

RESEARCH ARTICLE

Role of iron and oxidative stress in the exacerbation of allergic inflammation in murine lungs caused by urban particulate matter <2.5 μm and desert dust

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Abstract

Simultaneous exposure of lipopolysaccharide (LPS) and urban particulate matter <2.5 μm (PM_{2.5}) or desert dust exacerbated murine asthma. In the present study, the role of iron (Fe) contained in particles and oxidative stress was investigated using Fe chelator deferoxamine (DFO) and oxidative stress scavenger *N*-acetylcysteine (NAC) in a murine asthma model exacerbated by LPS + PM_{2.5} or LPS + Asian sand dust (ASD). When BALB/c mice were intratracheally challenged with ovalbumin (OVA) + LPS and either urban PM_{2.5} or ASD, LPS + PM_{2.5} and LPS + ASD caused exacerbation of OVA-induced lung eosinophilia along with T-helper 2 cytokine and eosinophil-relevant chemokine production in bronchoalveolar lavage fluid as well as the production of OVA-specific IgE in serum. LPS + PM_{2.5} with NAC tended to reduce the lung eosinophilia compared to the LPS + PM_{2.5} host, whereas LPS + PM_{2.5} with DFO did not reduce them. LPS + ASD with NAC moderately reduced the lung eosinophilia compared to the LPS + ASD host. LPS + ASD with DFO drastically reduced the lung eosinophilia compared to the LPS + ASD host. The concentration of Fe in urban PM_{2.5} and ASD were almost the same. However, the concentrations of trace metals Pb, Cu, As, Ni, Cr, Mo, Sb, Co, Se and Cd were greater in PM_{2.5} than in ASD. These results suggested that Fe and oxidative stress are at least partly involved in lung eosinophilia exacerbation caused by LPS + ASD. However, trace metals (except Fe) might also be involved in lung eosinophilia exacerbated by LPS + PM_{2.5}.

KEYWORDS

PM_{2.5}, desert dust, iron, lung eosinophilia, LPS, oxidative stress

1 | INTRODUCTION

Urban fine particulate matter less than 2.5 μm in size (PM_{2.5}) from anthropogenic activities and desert dust from sand storms have received much attention worldwide as particulate matters (PM) harmful to human health. For example, there is considerable evidence that PM has a tremendous adverse effect on respiratory health. PM contain a large number of toxic substances, which stay in the atmosphere for a prolonged time and travel long distances.

Consequently, they trigger asthma, bronchitis and other respiratory related diseases (Bi, 2012).

Anthropogenic PM_{2.5} contains large numbers of toxic chemicals formed from fossil fuel combustion. They are polycyclic aromatic hydrocarbons, polycyclic aromatic hydrocarbon-quinones, sulfates (SO_4^{2-}), nitrates (NO_3^-) and transition metals (i.e., Fe, Pb, Cu, Ni), as well as microbial materials such as lipopolysaccharide (LPS) (Toriba et al., 2003; Toriba et al., 2016). Desert dust also contains silica, microbial materials (LPS, β -glucan) and transition metals (He et al., 2013; Maki et al., 2010).

Epidemiological studies indicate that the PM_{2.5} level in ambient air is related to an increase in asthma incidences. Health problems related to asthma include an increase in emergency department visits for asthma at lag days 2 and 3 (Mar, Koenig, & Primomo, 2010), worsening wheezing and dyspnea (Vempilly et al., 2013), and hospital admissions for asthma in children aged 0–18 years (Iskandar et al., 2012). A recent report showed that asthma morbidity was positively associated with daily ambient PM_{2.5} concentrations, both in warm and cool seasons (Delfino et al., 2014). Furthermore, PM_{2.5} promotes sensitization to common aeroallergens and the subsequent development of allergic respiratory diseases (Gavett, Haykal-Coates, Copeland, Heinrich, & Gilmour, 2003).

Airborne desert dust (Asian sand dust [ASD]) also has various adverse effects on humans. These effects are evidenced by an increase in daily admissions and clinic visits for asthma in Taiwan (Yang, Tsai, Chang, & Ho, 2005), deterioration of pulmonary function in asthmatic patients and exacerbation of their symptoms at night in Korea (Park et al., 2005), and exacerbation of both adult (Watanabe et al., 2011) and child asthma in Japan (Kanatani et al., 2010).

Our previous studies using a murine asthma model demonstrated that urban PM_{2.5} or desert dust exacerbates allergic inflammation in the murine lung via a Toll-like receptor (TLR)2/TLR4/MyD88-signaling pathway. Another study reported that PM-bound trace microbial elements, such as LPS, might be strong candidates for exacerbation of murine lung eosinophilia (He, Ichinose, Song, et al., 2016; He, Ichinose, Yoshida, Arashidani, et al., 2017). We also reported that exposure to ultra pure LPS + desert dust—heated at 360°C to exclude toxic materials, such as microbial materials—exacerbated ovalbumin (OVA)-associated murine lung eosinophilia (Ren et al., 2014). However, the contamination level of LPS (1 ng) did not exacerbate OVA-induced allergic lung inflammation. We noticed that the presence of particles was necessary for exacerbation. However, the reason that the presence of ASD exacerbates allergies is not clear. It was hypothesized that the presence of metals in PM plays a role in this effect.

Transition metals present in ambient PM reportedly promote the formation of reactive oxygen species (ROS) and result in exacerbation of lung inflammation and symptoms of asthma (Gavett & Koren, 2001; Gehring et al., 2015; Godri Pollitt et al., 2016; Hamad, Schauer, Antkiewicz, Shafer, & Kadhim, 2016; Hou et al., 2016; Zhang, Cui, & Qi, 2001). However, the role of oxidative stress in urban PM_{2.5} and desert dust, and trace metals present in these PM, in particular iron, in the exacerbation of asthma, is not fully understood.

The aim of this study is to clarify the role of Fe and oxidative stress in allergen-induced lung eosinophilia exacerbated by PM (urban PM_{2.5} or desert dust) with LPS.

2 | MATERIALS AND METHODS

2.1 | Sample collection of particulate matter 2.5 µm and heat treatment

Beijing urban air dust (CRM no. 28) was obtained from the National Institute for Environmental Studies (Tsukuba, Ibaraki, Japan). Urban

air dust was recovered from the filters by mechanical vibration. After the coarse particles in the material recovered were removed with a 32 µm sieve filter using an impactor type classification apparatus, the sample was further sieved with 10 and 0.8 µm filters in series to obtain fine particles of PM_{2.5} (<0.8 µm).

Asian sand dust (no. 30 “Gobi Kosa Dust”) was acquired from the National Institute for Environmental Studies (Japan). After purification by the same way as the one used for urban air dust, they were pooled in a sterilized dry bottle and stored at –30°C with a desiccant.

The two samples were heated in air at 360°C for 30 minutes in an electric furnace model SSTR-25K (Isuzu Seisakusho Co., Ltd, Niigata, Japan). These particle samples were stored at –30°C in sterilized dry bottles with a desiccant until use for the experiments.

2.2 | Particle size

The particle sizes of PM were measured with KEYENCE all in one BZ-9000 fluorescence microscope (Osaka, Japan). A total of 2500 particles were counted. The median diameter was 1.26 ± 0.73 µm (mean $\pm$ SD) for heated urban PM_{2.5} and 2.87 ± 2.42 µm (mean $\pm$ SD) for heated ASD. The size distribution peaks of urban PM_{2.5} and ASD were 0.5–1.0 and 1.0–2.0 µm, respectively.

2.3 | Analysis of components in the samples

The elements in the samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, 61E Trace and ICP-750; Thermo Jarrell-Ash, MA, USA) after acid digestion with mixed acids (68% nitric, 38% hydrofluoric and 70% perchloric = 5:1:1) at 180°C for 3 hours.

The water-soluble components in the samples were analyzed using an ion chromatograph (DX-100; Dionex, Sunnyvale, CA, USA) and ICP-AES (61E Trace, Thermo Jarrell-Ash).

Both samples were measured for LPS and β -glucan activities using an Endospec ES test MK kinetic assay (Seikagaku Corp., Tokyo, Japan) and Fungitec G test MK kinetic assay (Seikagaku Corp.), respectively. Briefly, approximately 5 mg of PM_{2.5} was suspended in 1 mL water and allowed to stand on the bench top at room temperature for 2 hours. Supernatants were tested for LPS and β -glucan concentrations using a Pyro Color-MP: Chromogenic Diazo-Coupling Kit (Associates of Cape Cod, Inc., East Falmouth, MA, USA) for endpoint colorimetry. The detection limits for LPS and β -glucan were 0.001 EU/mL and 2 pg/mL, respectively.

2.4 | Animals

BALB/c mice (7 weeks of age) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Mice were checked for abnormal body weight or sickness for 1 week, and then used at 8 weeks of age. They were fed a commercial diet CE-2 (CLEA Japan, Inc., Tokyo, Japan) and given water ad libitum. They were housed in plastic cages lined with soft wood chips. The cages were placed in an air-conditioned room at 23°C with 55%–70% humidity and a light/dark (12/12 hour) cycle. The study adhered to the US National Institutes of Health Guidelines for the use of experimental animals. The animal care method was

approved by the Animal Care and Use Committee at Oita University of Nursing and Health Sciences in Oita, Japan.

2.5 | Study protocol

A total of 154 male BALB/c mice were divided into 11 groups ($n = 14$ per group), according to the treatment with the various particles chosen for investigation. The testing samples prepared were saline (control), OVA, OVA + LPS, OVA + PM2.5, OVA + PM2.5 + LPS, OVA + PM2.5 + LPS + NAC, OVA + PM2.5 + LPS + deferoxamine (DFO), and OVA + ASD, OVA + ASD + LPS, OVA + ASD + LPS + NAC and OVA + ASD + LPS + DFO. The suspension of PM2.5 and ASD for injection was prepared by sonicating in a β -glucan and LPS free sterile saline solution containing 0.9% NaCl (Otsuka Co., Kyoto, Japan) for 5 minutes with an ultrasonic disrupter, UD-201 type with micro tip (Tomy, Tokyo, Japan) under cooling conditions.

Mice were treated with the samples prepared for instillation according to a previously reported method (He, Ichinose, Kobayashi, et al., 2016; He, Ichinose, Song, et al., 2016; He, Ichinose, Yoshida, Ito, et al., 2017; He, Ichinose, Yoshida, Arashidani, et al., 2017; Ren et al., 2014). Grad VII OVA was bought from Sigma-Aldrich Co. (St. Louis, MO, USA) and dissolved in a sterile saline solution (Otsuka Co., Kyoto, Japan). The instillation volume of the suspension was 80 μ L/mouse. The instillation dose of PM and OVA was 0.1 mg and 4 μ g per mouse, respectively. The one time instillation volume was 0.08 mL/mouse. This OVA treatment dose, administration times and periods elucidated the potential of particles (0.1 mg) to exacerbate allergic reactions in mice. Ultrapure LPS (InvivoGen, San Diego, CA, USA), DFO and NAC (Sigma-Aldrich Co.) were dissolved in a saline solution for injection. The instillation dose of ultrapure LPS was 1 ng per mouse. Both PM samples (0.1 mg/80 μ L) were pretreated with 0.05 mg DFO for 6 hours at 37°C.

Mice were intratracheally instilled with OVA alone, a mixed solution of OVA, LPS, PM2.5 or ASD with/without DFO through a polyethylene tube under 4% halothane anesthesia (Takeda Chemical, Osaka, Japan) four times at 2 week intervals. The total instillation dose of PM2.5 or ASD was 0.4 mg/mouse. The control group was treated with an 80 μ L sterile saline solution.

An NAC sterile saline solution (5 mg/0.2 mL, pH 7.4) was administered intraperitoneally to mice in the OVA + PM2.5 + LPS + NAC and OVA + ASD + LPS + NAC groups 5 hours before intratracheal instillation.

One day after the last intratracheal administration, mice from all groups (age = 14 weeks) were anesthetized by intraperitoneal injections of pentobarbital and killed by exsanguination.

2.6 | Pathological evaluation

Six of 14 mice from each group were used for pathological examination. The lungs were fixed by 10% neutral phosphate-buffered formalin. After separation of the lobes, 2 mm thick blocks were taken for paraffin embedding and then sectioned at a thickness of 3 μ m. The samples were stained with May-Grunwald's stain solution (Nacalai Tesque, Inc., Kyoto, Japan) and Giemsa's azur eosin methylene blue

solution (Merck KGaA, Darmstadt, Germany) to evaluate the degree of infiltration of eosinophils or lymphocytes in the airways from proximal to distal. The sections were stained with periodic acid-Schiff to evaluate the degree of proliferation of goblet cells in the bronchial epithelium. A pathological analysis of inflammatory cells and epithelial cells in the airways was performed with a Nikon ECLIPSE light microscope (Nikon Co., Tokyo, Japan).

2.7 | Bronchoalveolar lavage fluid

After the pathological evaluation, the remaining eight mice were used to examine the free cell contents from bronchoalveolar lavage fluid (BALF). BALF and cell counts were conducted according to a previously reported method (He, Ichinose, Kobayashi, et al., 2016; He, Ichinose, Song, et al., 2016; He, Ichinose, Yoshida, Ito, et al., 2017; He, Ichinose, Yoshida, Arashidani, et al., 2017; Ren et al., 2014). Briefly, tracheas were cannulated after the collection of blood. The lungs were lavaged with two injections of 0.8 mL of sterile saline at 37°C by a syringe. The lavaged fluid was harvested by gentle aspiration. The mean volume retrieved was 90% of the amount instilled (1.6 mL). After fluids from the two lavages were pooled and cooled to 4°C, they were centrifuged at 210 g for 10 minutes. The total amount of lavages collected from each individual mouse was used to measure the protein levels of cytokines and chemokines in the BALF. The total cell count of a fresh fluid specimen was determined using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared by Cytospin (Sakura Co, Ltd., Tokyo, Japan) and stained with Diff-Quik (International Reagents Co, Kobe, Japan). A total of 300 cells were counted under oil immersion microscopy. The BALF supernatants were stored at -80°C until analyzed for cytokines and chemokines.

2.8 | Quantitation of cytokines and chemokines in bronchoalveolar lavage fluid

The cytokine protein levels in the BALF were measured using an enzyme-linked immunosorbent assay (ELISA). Interleukin (IL)-1 β , IL-4, IL-6, IL-13, interferon (IFN)- γ , eotaxin, keratinocyte chemoattractant (KC), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , regulated upon activation normal T-cell expressed, and presumably secreted (RANTES) and tumor necrosis factor (TNF)- α were measured by an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA). IL-5 and IL-12 were also measured using ELISA kits (Endogen, Cambridge, MA, USA). MCP-3 was measured with an additional ELISA kit (Bender MedSystems, Burlingame, CA, USA). BALF samples (50 μ L/well) were added to a plate with 96 wells. Subtractive readings of 550 nm from the reading at 450 nm were converted to pg/mL using the values obtained from standard curves prepared with varying concentrations of recombinant IL-1 β (<2.31 pg/mL), IL-4 (2 pg/mL), IL-6 (1.6 pg/mL), IL-13 (1.5 pg/mL), eotaxin (3 pg/mL), KC (2 pg/mL), MCP-1 (2 pg/mL), MIP-1 α (1.5 pg/mL), RANTES (2 pg/mL), TNF- α (1.88 pg/mL), IFN- γ (2 pg/mL), IL-5 (5 pg/mL), IL-12 (12 pg/mL) and MCP-3 (2.6 pg/mL). The values in parentheses were the limits of detection.

2.9 | Antigen-specific IgE and IgG1 antibodies

OVA-specific IgE and IgG1 antibodies were measured by a Mouse OVA-IgE ELISA kit and a mouse OVA-IgG1 ELISA kit (Shibayagi Co., Shibukawa, Japan). 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 (sub-wave length, 620 nm) for OVA-specific IgE and IgG1 antibodies was measured using a microplate reader (Spectrafluor; Tecan, Salzburg, Austria).

2.10 | Statistical analysis

Statistical analyses of cell numbers (macrophages, neutrophils, eosinophils and lymphocytes), cytokine/chemokine proteins in BALF were conducted by the Tukey test for pairwise comparisons, after the *t*-test and ANOVA test were performed. All analyses were performed with KyPlot Ver. 5 (Kyens Lab Inc., Tokyo, Japan). Differences among groups were determined as statistically significant at a level of $P < 0.05$.

3 | RESULTS

3.1 | Components of urban particulate matter <2.5 μm and Asian sand dust samples

To clarify the relationship between metals and the aggravation of allergic inflammatory responses caused by the PM_{2.5}, the elements in the two PM samples (urban PM_{2.5} and ASD) were analyzed. Table 1 shows the results of major elements analysis. A total of nine major elements were identified as well as carbon. In PM_{2.5}, the concentration of Si (228 $\mu\text{g}/\text{mg}$) was the highest, followed by Ca (98 $\mu\text{g}/\text{mg}$), Al (67 $\mu\text{g}/\text{mg}$), Fe (34 $\mu\text{g}/\text{mg}$), K (17 $\mu\text{g}/\text{mg}$), Ti (3.7 $\mu\text{g}/\text{mg}$) and Ba (1.2 $\mu\text{g}/\text{mg}$). In ASD, the concentration of Si (280 $\mu\text{g}/\text{mg}$) was the highest, followed by Al (82 $\mu\text{g}/\text{mg}$), Ca (45 $\mu\text{g}/\text{mg}$), Fe (37 $\mu\text{g}/\text{mg}$), K (22 $\mu\text{g}/\text{mg}$), Ti (4.7 $\mu\text{g}/\text{mg}$) and Ba (0.54 $\mu\text{g}/\text{mg}$). In a comparison between PM_{2.5} and ASD, concentrations of Ca, Zn, Mn and Ba were greater in PM_{2.5} than in ASD. Conversely, Si, Al and Ti were generally greater in ASD than in PM_{2.5}. Fe was somewhat greater in ASD.

Table 2 shows the results of 21 minor elements found in our analysis. In PM_{2.5}, the concentration of Pb (780 ng/mg) was the highest, followed by Cu (180 ng/mg), As (150 ng/mg), Sc (100 ng/mg), Ni (98 ng/mg), V (97 ng/mg) and Cr (91 ng/mg).

In PM_{2.5}, the concentrations found were, in decreasing order, Pb > Cu > As > Sc $\approx$ Ni $\approx$ V > Cr. In ASD, the concentration of Sc (150 ng/mg) was the highest, followed by Rb (110 ng/mg), V (96 ng/mg), Ce (92 ng/mg), Cr (62 ng/mg), Ni (55 ng/mg) and Cu (41 ng/mg). In ASD, the concentrations found were, in decreasing order, Sc > Rb > V > Ce > Cr > Ni > Cu. Comparing the PM_{2.5} and ASD samples, we see that the concentrations of Pb, Cu, As, Ni, Cr, Mo, Sb, Co, Se and Cd were greater in PM_{2.5} than in ASD. Conversely, concentrations of Sc and Rb were greater in ASD than in PM_{2.5}. The same levels of V, Ce, Cs, La, Sm, Sm, Hf, Ta and Th were found in both samples.

Table 3 shows concentrations of water-soluble components and elemental carbon in the two samples. The concentration of SO_4^{2-} ,

TABLE 1 Concentration ($\mu\text{g}/\text{mg}$) of major elements in 360°C-heated PM_{2.5} and ASD

Element	PM _{2.5} , 360°C	ASD, 360°C
Si	228	280
Al	67	82
K	17	22
Ca	98	45
Fe	34	37
Zn	1.9	1.1
Ti	3.7	4.7
Mn	0.99	0.79
Ba	1.20	0.54

ASD, Asian sand dust; PM_{2.5}, particulate matter <2.5 μm .

TABLE 2 Concentration (ng/mg) of minor elements in 360°C-heated PM_{2.5} and ASD

Element	PM _{2.5} , 360°C	ASD, 360°C
Pb	780	25
Sc	100	150
V	97	96
Cr	91	62
Co	36	16
Ni	98	55
Cu	180	41
As	150	16
Se	26	<2.7
Rb	83	110
Mo	46	1.4
Sb	37	1.2
Cs	7.4	8.4
La	44	39
Ce	91	92
Sm	6.8	6.1
Hf	4.9	5
W	8.5	4.6
Ta	1.1	0.99
Th	16	13
Cd	9.5	<0.39

ASD, Asian sand dust; PM_{2.5}, particulate matter <2.5 μm .

Cl^- , Na^+ , K^+ , Ca^{2+} and elemental carbon were higher in PM_{2.5} than in ASD. The concentration of NO_3^- , NH_4^+ and Mg^{2+} were the same in both samples. LPS and β -glucan were not detected in either sample.

3.2 | Effects of N-acetylcysteine and deferoxamine on bronchoalveolar lavage fluid cell numbers

To evaluate the effects of NAC and DFO on OVA-induced lung inflammation exacerbated by PM_{2.5} and ASD with LPS, the cellular profile of BALF was investigated 24 hours after the last intratracheal instillation. Figure 1 shows the effects of NAC and DFO on the cellular

profile of BALF. OVA + PM2.5 + LPS and OVA + ASD + LPS markedly increased the number of macrophages, neutrophils, eosinophils and lymphocytes over both the control and OVA alone.

OVA + PM2.5 + LPS + NAC significantly decreased macrophages, but only tended to decrease the number of eosinophils and

lymphocytes compared to OVA + PM2.5 + LPS counterparts. The number of neutrophils in both groups was similar. OVA + PM2.5 + LPS + DFO tended to decrease the number of macrophages, eosinophils and lymphocytes, but tended to increase neutrophils compared to OVA + PM2.5 + LPS counterparts.

OVA + ASD + LPS + NAC significantly decreased the number of eosinophils and lymphocytes and tended to decrease neutrophils compared to OVA + ASD + LPS counterparts. OVA + ASD + LPS + DFO significantly decreased the number of eosinophils and lymphocytes compared to OVA + PM2.5 + LPS counterparts.

The number of macrophages and neutrophils in both groups was similar.

TABLE 3 Concentration of water-soluble components ($\mu\text{g}/\text{mg}$), EC ($\mu\text{g}/\text{mg}$) and microbial elements (ng/mg) in 360°C-heated PM2.5 and ASD

Element	PM2.5, 360°C	ASD, 360°C
SO_4^{2-}	16	0.68
NO_3^-	0.89	0.71
Cl^-	4.0	<0.042
Na^+	3.5	2.9
NH_4^+	<0.45	<0.32
K^+	3.4	1.2
Mg^{2+}	4.5	4.9
Ca^{2+}	78	21
EC	3.93	2.4
Microbial element		
LPS	ND	ND
β -glucan	ND	ND

ASD, Asian sand dust; EC, elemental carbon; LPS, lipopolysaccharide; ND, not detectable; PM2.5, particulate matter <2.5 μm .

3.3 | Effects of N-acetylcysteine and deferoxamine on bronchoalveolar lavage fluid cell cytokine and chemokine

To investigate the effects of NAC and DFO on OVA-induced lung inflammation exacerbated by PM2.5 and ASD with LPS, the protein levels of proinflammatory molecules in BALF were measured 24 hours after the final instillation.

Figure 2 shows the expressions of eosinophil relevant cytokines and chemokines.

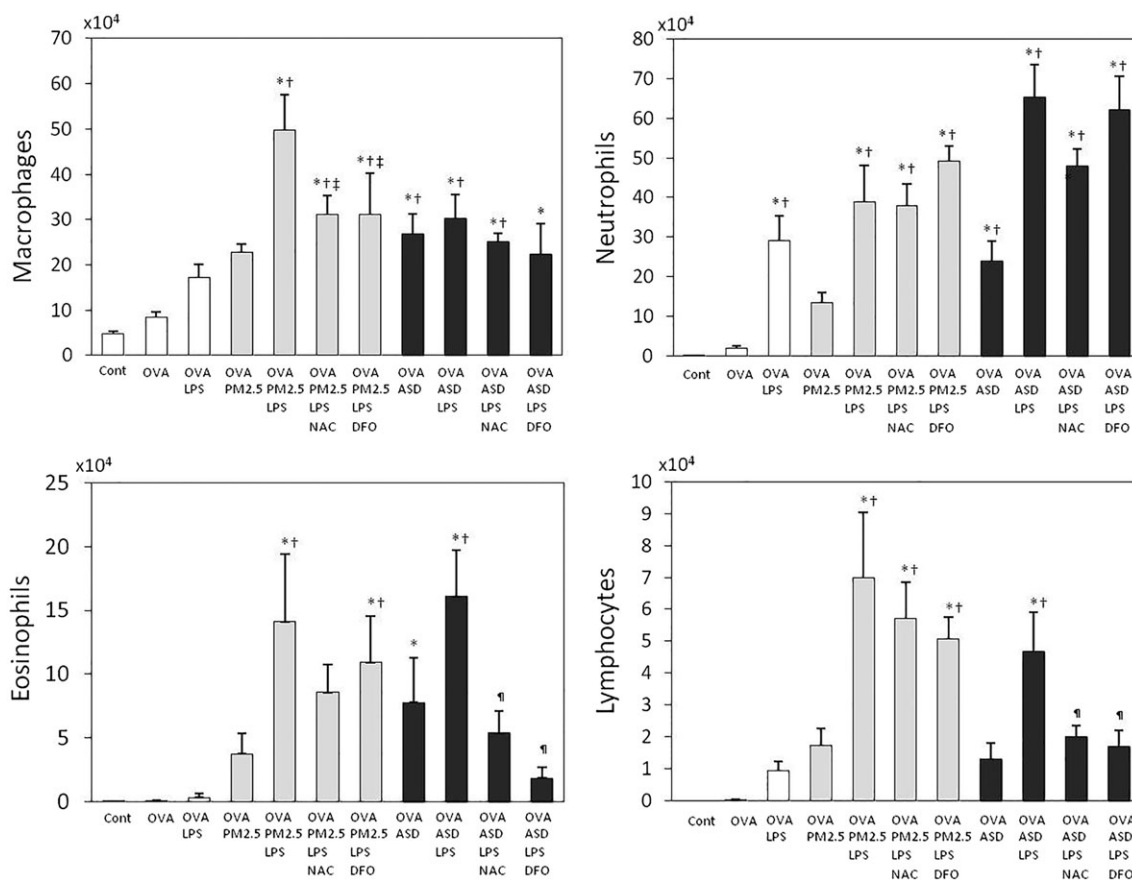


FIGURE 1 Effects of NAC and DFO on cellular profile of bronchoalveolar lavage fluid. Eleven groups of mice were treated intratracheally with the various test samples four times at 2 week intervals. Differential cell counts were assessed in cytologic preparations stained with Diff-Quik. All values are expressed as mean $\pm$ SE ($n = 8$). * $P < 0.05$ vs. Cont, $^\dagger P < 0.05$ vs. OVA, $^\ddagger P < 0.05$ vs. OVA + PM2.5 + LPS, $^\S P < 0.05$ vs. OVA + ASD + LPS. ASD, Asian sand dust; Cont, control; DFO, deferoxamine; LPS, lipopolysaccharide; NAC, N-acetylcysteine; OVA, ovalbumin; PM2.5, particulate matter <2.5 μm

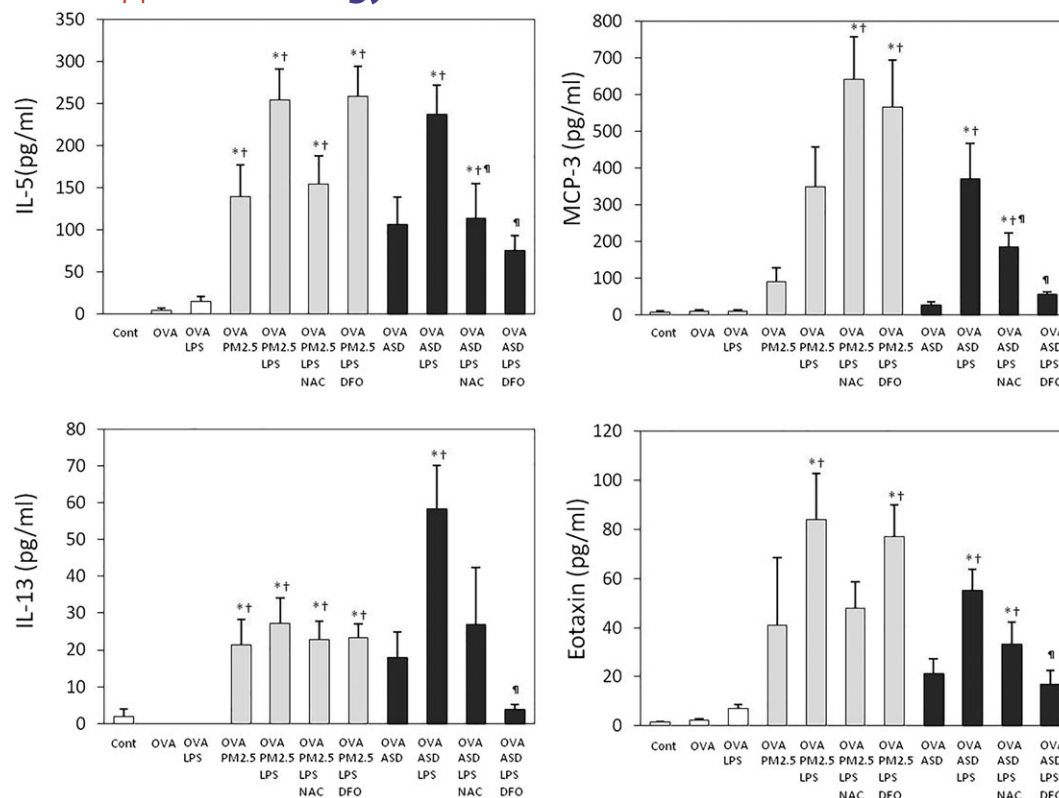


FIGURE 2 Effects of NAC and DFO on expression of IL-5, IL-13, MCP-3 and eotaxin in bronchoalveolar lavage fluid. Eleven groups of mice were treated intratracheally with the various test samples four times at 2 week intervals. All values are expressed as mean $\pm$ SE ($n = 8$). * $P < 0.05$ vs. Cont, $^{\dagger}P < 0.05$ vs. OVA, $^{\ddagger}P < 0.05$ vs. OVA + PM2.5 + LPS, $^{\S}P < 0.05$ vs. OVA + ASD + LPS. ASD, Asian sand dust; Cont, control; DFO, deferoxamine; IL, interleukin; LPS, lipopolysaccharide; NAC, N-acetylcysteine; MCP, monocyte chemotactic protein; ND, not detectable; OVA, ovalbumin; PM2.5, particulate matter $<2.5 \mu\text{m}$

OVA + PM2.5 + LPS and OVA + ASD + LPS significantly increased the levels of IL-5, IL-13 and eotaxin, and they tended to increase the MCP-3 level compared to the control and OVA-alone counterparts.

OVA + PM2.5 + LPS + NAC decreased the levels of IL-5 and eotaxin, but increased MCP-3 compared to OVA + PM2.5 + LPS counterparts. The expression level of IL-13 in both groups was similar. OVA + PM2.5 + LPS + DFO increased MCP-3 levels over those found in the case of the OVA + PM2.5 + LPS host alone. The expression levels of IL-5, IL-13 and eotaxin in both groups were similar. OVA + ASD + LPS + NAC significantly decreased IL-5, MCP-3 and tended to decrease IL-13 and eotaxin compared to the OVA + PM2.5 + LPS counterparts. OVA + ASD + LPS + DFO markedly decreased IL-5, MCP-3, IL-13 and eotaxin compared to their OVA + PM2.5 + LPS counterparts.

OVA + ASD + LPS + DFO suppressed the expression of IL-5, MCP-3, IL-13 and eotaxin less than their OVA + ASD + LPS + NAC counterparts.

Figure 3 shows the expressions of IL-1 β , IL-6, IL-12 and TNF- α .

OVA + PM2.5 + LPS and OVA + ASD + LPS significantly increased IL-1 β , IL-12 and TNF- α , and moderately increased IL-6 compared to the control and OVA-alone counterparts. OVA + PM2.5 + LPS + NAC increased IL-1 β and IL-6 compared to OVA + PM2.5 + LPS counterparts. The expression levels of IL-12 and IL-1 β in both groups were similar. OVA + ASD + LPS + NAC considerably decreased IL-1 β , IL-6, IL-12, and moderately decreased TNF- α compared to OVA + PM2.5 + LPS counterparts. OVA + ASD + LPS + DFO

decreased IL-1 β , IL-6, IL-12 and TNF- α compared to their OVA + PM2.5 + LPS counterparts.

Figure 4 shows the expressions of MIP-1 α , KC, MCP-1 and RANTES.

OVA + PM2.5 + LPS and OVA + ASD + LPS markedly increased MIP-1 α , KC, MCP-1, and RANTES over the control and OVA alone counterparts. OVA + PM2.5 + LPS + NAC increased MIP-1 α , KC and MCP-1 compared to OVA + PM2.5 + LPS counterparts. The expression level of RANTES in both groups was similar. OVA + PM2.5 + LPS + DFO increased MIP-1 α , KC and MCP-1, and significantly increased RANTES compared to OVA + PM2.5 + LPS counterparts. OVA + ASD + LPS + NAC and OVA + ASD + LPS + DFO significantly decreased MIP-1 α , KC and MCP-1, and moderately decreased RANTES compared to their OVA + PM2.5 + LPS counterparts. OVA + ASD + LPS + DFO suppressed the expression of KC, MIP-1 α and RANTES to levels less than their OVA + ASD + LPS + NAC counterparts. IFN- γ was not detected.

3.4 | Effects of N-acetylcysteine and deferoxamine on serum ovalbumin-specific IgE

To investigate the effects of NAC and DFO on OVA-induced lung inflammation exacerbated by PM2.5 and ASD with LPS, OVA-specific IgE antibody in the serum was measured.

Figure 5 shows the effects of NAC and DFO on OVA-specific IgE production in serum. OVA + PM2.5 + LPS and OVA + ASD + LPS

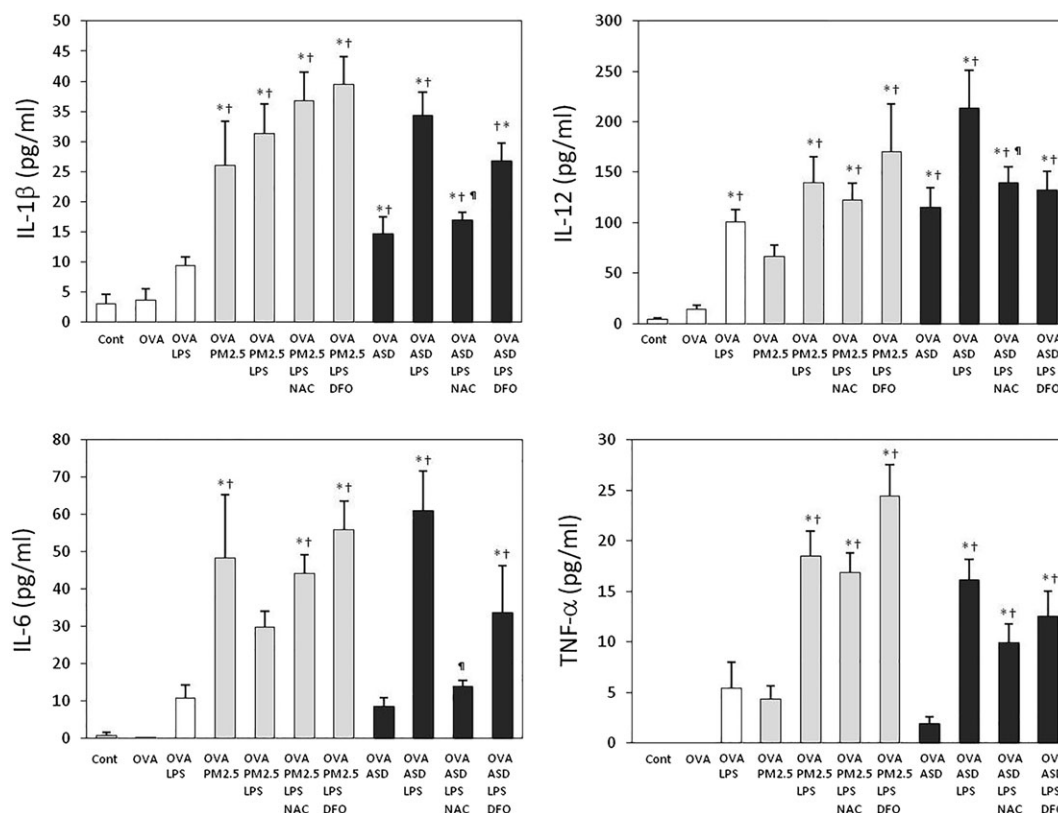


FIGURE 3 Effects of NAC and DFO on expression of IL-1 β , IL-6, IL-12 and TNF- α in bronchoalveolar lavage fluid. Eleven groups of mice were treated intratracheally with the various test samples four times at 2 week intervals. All values are expressed as mean $\pm$ SE ($n = 8$). * $P < 0.05$ vs. Cont, † $P < 0.05$ vs. OVA, ‡ $P < 0.05$ vs. OVA + PM2.5 + LPS, § $P < 0.05$ vs. OVA + ASD + LPS. ASD, Asian sand dust; Cont, control; DFO, deferoxamine; IL, interleukin; LPS, lipopolysaccharide; NAC, N-acetylcysteine; ND, not detectable; OVA, ovalbumin; PM2.5, particulate matter <2.5 μ m; TNF, tumor necrosis factor

obviously increased IgE over the control and OVA alone. OVA + PM2.5 + LPS + NAC and OVA + PM2.5 + LPS + DFO considerably decreased IgE compared to the OVA + ASD + LPS host. OVA + ASD + LPS + NAC remarkably decreased IgE compared to OVA + ASD + LPS host but only slightly in OVA + ASD + LPS + DFO.

3.5 | Effects of N-acetylcysteine and deferoxamine on pathologic changes in the airways caused by particulate matter <2.5 μ m and Asian sand dust

To evaluate the effects of NAC and DFO on OVA-induced lung inflammation exacerbated by PM2.5 and ASD with LPS, lung pathology in mice exposed to each test sample was examined.

Figure 6 shows the bronchus in the lungs stained with periodic acid-Schiff. No pathologic alterations were found in the lungs of the control group (Figure 6A). OVA alone and OVA + LPS caused very slight to mild peribronchiolar inflammation and proliferation of goblet cells in the airways epithelium (Figure 6B and 6C). OVA + PM2.5 and OVA + ASD caused mild to moderate peribronchiolar inflammation and proliferation of goblet cells in the airways epithelium (Figure 6D and 6H). However, with OVA + PM2.5 + LPS and OVA + ASD + LPS these pathological changes were marked (Figure 6E and 6I). These pathological changes were slightly alleviated in the OVA + PM2.5 + LPS + NAC group (Figure 6F) and the

OVA + ASD + LPS + NAC group (Figure 6J). However, the pathological changes in the OVA+PM2.5 + LPS + DFO group were almost the same as those in the OVA + ASD + LPS group (Figure 6G). On the other hand, the pathological changes in the OVA + ASD + LPS + DFO group were significantly alleviated compared to those in the OVA + ASD + LPS group (Figure 6K).

Figure 7 shows the airways submucosa in the lungs by May-Giemsa stain. OVA + PM2.5 + LPS and OVA + ASD + LPS caused marked infiltration of eosinophils, neutrophils and lymphocytes into the airways submucosa (Figure 7A and 7D). These inflammatory cells were slightly alleviated in the OVA + PM2.5 + LPS + NAC group and the OVA + ASD + LPS + NAC group (Figure 7B and 7E). However, OVA + PM2.5 + LPS + DFO did not alleviate the infiltration of these inflammatory cells into the airways submucosa (Figure 7C). OVA + ASD + LPS + DFO markedly suppressed the infiltration of these inflammatory cells (Figure 7F).

4 | DISCUSSION

Transition metals, in particular Fe present in PM, and oxidative stress might relate to the induction of lung inflammation and exacerbation of asthma. In the present study, the roles of Fe in PM and oxidative stress were investigated using a murine asthma model enhanced by urban PM2.5 or ASD with LPS. Results of the present study suggested

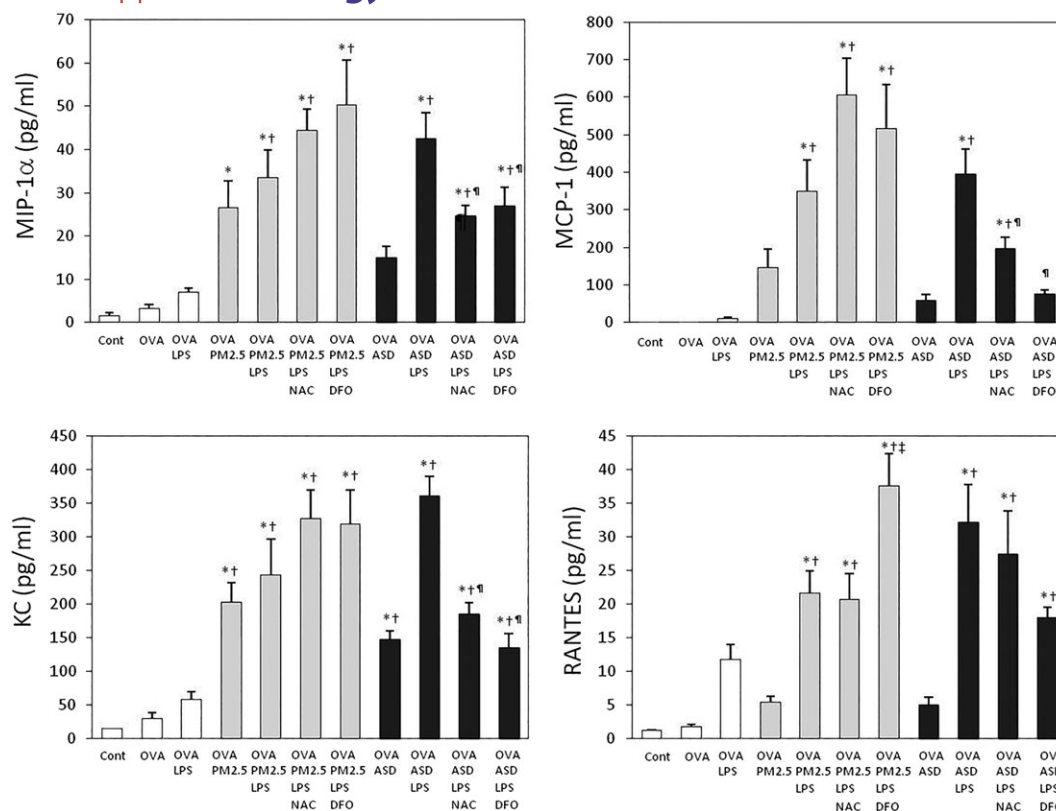


FIGURE 4 Effects of NAC and DFO on expression of MIP-1 α , KC, MCP-1 and RANTES in bronchoalveolar lavage fluid. Eleven groups of mice were treated intratracheally with the various test samples four times at 2 week intervals. All values are expressed as mean $\pm$ SE ($n = 8$). * $P < 0.05$ vs. Cont, $^{\dagger}P < 0.05$ vs. OVA, $^{\ddagger}P < 0.05$ vs. OVA + PM2.5 + LPS, $^{\S}P < 0.05$ vs. OVA + ASD + LPS. ASD, Asian sand dust; Cont, control; DFO, deferoxamine; KC, keratinocyte chemoattractant; LPS, lipopolysaccharide; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NAC, N-acetylcysteine; ND, not detectable; OVA, ovalbumin; PM2.5, particulate matter $<2.5 \mu\text{m}$

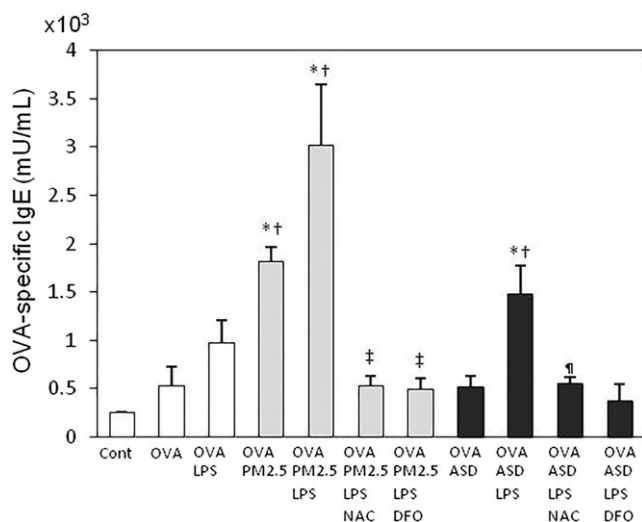


FIGURE 5 Effects of NAC and DFO on OVA-specific IgE production in serum. Eleven groups of mice were treated intratracheally with the various test samples four times at 2 week intervals. All values are expressed as mean $\pm$ SE ($n = 8$). * $P < 0.05$ vs. Cont, $^{\dagger}P < 0.05$ vs. OVA, $^{\ddagger}P < 0.05$ vs. OVA + PM2.5 + LPS, $^{\S}P < 0.05$ vs. OVA + ASD + LPS. ASD, Asian sand dust; Cont, control; DFO, deferoxamine; LPS, lipopolysaccharide; NAC, N-acetylcysteine; ND, not detectable; OVA, ovalbumin; PM2.5, particulate matter $<2.5 \mu\text{m}$

that Fe in ASD is involved in lung eosinophilia enhancement. On the other hand, PM2.5 activity might involve the presence of trace metals, such as Pb, Cu, As, Mo and Cd.

T-helper (Th)2 cytokine IL-5 causes eosinophil infiltration into the airways submucosa and airways hyper-responsiveness (Ok, Kim, Kim, Lee, & Lee, 2009), selective differentiation, regulating growth, activation and survival of eosinophils (Brito et al., 2014). Th2 cytokine IL-13 causes airways hyper-reactivity, goblet cell proliferation and eotaxin production (Grünig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999). In addition, eotaxin and MCP-3 induced the recruitment of eosinophils into the lungs (Dahinden et al., 1994). Both PM + LPS exacerbated OVA-associated eosinophilic lung inflammation along with various other effects, including an increase in Th2 cytokine (IL-5) and eosinophilic relevant chemokines (MCP-3, eotaxin), eosinophil numbers in BALF and pathological changes in the lungs. Pathological pulmonary changes included infiltration of eosinophils into the airways submucosa and proliferation of goblet cells in the airways epithelium as well as the production of OVA-specific IgE in serum. Pretreatment with NAC tended to attenuate the eosinophilic lung inflammation in both PM. Interestingly, DFO attenuated the allergic inflammation exacerbated by OVA + ASD + LPS, but not by OVA + PM2.5 + LPS.

LPS is a toxic component of gram-negative bacteria and a ligand of TLR4, which recruits and activates neutrophils (Schwandner, Dziarski, Wesche, Rothe, & Kirschning, 1999).

OVA + PM2.5 + LPS and OVA + ASD + LPS markedly increased the number of neutrophils in BALF in this allergic inflammatory event. In the case of PM2.5, NAC and DFO treatment did not reduce the neutrophil numbers and neutrophil relevant proinflammatory

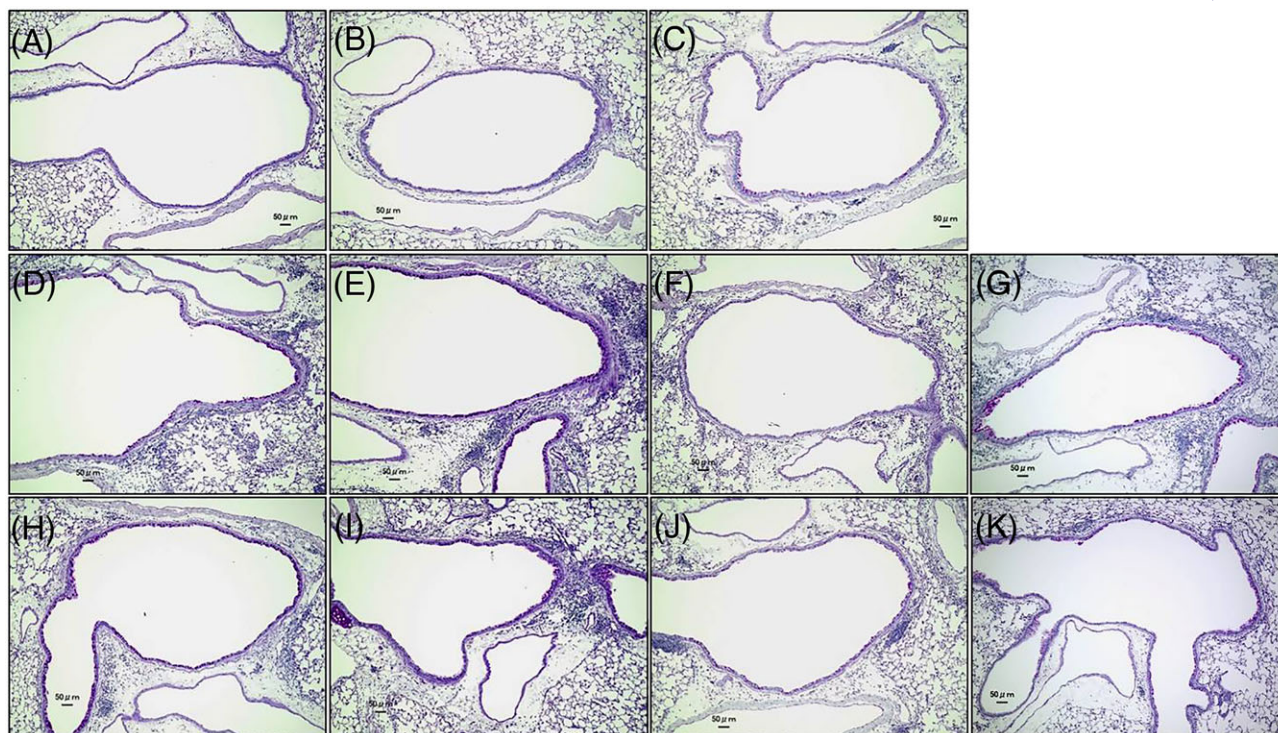


FIGURE 6 Effects of NAC and DFO on pathological changes in the lung. Sections of the lung from six mice were stained with periodic acid-Schiff. A, No pathologic alterations were found in the lungs of the control group. B, OVA alone caused very slight to mild peribronchiolar inflammation and proliferation of goblet cells in the airways epithelium. C, OVA + LPS caused pathological changes similar to those of OVA alone. D, OVA + PM2.5 caused mild to moderate peribronchiolar inflammation and proliferation of goblet cells in the airways epithelium. H, OVA + ASD caused pathological changes similar to those of OVA + PM2.5 seen in (D). E, OVA + PM2.5 + LPS caused marked these pathological changes. Many goblet cells stained pink by periodic acid-Schiff solution were seen in the airways epithelium. Numerous inflammatory cells were found around the bronchus. I, OVA + ASD + LPS caused pathological changes similar to those of OVA + PM2.5 + LPS seen in (E). Proliferation of pink goblet cells and numerous inflammatory cells were seen in the airways. F, OVA + PM2.5 + LPS + NAC slightly alleviated these pathological changes. J, OVA + ASD + LPS + NAC slightly alleviated these pathological changes. G, OVA + PM2.5 + LPS + DFO did not alleviate marked peribronchiolar inflammation and proliferation of goblet cells in the airways epithelium by OVA + PM2.5 + LPS seen in (E). K, OVA + ASD + LPS + DFO alleviated marked pathological changes by OVA + ASD + LPS seen in (I). ASD, Asian sand dust; Cont, control; DFO, deferoxamine; LPS, lipopolysaccharide; NAC, N-acetylcysteine; OVA, ovalbumin; PM2.5, particulate matter <2.5 μm

mediators (IL-1 β , IL-6, IL-12, TNF- α , MIP-1 α , KC, RANTES) compared to the OVA + PM2.5 + LPS host. Proinflammatory mediators were increased by a DFO treatment. These results indicate that DFO does not reduce neutrophil-related responses, at least, in allergic events caused by OVA + PM2.5 + LPS.

In the case of ASD, NAC treatment reduced the neutrophil number and significantly reduced proinflammatory mediators (IL-1 β , IL-6, IL-12, MIP-1 α , KC, MCP-1) compared to the OVA + ASD + LPS host. Thus, NAC also suppressed neutrophil-related responses. DFO did not reduce neutrophil numbers, whereas some cytokines (MIP-1 α , KC, MCP-1) were remarkably reduced. Therefore, it is suggested that neutrophil-related responses are suppressed by DFO. The neutrophil-related responses do not seem to be a direct effect of LPS. The responses detected in PM2.5 and ASD might occur by linking to allergic reactions with OVA.

TLRs in antigen-presenting cells, like macrophages and dendritic cells, play an important role in innate immunity as well as adaptive immunity (Bellocchio et al., 2004). It is well known that the low levels of LPS causes TLR4-dependent Th2 responses to OVA (Eisenbarth et al., 2002) through MyD88 (Piggott et al., 2005). However, we found that the contamination level of LPS (1 ng) did not exacerbate OVA-

induced allergic lung inflammation but LPS + ASD did (Ren et al., 2014). In this study, the same result was obtained in the case of PM2.5. The exacerbation of allergic lung inflammation caused by LPS + PM may occur via TLR4 because mice that lacked TLR4 showed markedly attenuated exacerbation of allergic lung inflammation by ASD and PM2.5 containing LPS (He, Ichinose, Song, et al., 2016; He, Ichinose, Yoshida, Arashidani, et al., 2017). As LPS + PM is presumed to activate the OVA-induced Th2 response through the TLR4/MyD88 pathway, the mechanism by which the presence of PM exacerbates allergy is not clear. It is hypothesized that the metal present in PM is related to that phenomenon.

The processes of LPS-induced mitogen-activated protein kinases (MAPKs) activation and MAPK-mediated TNF- α production reportedly were regulated, at least in part, by ROS. NADPH oxidase (NOX) was involved in this process of ROS generation. NAC, which induced the accumulation of glutathione (GSH), reduced the TNF- α secretion by scavenging ROS (Haddad & Land, 2002). LPS also induced ROS production through TLR4/MyD88/c-Src/p47phox/NOX in human renal mesangial cells (Lee, Shih, Lin, Chen, & Yang, 2012).

Hepatocarcinogen 2-acetylaminofluorene-induced ROS caused activation of IKK kinases and increased nuclear factor kappaB (NF- κ B)

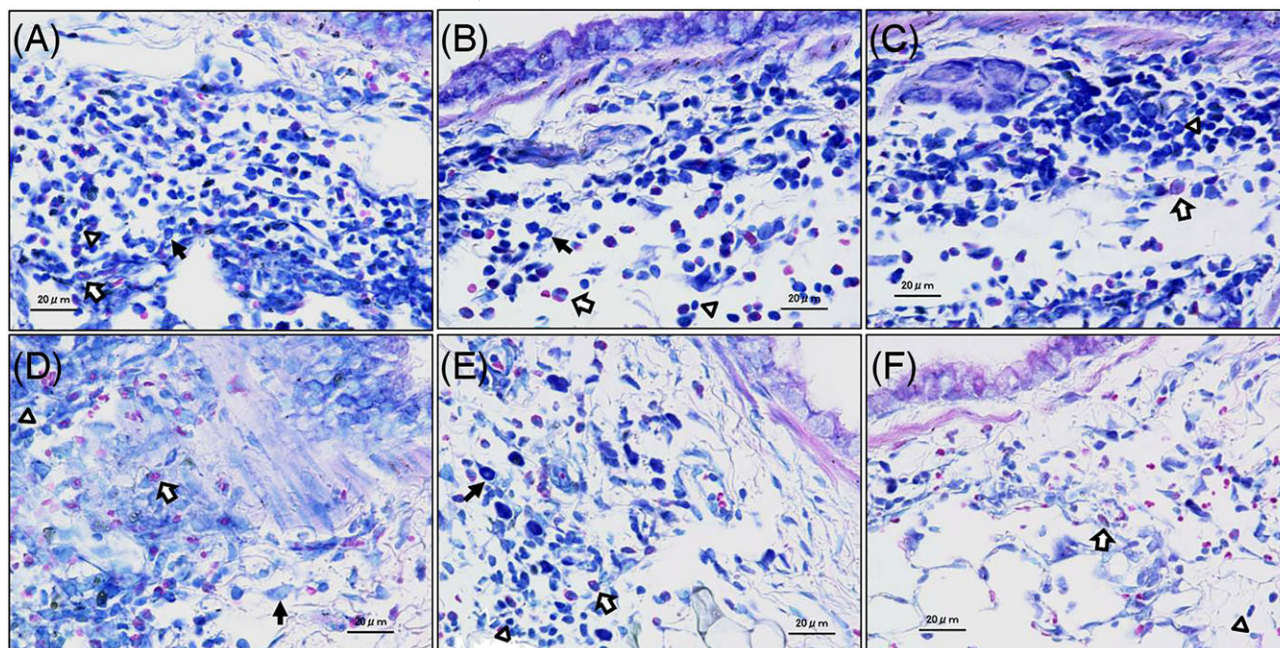


FIGURE 7 Effects of NAC and DFO on infiltration of inflammatory cells in the airways. Sections of the lung from six mice were stained with May-Giemsa's solution. Triangles show lymphocytes; thin arrows show tissue macrophages; thick arrows show eosinophils with red granules. A, OVA + PM2.5 + LPS caused marked infiltration of eosinophils, neutrophils and lymphocytes into the airways submucosa. Many eosinophils were seen in the airways submucosa. D, OVA + ASD + LPS caused pathological changes similar to those of OVA + PM2.5 + LPS in (A). Numerous eosinophils were observed in the airways submucosa (thick arrow). B, OVA + PM2.5 + LPS + NAC slightly alleviated the infiltration of these inflammatory cells into the airways submucosa. E, OVA + ASD + LPS + NAC also slightly alleviated these inflammatory changes in the airways submucosa. C, OVA + PM2.5 + LPS + DFO did not alleviate the infiltration of these inflammatory cells into the airways submucosa. F, OVA + ASD + LPS + DFO markedly suppressed the infiltration of these inflammatory cells into the airways submucosa. ASD, Asian sand dust; DFO, deferoxamine; LPS, lipopolysaccharide; NAC, N-acetylcysteine; OVA, ovalbumin; PM2.5, particulate matter <2.5 μm

DNA binding activity. Overproduction of GSH by NAC blocked the activation of IKK kinase complex and NF- κB DNA binding (Song, Lee, & Chan, 2005). Therefore, LPS causes NOX activation and intracellular ROS generation, which activates MAPKs and IKK kinases. NAC blocks the intracellular ROS generation.

In the present study, the suppressive effects of NAC on Th2 responses by both PM might be caused by scavenging ROS in antigen-presenting cells. Therefore, in the context of allergy exacerbation by LPS + PM, we speculate that intracellular ROS accelerates LPS/TLR4/TAK1/MAPK and NF- κB signal pathways in the antigen-presenting cell and that the mediated signal might activate the Th2 responses to OVA.

Figure 8 shows a schematic diagram of activation of the signaling pathways by ROS and suppression by NAC. In the present study, the Fe concentration in the tested urban PM2.5 and ASD was the same, whereas the concentrations of Pb, Cu, As, Ni, Cr, Mo, Sb, Co, Se and Cd were greater in PM2.5 than in ASD. Metal-rich PM2.5 are derived from fossil fuel combustion and vehicular emissions (Primbs et al., 2007). Redox active metals Fe, Ni, Cr, Cu and Co trigger ROS (H_2O_2 , $\bullet\text{O}_2^-$, $\bullet\text{OH}$) generation through Fenton-type and Harber-Weiss reactions. Redox inactive metals Pb, Cd and As caused their toxic effects via binding to a sulfhydryl group of proteins and subsequent depletion of GSH (Jomova & Valko, 2011).

DFO, which is an iron chelator, suppresses ROS production, neuronal death and neutrophil infiltration by reducing Fe accumulation after intracerebral hemorrhage in rats and mice (Nakamura et al.,

2003; Wu, Wu, Xu, Wang, & Wang, 2011). In the case of ASD, Fe in the ASD incorporated into the cell by phagocytosis might act as a catalyst of the ROS production promoted by LPS-activated NOX. DFO reduced ROS production by chelating Fe in ASD, subsequently suppressing the activation of the LPS/TLR4/TAK1/MAPKs and NF- κB pathway (Figure 8). The reason that ASD is required for exacerbation of OVA-induced allergy might be the presence of Fe, which is incorporated into cells. In our previous study, ASD containing Fe caused exacerbation of OVA-induced lung allergy, whereas aluminum trioxide (Al_2O_3) particles did not (Ichinose et al., 2008). A schematic diagram of the activation of the inflammation-induced signaling pathways by Fe in ASD incorporated into antigen-presenting cells is also shown in Figure 8.

In the case of PM2.5, the reason that DFO has no suppressive effect on lung allergy is probably because it contains larger amounts of redox active metals (e.g., Ni, Cr, Cu, Co, but not Fe) that generate ROS than ASD. Redox of inactive metals (Pb, Cd and As) might consume intracellular GSH and promote ROS generation.

According to one epidemiological study on the relationship between the levels of heavy metals in PM2.5 or in blood and the prevalence of respiratory symptoms and asthma in children, Pb and Cd in blood were positively associated with coughing. Pb levels in blood higher than 5 $\mu\text{g}/\text{dL}$ were significantly associated with asthma, and high blood Cr and Mn levels were associated with coughing and wheezing (Zeng et al., 2016). Another epidemiological result has shown that PM constituents, in particular Fe, Cu and Zn, resulting

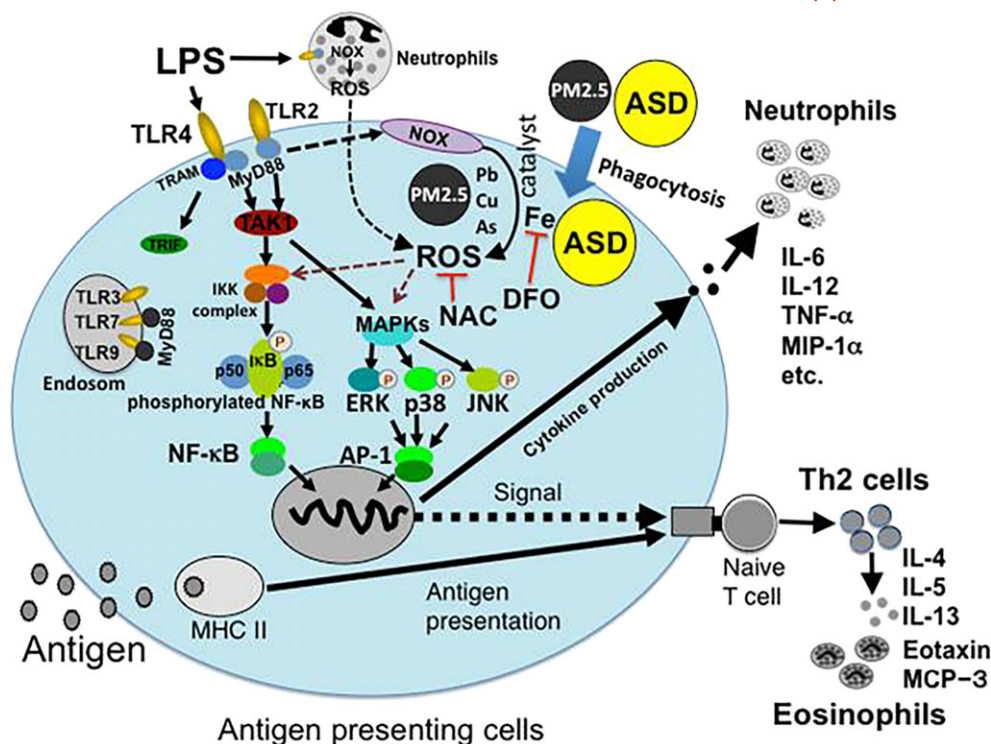


FIGURE 8 Schematic diagram of activation of the inflammation-induced signaling pathways by metals present in particles incorporated into antigen-presenting cells. LPS induces activation of MAPKs and IKK kinases and then causes AP-1 and NF- κ B activation and produces proinflammatory mediators. LPS activates NOX via TLR4. PM2.5 and ASD are incorporated into cells by phagocytosis. Fe in ASD and redox active metals in PM2.5 incorporated into the cell act as catalysts in the ROS production process by LPS-activated NOX. ROS accelerates the LPS/TLR4/TAK1/MAPKs and NF- κ B signaling pathways and the mediated signal activates the Th2 responses to OVA. DFO reduces ROS production by chelating of the Fe in ASD, partially suppressing the activation of the LPS-mediated signaling pathway and the signal to Th2 cells. NAC also partially reduces the activation of these signaling pathways by scavenging ROS. AP, activator protein; ASD, Asian sand dust; DFO, deferoxamine; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP, monocyte chemotactic protein; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; NAC, N-acetylcysteine; NF- κ B, nuclear factor kappaB; NOX, NADPH oxidase; OVA, ovalbumin; PM2.5, particulate matter <2.5 μ m; ROS, reactive oxygen species; Th2, T-helper 2; TLR, Toll-like receptor; TNF, tumor necrosis factor

from poorly regulated non-tailpipe road traffic emissions, may increase the risk of asthma and allergy in schoolchildren (Gehring et al., 2015). PM2.5-associated metals have a great impact on not only asthma, but also exacerbation of mycoplasma pneumonia related to asthma (Hou et al., 2016). Thus, transition metals may be considered as the key indicators with which to evaluate the exacerbating effects of PM on bronchial asthma. We hope that our results support these epidemiological findings.

In conclusion, we found that PM2.5 and ASD had different effects related to the Fe chelator DFO. The lung eosinophilia exacerbated by LPS + ASD was suppressed by DFO. On the other hand, it was not suppressed in the case of LPS + PM2.5. The lung eosinophilia exacerbated by LPS + both PM was suppressed slightly by NAC. These results suggested that Fe present in ASD and oxidative stress are at least partly involved in lung eosinophilia exacerbation. However, trace metals, such as Pb, Cu, As, Ni, Cr, Co and Cd, which are found at higher levels in PM2.5 than in ASD, might be involved in lung eosinophilia exacerbation. That PM is required for exacerbation of OVA-induced allergy might be due to transition metals in particles incorporated into cells. Further study to clarify the effects of metals in PM in cell culture experiments is in order. Our results indicate that transition metals in PM2.5 and desert dust are a significant risk factor for human respiratory health.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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