg) group; e, OVA/PM2.5 (31.6 µg) group; f, OVA/PM2.5 (100 µg) group; g, OVA/DXM/PM2.5 (10 µg) group; h, OVA/DXM/PM2.5 (31.6 µg) group; i, OVA/DXM/PM2.5 (100 µg) group. OVA, ovalbumin; DXM, dexamethasone; PM2.5, fine particulate matter.

56±8.4x10⁴, 62.6±7.4x10⁴]. The number of total inflammatory cell in the OVA/DXM/PM2.5 (10 and 31.6 µg) group was significantly reduced compared with the OVA group (P<0.05), while there were no significant differences between the OVA/DXM/PM2.5 (100 µg) and OVA groups (P>0.05, Fig. 2A). Importantly, there was a significant increase in the percentage of eosinophils in all OVA/PM2.5 groups (11.3±3.6, 13.6±1.8 and 16.1±2.4) compared with the OVA group (10.5±1.6, Fig. 2B). The percentage of neutrophils in the BALF was also significantly increased in the OVA/PM2.5 groups (11.3±3.6, 13.6±1.8 and 16.1±2.4) compared with the OVA

group (7.6±0.5). Furthermore, the percentage of neutrophils in the OVA/DXM/PM2.5 (100 µg) group was significantly increased compared with the OVA/DXM group (Fig. 2C). The percentage of lymphocytes in the BALF was similarly increased (Fig. 2D) and that of the macrophages was correspondingly decreased in the OVA/PM2.5 groups (10, 31.6 and 100 µg) compared with the OVA group (Fig. 2E).

Effect of PM2.5 on the expression of IL-4 and IL-13 in the BALF and total IgE in serum. In the OVA group, the levels of the T-helper 2 (Th2) cell cytokines IL-4 (78.1±9.9 pg/ml) in
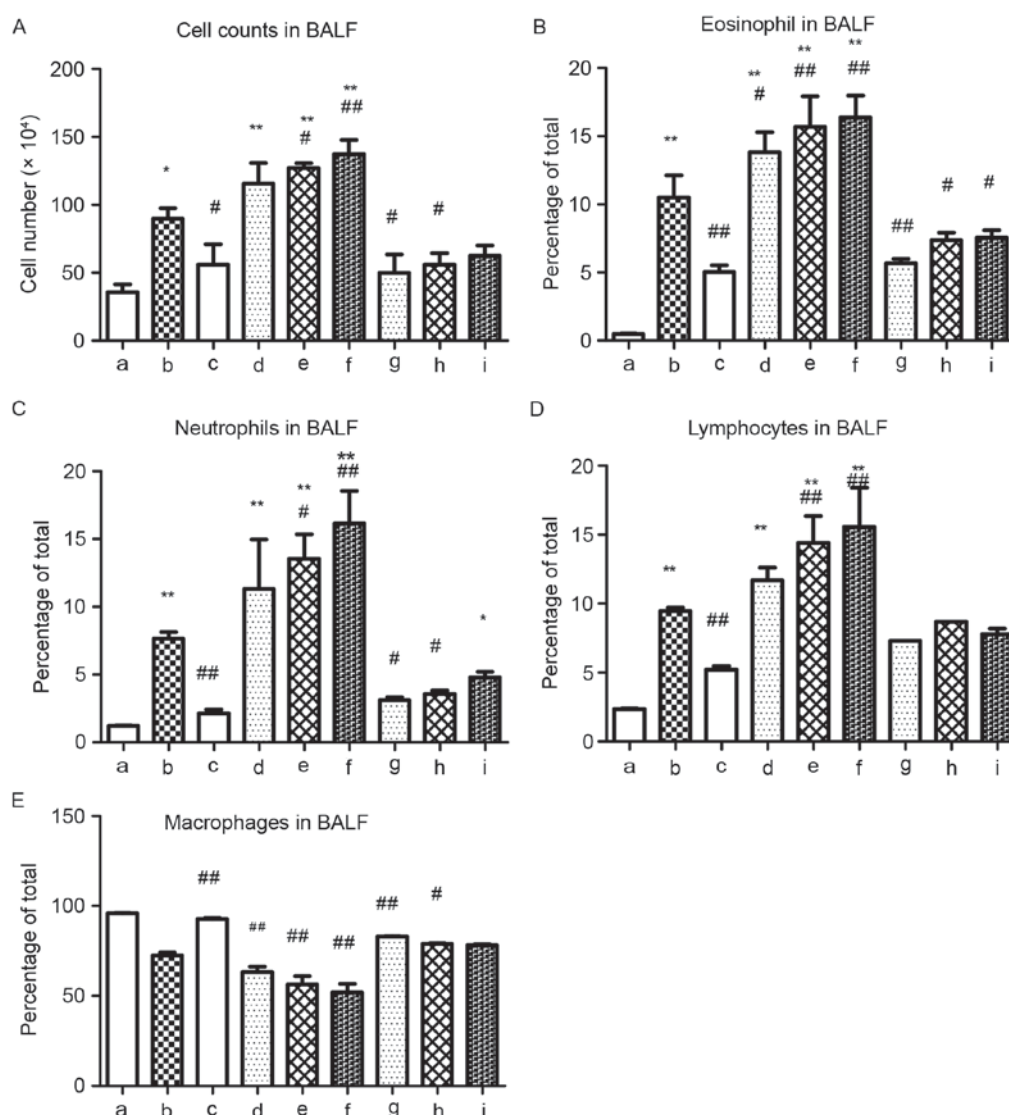
Figure 2. Inflammatory cell accumulation in BALF in a murine model of asthma. (A) Cell counts and levels of (B) eosinophil, (C) neutrophils, (D) lymphocytes and (E) macrophages. Data are expressed as the mean ± standard error (n=6-8/group). *$P<0.05$ and **$P<0.01$ vs. control group; #$P<0.05$ and ##$P<0.01$ vs. OVA group. a, control group; b, OVA group; c, OVA/DXM group; d, OVA/PM2.5 (10 μg) group; e, OVA/PM2.5 (31.6 μg) group; f, OVA/PM2.5 (100 μg) group; g, OVA/DXM/PM2.5 (10 μg) group; h, OVA/DXM/PM2.5 (31.6 μg) group; i, OVA/DXM/PM2.5 (100 μg) group. BALF, broncho-alveolar lavage fluid; OVA, ovalbumin; DXM, dexamethasone.

BALF, IL-13 (21.5±0.6 pg/ml) in the BALF and total IgE in serum (16.8±2.6 ng/ml) were significantly increased compared with that in the control group (78.1±9.9 vs. 26.9±1.3 pg/ml, 21.5±0.6 vs. 9.8±0.5 pg/ml and 16.8±2.6 vs. 1.3±0.5 ng/ml; Fig. 3A-C, respectively). IL-4 levels in the OVA/PM2.5 (100 μg) group were significantly increased compared with the OVA group (98.8±4.9 vs. 78.1±9.9 pg/ml; $P<0.05$; Fig. 3A). Levels of IL-13 (27.9±1.3 pg/ml) in the BALF and IgE (24.2±3.0 ng/ml) in the serumwere significantly increased in the OVA/PM2.5 (31.6 μg) group compared with the OVA group (Fig. 3B-C, $P<0.05$). Furthermore, the levels of IgE (26.9±6.9 ng/ml) and IL-13 (34.5±2.6 pg/ml) in the OVA/PM2.5 (100 μg) group were significantly increased compared with that in the OVA group ($P<0.01$). However, the levels of IL-4, IL-13 and IgE in glucocorticoid-treatedasthmatic mice exposed to 10 and 31.6 μg concentration PM2.5 were not statistically different from the OVA/DXM group. However, those of the glucocorticoid-treatedasthmatic mice exposed to 100 μg PM2.5 were

revealed to be increased compared with the OVA/DXM group ($P<0.05$).

Effect of PM2.5 on the protein expression levels of TSLP. As presented in Fig. 4, Compared with the OVA groups, TSLP protein expression levels were upregulated in mice exposed to PM2.5 in a dose-dependent manner. TSLP expression levels were also upregulated in the OVA/DXM/PM2.5 (100 μg) group compared with that in the OVA groups (Fig. 4, $P<0.05$).

Discussion

The chemical composition of PM2.5 mainly consists of various types of the polycyclic aromatic hydrocarbons (PAHs; including phenanthrene, fluoranthene, naphthalene and acenaphthylene), sodium and other metallic elements (iron and zinc) (11,12). Of these chemical constituents, the PAHs and metallic elements may be associated not only
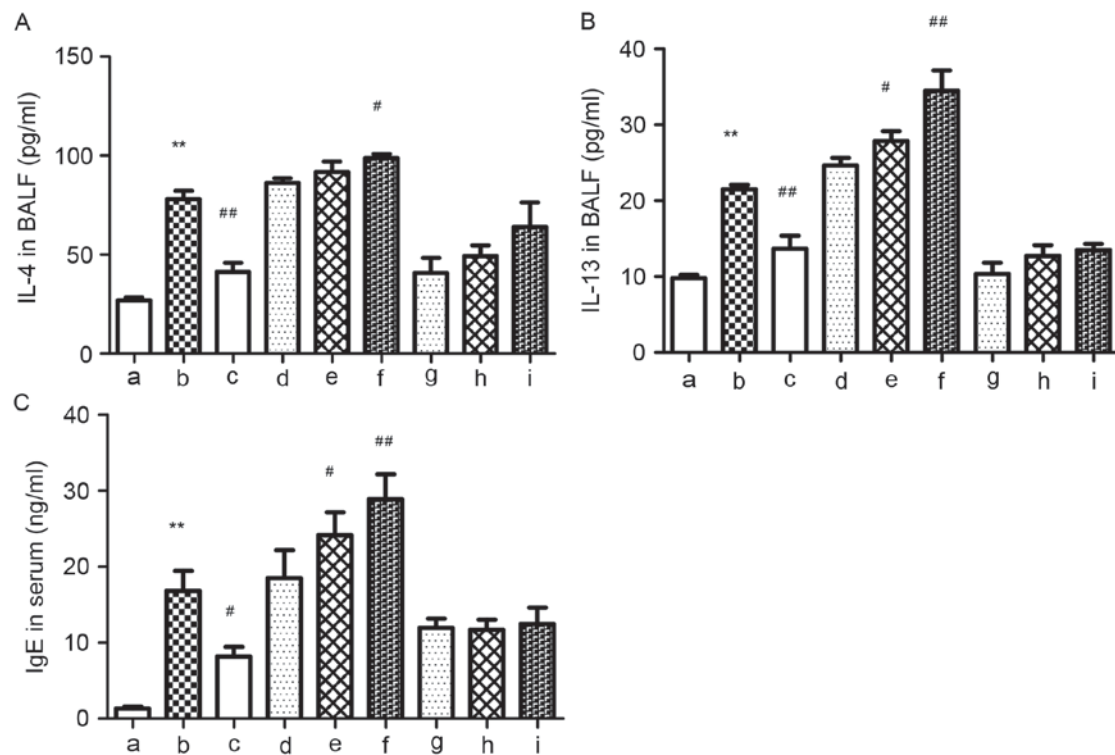
Figure 3. ELISA measurements. (A) IL-4 and (B) IL-13 levels in the BALF, and (C) total IgE in serum were measured by ELISA. Data are expressed as the mean ± standard error (n=6-8/group). **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. OVA group. a, control group; b, OVA group; c, OVA/DXM group; d, OVA/PM2.5 (10 µg) group; e, OVA/PM2.5 (31.6 µg) group; f, OVA/PM2.5 (100 µg) group; g, OVA/DXM/PM2.5 (10 µg) group; h, OVA/DXM/PM2.5 (31.6 µg) group; i, OVA/DXM/PM2.5 (100 µg) group. BALF, broncho-alveolar lavage fluid; IL, interleukin; OVA, ovalbumin; DXM, dexamethasone; IgE, immunoglobulin E.
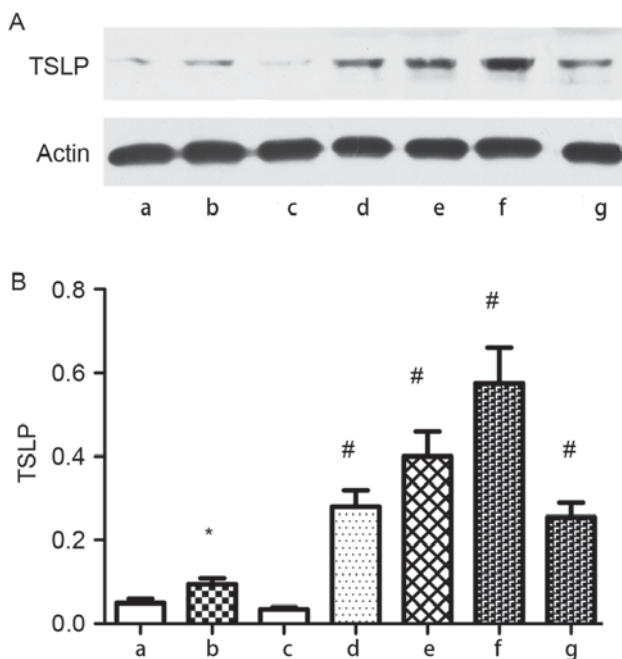

Figure 4. TSLP expression in the lungs of asthmatic mice. (A) Representative western blot images and (B) quantification of TSLP protein expression levels in lung tissue of mice. Actin served as an internal control. Data are expressed as the mean ± standard error (n=6-8/group). *P<0.05 vs. control group; #P<0.05 vs. OVA group. a, control group; b, OVA group; c, OVA/DXM group; d, OVA/PM2.5 (10 µg) group; e, OVA/PM2.5 (31.6 µg) group; f, OVA/PM2.5 (100 µg) group; g, OVA/DXM/PM2.5 (100 µg) group. OVA, ovalbumin; DXM, dexamethasone; TSLP, thymic stromal lymphopoietin.

with oxidative stress but also with many of the phenotypic changes associated with asthma. Furthermore, PM2.5 also includes various immunogenic substances, including fungal spores and pollen, which have been independently associated with exacerbations of asthma symptoms (13,14). Exposure to PM2.5 can result in oxidative stress and lung function abnormalities including increased airflow obstruction and airway hyperresponsiveness, either alone or in combination with allergic sensitization (8,15). Although the mechanisms by which exposure to PM2.5 exacerbates asthma remain unclear, the framework proposed by the UK Committee on the medical effects of air pollutants identified four main mechanisms: Oxidative stress and damage, inflammatory pathways, airway remodeling and immunological responses, and enhancement of respiratory sensitization to aeroallergens (16,17).

In terms of the effects of PM2.5 on allergic airway inflammation, the present study demonstrated that compared with the OVA group, a significant increase in inflammatory cell infiltration was identified in the OVA/PM2.5 (31.6 and 100 µg) groups. These data demonstrated that exposure to PM2.5 results in aggravation of allergic airway inflammation and the infiltration of inflammatory cells including eosinophils, lymphocytes and neutrophils. Eosinophils are the most important inflammatory effector cells. They infiltrate and accumulate at the sites of allergic inflammation, where they release cytotoxic and inflammatory molecules and cause airway epithelial damage, which results in the manifestation of allergic asthma (18,19). Furthermore, the present study revealed that compared with the OVA group, there were statistically significant changes in the

levels of IL-13 and IL-4 in the OVA/PM2.5 (31.6 and 100 μg) groups. This indicated that exposure to PM2.5 can cause activation of the inflammatory cells, thereby promoting release of the IL-4 and IL-13 cytokines. It is known that although the T cell-derived cytokines IL-4 and IL-13 have distinct functions, they are structurally similar and share a functional signaling receptor chain (the IL-4Ra chain) (20,21). These mediators are critical in regulating certain inflammatory and immune responses. Various functions, including regulation of the expression of the eosinophil-activating molecules, promotion of IgE isotype switching, enhancement of the expression of major histocompatibility class II antigens on B cells, and regulation of the expression of pro-inflammatory cytokines and chemokines by monocytes, may be attributed to IL-4 and IL-13 (22). Therefore, the underlying mechanism by which PM2.5 aggravates asthmatic airway inflammation may be associated with the upregulation of IL-4 and IL-13 expression.

The present study demonstrated that serum IgE levels were increased following 100 μg PM2.5 administration. Therefore, the mechanism by which PM2.5 aggravates asthmatic airway inflammation may be associated with the expression of serum IgE. IgE is a trigger and mediator of type 2 inflammation. It acts as part of a protein network that includes its two principal receptors, FcεRI (high-affinity Fc receptor for IgE) and CD23 (also known as FcεRII), and the IgE-and-FcεRI-binding protein (23,24). FcεRI is expressed by mast cells and basophils, in which its activation mediates cellular degranulation, eicosanoid production and cytokine production, whereas FcεRII is expressed by B cells, in which it regulates IgE production and facilitates antigen processing and presentation (25). IgE can mediate the type I hypersensitivity reactions that contribute to the pathogenesis of allergic asthma. The levels of serum IgE in healthy humans are very low (50-200 ng per ml of blood) compared with other immunoglobulin isotypes (1-10 mg per ml of blood); similar relative values are also present in mice (25,26). Additionally, compared with the OVA group, 31.6 and 100 μg PM2.5 administration significantly altered these levels in mice; however, 10 μg did not. Furthermore, the changes in the OVA/PM2.5 (100 μg) group were greater. Therefore, the development of airway inflammation in the asthmatic mice may be closely associated with the dose of PM2.5. PM2.5 in the bronchi and alveoli following deposition promotes macrophages to release pro-inflammatory cytokines and causes oxidative damage of the airway epithelium (27,28).

The percentage of neutrophils and the levels of IL-4/IL-13 and total IgE in the OVA/DXM/PM2.5 (100 μg) group were significantly different from the OVA/DXM group. Furthermore, these results corresponded with TSLP protein expression levels, as assessed by western blotting. These data demonstrated that exposure to a high dose of PM2.5 (100 μg) may inhibit the activity of the DXM-inducible glucocorticoid response element after OVA-sensitization and OVA-challenge. This result highlights a novel mechanism by which a high concentration of PM2.5 promotes glucocorticoid insensitivity. It is also clear that there is a need for improved understanding of the pathogenesis, which may lead to a greater range of treatment options for asthma. TSLP is now recognized as a protein released primarily from epithelial cells in response to proinflammatory stimuli. The activity of this protein drives

the allergic inflammatory response in asthmatic mice. TSLP serves a major role in the allergen-induced airway response and persistent airway inflammation driven by Th2 cells (29,30). Numerous mechanisms have been demonstrated. First, elevated levels of TSLP expression have been identified in the lungs of asthmatics (31). Second, TSLP is an epithelial cell (EC)-derived cytokine, and asthma develops at EC surfaces. Over the past few years, a role for TSLP in allergic inflammation has been demonstrated in both humans and mice (32,33). The present study demonstrated that a high concentration of PM2.5 induces glucocorticoid insensitivity. In addition to the TSLP functions, reduced levels of HDAC2 also serve a critical role in glucocorticoid insensitivity.

As mentioned above, the aspirated doses of 10, 31.6 and 100 μg PM2.5 in mice are considered to be equivalent to low, moderate and severe pollution. Exposure to concentrations of PM2.5 equivalent to moderate pollution and severe pollution has been linked to acute exacerbations of allergic airway inflammation in mice. In contrast, concentrations of PM2.5 equivalent to low pollution levels resulted in no significant effects (6,34,35).

In conclusion, the results from the present study suggested the exacerbation of allergic airway inflammation in mice from exposure to PM2.5 are closely associated with increased infiltration of asthmatic airway inflammatory cells, upregulation of IL-4/IL-13 expression, elevated levels of serum IgE and damage to the airway epithelial cells and oxidative stress, following deposition of PM2.5 in the bronchi and alveoli. Concentrations of PM2.5 equivalent to moderate and severe pollution may cause acute exacerbations of allergic airway inflammation in mice. When the concentrations of PM2.5 were equivalent to low pollution, no significant effects were observed. It is necessary to further examine the specific mechanisms of these responses. The results of the present study serve as a basis for further study on the pathogenesis of disease, and implicate potential interventions to prevent the exacerbation of asthma.

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