The effect of size-segregated ambient particulate matter on Th1/Th2-like immune responses in mice

Kuo-Liang Huang[1,2], Szu-Yuan Liu[2], Charles C. K. Chou[3], Yi-Hsin Lee[4], Tsun-Jen Cheng[2]*

1 Division of Pulmonary Medicine, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, New Taipei City, Taiwan, **2** Institute of Occupational Medicine and Industrial Hygiene, College of Public Health, National Taiwan University, Taipei, Taiwan, **3** Research Center for Environmental Change, Academia Sinica, Taipei, Taiwan, **4** Department of Pathology, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, New Taipei City, Taiwan

* tcheng@ntu.edu.tw

Abstract

Background

Particulate matter (PM) has been associated with increased pulmonary and cardiovascular mortality and morbidity. Additionally, PM is known to exacerbate asthma. However, whether ambient PM exposure contributes to the onset of asthma, especially in non-atopic children and adults, is less conclusive. The current study aimed to evaluate the effects of size-fractioned PM on lung immune responses in healthy BALB/c mice.

Methods and principal findings

We collected PM_{10}, $PM_{2.5}$, PM_1 and $PM_{0.1}$ samples from October 2012 to August 2013 in the Taipei Basin. These PM samples were representative of urban traffic pollution. The samples were extracted and sonicated in phosphate-buffered saline (PBS). Female BALB/c mice were exposed to the samples via intratracheal instillation at three different doses: 1.75 mg/kg (35 μg/per mouse), 5 mg/kg (100 μg/per mouse), and 12.5 mg/kg (250 μg/per mouse). The mice were exposed on days 0 and 7, and PBS alone was used as a control. Following the exposures, the expression profiles of inflammatory cells and cytokines in bronchoalveolar lavage fluid (BALF) were assessed. Exposure to PM_{10} resulted in inflammatory responses, including the recruitment of neutrophils and the induction of T helper 1 (Th1) cell-related cytokine release, such as TNF-α and IFN-γ. Furthermore, an allergic immune response, including the recruitment of eosinophils and the up-regulation of T helper 2 (Th2) cell-related cytokine release, such as IL-5 and IL-13, was also observed in the BALF of mice exposed to PM_{10}.

Conclusions

Our study showed that exposure to PM alone caused mixed Th1/Th2 inflammatory responses in healthy mice. These findings support the hypothesis that PM may contribute to the onset of asthma.

Introduction

Particulate matter (PM) is the major component of air pollution, which includes emissions from both anthropogenic and natural sources. Based on its aerodynamic diameter, PM is crudely categorized as coarse PM, which has an aerodynamic diameter of 2.5–10 μm; fine PM, which has an aerodynamic diameter of <2.5 μm; and ultrafine PM, which has an aerodynamic diameter of <0.1 μm. Fine PM is small enough to penetrate alveoli and terminal bronchioles, while coarse PM is primarily deposited in large conducting airways [1]. PM_{10} (aerodynamic diameter <10 μm) includes coarse, fine and ultrafine PM. The sizes and components of the PM depend on its source. For example, ultrafine PM is predominantly derived from primary combustion emissions or gas-to-particle conversion processes and consists of sulfates, nitrates, organic carbon (OC) and elemental carbon (EC). Coarse PM contributes the major proportion of total particle mass in smoke, soil from roads and construction sites. It can also contain sea salts, molds, dust mites, pollen and spores [2]. As industrialization and urbanization have increased, diesel exhaust particles (DEPs) have become a major source of ambient PM in modern cities. DEPs are composed of an elemental carbon core to which hundreds of chemicals and transition metals are attached [3]. Evidence suggests that PM is associated with increased pulmonary and cardiovascular morbidity and mortality [4–6].

Asthma is a chronic inflammatory disease of the airways and the most common chronic disease among children [7]. The World Health Organization (WHO) estimates that 1–2% of total health care expenditures in developed countries are associated with treating asthma [8]. The prevalence of asthma is also increasing in many countries, including Taiwan [9]. A study conducted by the International Study of Asthma and Allergies in Childhood (ISAAC) reported that the prevalence of asthma symptoms shows a high degree of global variation, even within genetically similar groups [10]. This suggests that environmental factors, such as allergens, viruses and PM, may be responsible for some of this variation.

Epidemiological reports have shown that PM can exacerbate asthma [11–13]. However, whether ambient PM exposure contributes to the onset of asthma, especially in non-atopic children and adults, is less conclusive. An epidemiological study showed that exposure to PM may adversely affect lung function development between the ages of 10 and 18 years [14]. Exposure to PM_{10} in utero and during the first year of life has also increased the risk of asthma development [15]. A cross-sectional epidemiological study conducted in Germany suggested that the composition of PM in the study area may have contributed to the high asthma prevalence there [16]. In a heavily industrialized province of China, investigators found that PM_{10} was related to asthma prevalence among children without an allergic predisposition [17]. In a prospective study of the World Trade Center (WTC) disaster, those who were exposed to WTC dust showed bronchial hyperactivity, persistent cough and increased risk of asthma [18]. A human challenge study found that inhaled coarse fraction PM may activate monocytic cells and potentially result in an allergic response in airways [19]. These studies suggest that long-term time-weighted averages of or short-term peak exposure to PM may be responsible for new-onset asthma.

Murine asthma models exhibit important features of allergic asthma, including the development of airway hyper-responsiveness, increased serum IgE levels, increased eosinophil counts and augmented T helper 2 cell (Th2) cytokine expression (e.g., IL-4, IL-5, and IL-13). Therefore, these models have been widely used to study the impact of environmental factors on asthma development [20]. For example, an ovalbumin (OVA)-induced mouse model of allergic airway disease was used to validate findings from an epidemiological study demonstrating that $PM_{2.5}$ composition may influence the severity of allergic airway inflammation both during sensitization and the challenge phase [21]. Li et al. [22] found that the inhalation of ambient

ultrafine particles enhanced the secondary immune response to OVA in sensitized mice, suggesting that exposure to vehicle exhaust can exacerbate allergic inflammation in asthmatic subjects. Most previous *in vivo* studies have addressed the adjuvant effects of PM on allergic airway inflammation [21–24], whereas few have investigated the pure PM effect. We hypothesized that repeated exposure to ambient PM without additional OVA treatment would promote allergic airway inflammation.

In this study, we used a murine intratracheal sensitization model and different doses of size-fractionated PM collected from air samplers in the Taipei Basin to determine how PM contributes to the development of Th2 immune responses in healthy mice.

Materials and methods

PM collection and preparation

PM samples were collected at the Atmospheric Science Building of the National Taiwan University (NTU) (25.0˚N, 121.5˚E) in Taipei, Taiwan from October 2012 to August 2013. The NTU is located in the downtown area of the Taipei Basin and therefore provides representative data for the PM that exists in a subtropical urban area. PQ200 Air Samplers (Model PQ200, BGI Inc., MA, USA) were used to collect PM_{10}, $PM_{2.5}$, and PM_1 (aerodynamic diameter <1.0 µm) samples at a flow rate of 16.67 L/min. MOUDIs (Model 110, MSP Corp., MN, USA) were used to collect $PM_{0.1}$ samples at a flow rate of 30.0 L/min in the sampling campaigns. Two aerosol samples were collected for each condition on polytetrafluoroethylene (PTFE) and quartz fiber filters. The sampling period for each condition was 22 h: from 13:00 LST to 11:00 LST the next day. The PTFE samples were used for animal studies, and the quartz filter samples were used for composition analysis.

Chemical characterization of PM samples. All quartz filters were pre-heated at 900˚C for 3 h and then stored in aluminum foil before sampling. After sampling, the filters were immediately placed in a cooler packed with ice and transported to the laboratory within 30 min. The samples were then stored in a freezer at -18˚C until OC/EC analysis, which occurred within 3 days. All loaded filters for PM_{10}, $PM_{2.5}$, PM_1 and $PM_{0.1}$ were analyzed for OC and EC content using a DRI Model 2001 Thermal/Optical Carbon Analyzer (Atmoslytic Inc., Calabasas, CA, USA). Additionally, the following ion species were analyzed using an ion chromatograph (IC, Model DX-120, Dionex Corp, Sunnyvale, CA): Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NH_4^+, NO_3^-, PO_4^{3-} and SO_4^{2-}. Endotoxin levels in PBS-eluted PM samples collected on PTFE filters were determined using a Limulus amebocyte lysate assay according to the manufacturer's instructions (QCL-1000, CAPE COD, USA).

Preparation of PM samples for animal studies. Pre-weighed PTFE filters stored at −18˚C were acclimated to room temperature (16–20˚C, 30–40% relative humidity) prior to the elution of PM. The filters were placed into labeled 15 ml conical vials. A 1 ml aliquot of phosphate-buffered saline (PBS) was added to each conical vial. Elution was performed in water with water bath sonication for 30 min. The filters were weighed before and after elution using an analytical balance. The eluted contents were pooled into one vial and then diluted in PBS to final concentrations of 35 µg/100 µL, 100 µg/100 µL, and 250 µg/100 µL. All eluted PM samples were stored as aliquots at −80˚C until 30 min prior to experimentation.

Animals

Eight-week-old female BALB/c mice (~20 g each) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and maintained under a 12 h light/dark cycle at a constant temperature of 22 ± 2˚C and 55 ± 10% relative humidity throughout the study. The animals were housed in plastic cages and had access to LabDiet 5001 food (PMI Nutrition International,

Brentwood, MO, USA) and water *ad libitum*. The animals were acclimated for one week prior to the initiation of the study. The care and handling of the animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at National Taiwan University. This study was approved by the Institutional Animal Care and Use Committee of NTU (Permit Number: 20110508).

Experimental design

The mice (10–15 animals per group) were divided into the following five groups: PBS, $PM_{0.1}$, PM_1, $PM_{2.5}$, and PM_{10}. Each group was further divided based on treatment dose; three different doses were evaluated: 1.75 mg/kg (low dose, 35 μg/per mouse), 5 mg/kg (medium dose, 100 μg/per mouse) and 12.5 mg/kg (high dose, 250 μg/per mouse). We referred to previous PM studies to determine the exposure doses [25,26]. Due to the limited amount of PM collected, a high-dose group was not established for $PM_{0.1}$. On days 0 and 7, the mice were exposed to 100 μL of the appropriate solution by intratracheal instillation (IT) under light anesthesia (Sevoflurane; Abbott Laboratories, UK). Briefly, each mouse was placed in a supine position at an elevation of 45–60 degrees, and its mouth was opened by hanging its incisors on a small wire. The solution was then instilled into the trachea via gavage needle under a laryngoscope. After the instillation, the mouse was closely observed until it fully recovered to ensure that suffocation was avoided. The animals were sacrificed 3 days after the second treatment. Blood, bronchoalveolar lavage fluid (BALF) and lung samples were collected (Fig 1). To achieve the minimum amount of experimental animals, we measured eosinophils and Th2 cytokines in the lung lavage fluid at same time because they play major roles in allergic airway inflammation. The protocol was modified from our previous study, which showed that the Th2 cytokine levels increased at 24 h, and eosinophilia peaked at 7 days in the BALF after ZnO nanoparticle exposure [27]. We conducted these independent experiments at intervals of 1 month apart (20–25 mice per independent experiment).

Determination of total serum IgE and IL-6 levels. We obtained blood samples from the facial veins of the mice. Total serum IgE was measured using a Mouse IgE ELISA Kit (BD Biosciences, San Jose, CA, USA), and serum IL-6 concentrations were measured using mouse-specific IL-6 ELISAs (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Animal sacrifice, sample collection and analysis. The animals were sacrificed using an overdose of sodium pentobarbital (0.1 ml at 200 mg/ml) administered via intraperitoneal injection. Samples of BALF and left lung tissue were collected. Then, BALF was prepared for the subsequent analysis. The total protein concentration was determined using a total protein assay kit (Bio-Rad Hercules, CA, USA). Total cell counts were determined from fresh fluid
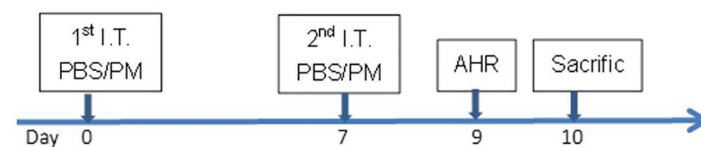

Fig 1. Overview of the experimental design used to expose mice to PM. Mice (10–15 animals per group) were divided into the following five groups: PBS, $PM_{0.1}$, PM_1, $PM_{2.5}$, and PM_{10}. Each group was further divided according to dose: either two or three doses of 1.75 mg/kg (35 μg/per mouse), 5 mg/kg (100 μg/per mouse) or 12.5 mg/kg (250 μg/per mouse) were administered. On days 0 and 7, the mice were exposed to 100 μL of the appropriate solution by intratracheal instillation under light anesthesia. The animals were sacrificed 3 days after the second treatment, and samples of blood, BALF and lung were collected.

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specimens using a hemocytometer after trypan blue staining. Differential cell counts were measured on cytological slides with Liu's stain (Tonyar Biotech, Taiwan). Inflammatory cytokines in the BALF were measured using a Cytometric Bead Array Mouse Enhanced Sensitivity Flex Set system (BD BioSciences, San Diego, CA, USA). The detailed procedures were previously described by us [27].

Lung histology. The left lung lobes of the mice were excised, fixed with 10% neutral phosphate-buffered formalin, embedded in paraffin, sectioned at 5-μm thickness and stained with hematoxylin and eosin (H&E). The histological samples were examined in a blinded manner under a light microscope by an experienced histopathologist.

Statistical analysis

All animal study results are expressed as the mean ± SEM. Differences between groups were evaluated by analysis of variance (ANOVA), and Turkey's post hoc t-test for multiple comparisons was used to distinguish between pairs of groups. Relationships between PM constituents and inflammatory variables of all tested doses were analyzed using Spearman's rank correlation test. All statistical analyses in this study were performed with SAS software (SAS Institute, Cary. NC, USA), version 9.3. The level of statistical significance was set at $p < 0.05$.

Results

Characterization of PM

The chemical mass constituents found in the size-segregated ambient PM samples collected between the 2012 winter and 2013 summer seasons are shown in Table 1. Sulfate was identified as the major constituent in the samples, accounting for 25.65% of the PM_{10} mass, followed by OC, NH_4^+ and NO_3^-. The $PM_{2.5}$ and PM_1 samples showed similar mass proportions of major constituents to those found in PM_{10}. OC and EC contents were highly enriched in the $PM_{0.1}$ sample. The characteristics and compositions of PM samples collected at a nearby central air monitor station in Taipei Basin have been previously reported [28]. In general, soil dust was the major source of PM_{10}, followed by vehicle emissions, secondary aerosols, sea salts and industrial emissions. For $PM_{2.5}$, vehicle emissions were the major aerosols, followed by

Table 1. Chemical mass constituents in size-segregated ambient PM collected between the 2012 winter and 2013 summer seasons.

	PM0.1	PM1	PM2.5	PM10
Collected mass (mg)	1.87	19.48	26.17	40.88
Ionic components (μg/mg)				
Na^+	3.44	3.84	13.72	31.73
K^+	6.92	13.19	12.67	10.78
Mg^{2+}	1.33	0.59	2.08	4.92
Ca^{2+}	7.87	1.49	3.34	14.68
Cl^-	5.12	2.78	9.77	18.90
NH_4^+	11.31	125.34	119.93	75.76
NO_3^-	17.46	49.37	87.91	96.19
PO_4^{3-}	7.47	1.37	1.68	1.45
SO_4^{2-}	45.28	349.90	318.69	240.19
Carbon species				
OC (μg/mg)	314.02	120.51	166.74	126.14
EC (μg/mg)	47.53	53.05	46.31	41.60
Endotoxin (EU/ml)	62.44	53.66	55.05	122.27

Table 2. Serum total IgE, serum IL-6 and total protein in BALF.

	N	Serum IgE (ng/ml)	Serum IL-6 (pg/ml)	Total Protein (mg/ml)
PBS	5	676.4 (±220.8)	5.41 (±0.64)	0.28 (±0.02)
Low dose PM				
$PM_{0.1}$ 35 µg	5	968.0 (±311.9)	6.68 (±1.16)	0.30 (±0.03)
$PM_{1.0}$ 35 µg	5	575.1 (±165.7)	6.43 (±0.65)	0.31 (±0.04)
$PM_{2.5}$ 35 µg	5	424.7 (±113.1)	6.41 (±0.91)	0.27 (±0.03)
PM_{10} 35 µg	5	513.9 (±164.5)	6.09 (±0.43)	0.23 (±0.02)
Medium dose PM				
$PM_{0.1}$ 100 µg	5	630.6 (±98.7)	6.58 (±1.04)	0.31 (±0.03)
$PM_{1.0}$ 100 µg	5	419.0 (±64.5)	6.64 (±1.29)	0.35 (±0.05)
$PM_{2.5}$ 100 µg	5	445.9 (±60.2)	5.97 (±0.95)	0.33 (±0.04)
PM_{10} 100 µg	5	547.4 (±118.5)	5.42 (±1.23)	0.29 (±0.05)
High dose PM				
$PM_{1.0}$ 250 µg	5	440.7 (±95.5)	8.27 (±1.46)	0.22 (±0.01)
$PM_{2.5}$ 250 µg	5	465.9 (±87.5)	6.83 (±0.98)	0.34 (±0.03)
PM_{10} 250 µg	5	406.9 (±64.1)	5.64 (±0.94)	0.34 (±0.02)

The mice were exposed to 100 µL solutions by intratracheal instillation on days 0 and 7 as described in the Materials and Methods section.

The data are presented as the mean ± SEM.

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industrial emissions and secondary aerosols. Secondary aerosols and vehicle emissions were the major sources of $PM_{0.1}$. The highest endotoxin levels were detected in the PM_{10} samples.

Serum total IgE, serum IL-6 and total protein in BALF

Total serum IgE and IL-6 levels did not significantly increase following the intratracheal instillation of different doses or different sizes of PM. BALF total protein content did not significantly differ between any of the treatment groups (Table 2).

Inflammatory cell profiles in BALF

BALF inflammatory cell profiles were analyzed three days after the second PM exposure. Neutrophils were significantly increased only in the group exposed to the medium dose of PM_{10}. Eosinophils were significantly increased in the medium and high dose PM_{10} exposure groups (Table 3). However, the percentage of neutrophils was significantly increased in the mice treated with a high dose of $PM_{2.5}$ and medium and high doses of PM_{10}. The percentage of lymphocytes was significantly increased in the mice treated with a medium dose of PM_1 and high doses of PM_1, $PM_{2.5}$, and PM_{10}. The percentage of eosinophils was only significantly elevated in the BALF samples of the mice exposed to medium and high doses of PM_{10} (Fig 2).

Cytokine expression profiles in BALF

We next determined the cytokine expression profiles in the BALF samples (Fig 3). We found significantly increased levels of IL-5, IL-13, IL-17A, IL-6, TNF-α and IFN-γ in the high-dose PM_{10} treatment group compared to the control and other treatment groups. TNF-α levels also increased after exposure to a medium dose of PM_{10}.

Table 3. Inflammatory cell profiles in BALF following the 2nd exposure to PM.

Treatment	Total cell	Neutrophil	Macrophage	Lymphocyte	Eosinophil
Control	29.6 (±9.2)	0.59 (±0.38)	28.0 (±8.6)	0.95 (±0.33)	0 (±0)
Low dose PM					
$PM_{0.1}$ 35 µg	36.8 (±6.4)	0.32 (±0.06)	34.5 (±6.1)	1.8 (±0.3)	0.14 (±0.09)
$PM_{1.0}$ 35 µg	35.8 (±15.4)	0.23 (±0.09)	34.5 (±14.7)	1.1 (±0.6)	0 (±0)
$PM_{2.5}$ 35 µg	24.9 (±7.4)	0.48 (±0.11)	23.6 (±7.1)	0.9 (±0.2)	0 (±0)
PM_{10} 35 µg	49.8 (±6.1)	4.61 (±1.63)	43.5 (±5.7)	3.5 (±1.4)	0 (±0)
Medium dose PM					
$PM_{0.1}$ 100 µg	62.0 (±12.9)	5.11 (±1.60)	53.0 (±10.8)	3.6 (±0.9)	0.32 (±0.08)
$PM_{1.0}$ 100 µg	26.5 (±2.1)	1.43 (±0.41)	22.6 (±2.0)	2.5 (±0.4)	0.08 (±0.03)
$PM_{2.5}$ 100 µg	43.7 (±10.2)	4.66 (±1.06)	35.6 (±8.5)	2.9 (±0.7)	0.30 (±0.10)
PM_{10} 100 µg	65.9 (±9.0)	13.08 (±1.25)* #	45.2 (±6.8)	4.6 (±1.1)*	3.01 (±0.46)* #
High dose PM					
$PM_{1.0}$ 250 µg	16.6 (±4.4)	1.28 (±0.73)	13.7 (±3.7)	1.5 (±0.4)	0 (±0.0)
$PM_{2.5}$ 250 µg	25.5 (±6.7)	6.83 (±4.28)	16.3 (±2.5)	2.1 (±0.4)	0.19 (±0.06)
PM_{10} 250 µg	28.3 (±6.1)	6.43 (±0.92)	18.2 (±4.3)	2.5 (±0.7)	1.03 (±0.31)* #

The mice were exposed to 100 µL aliquots of solutions by intratracheal instillation on days 0 and 7 as described in the *Materials and Methods* section. The data are presented as the mean ± SEM (10^4/ml).

*$p < 0.05$ compared to the PBS group

#$p < 0.05$ compared to the other groups at the same dose (n = 5 for each treatment group)

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Histological analysis

Representative images of H&E-stained lung tissues are shown in Fig 4. Exposure to a high dose of PM_{10} resulted in focal infiltration of neutrophils, lymphoplasma cells and foamy histiocytes. Exposure to a medium dose of PM_{10} resulted in focal infiltration of lymphocytes and foamy histiocytes into the alveolar space. Exposure to a high dose of $PM_{2.5}$ also resulted in focal aggregation of lymphocytes and foamy histiocytes in the alveolar space. The histological changes were minimal in the other exposure groups.

Associations between PM constituents and inflammatory responses

The coefficients for the correlations between the constituents of the PM samples and the measured inflammatory responses are shown in Table 4. Several ions (Mg^{2+}, Ca^{2+}, and Cl^-) had statistically significant positive correlations with elevated percentages of neutrophils, eosinophils and inflammatory cytokines (TNF-α, IFN-γ, and IL-5). Endotoxin levels were also positively associated with elevated percentages of neutrophils and eosinophils as well as augmented TNF-α expression in BALF.

Discussion

In this study, we evaluated the effects of PM on the sensitization phase of a murine asthma model. For these experiments, on day 0 and day 7, mice were administered either PBS or different doses of $PM_{0.1}$, PM_1, $PM_{2.5}$ and PM_{10} samples for allergic sensitization. We found that the percentages of neutrophils in BALF were significantly increased in the mice that were treated with a high dose of $PM_{2.5}$ and medium and high doses of PM_{10}. Furthermore, increased levels of TNF-α, IFN-γ, IL-5, IL-13, IL-17A, and IL-6 were found in the BALF samples of the mice that underwent high-dose PM_{10} exposure compared to those of the control and other treatment groups. BALF analysis also showed that exposure to high doses of PM_{10} alone led to elevated
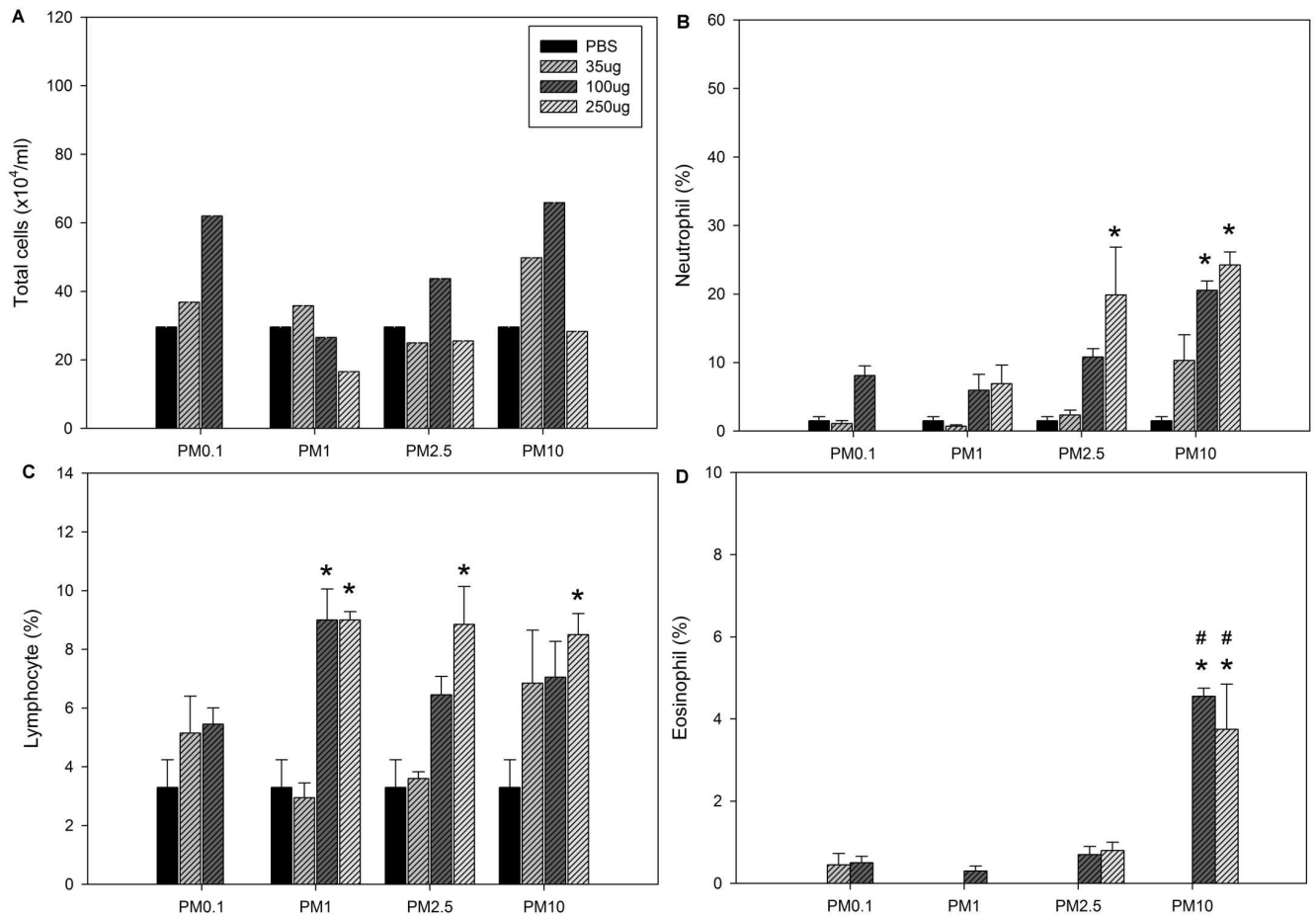
Fig 2. Inflammatory cell profiles in BALF following the 2nd exposure to PM. A: Total cells. B: Neutrophils. C: Lymphocytes. D: Eosinophils. The mice were exposed to 100 μL aliquots of solutions by intratracheal instillation on days 0 and 7 as described in the *Materials and Methods* section. The data are presented as the mean ± SEM (n = 5 per treatment group). Neutrophil, lymphocyte and eosinophil counts are expressed as percentages of total cell counts. *$p<0.05$ compared to the PBS group; #$p<0.05$ compared to the other groups at the same dose.

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numbers and percentages of eosinophils and increased inflammatory cytokine expression (IL-5 and IL-13). Extensive testing of individual $PM_{2.5}$, PM_1 and $PM_{0.1}$ samples did not reveal an effect on eosinophilic inflammation.

Our results showed that neutrophils and expression levels of inflammatory cytokines (TNF-α, IFN-γ, and IL-6) in BALF were significantly increased in mice treated with high doses of PM_{10} compared with those of the control and other treatment groups. Several epidemiological studies have suggested that coarse PM has a similar or stronger short-term effect on respiratory diseases compared to that of fine PM [29]. A previous study investigating healthy mice submitted to oropharyngeal exposure to PM of different sizes collected from six US cities at doses of 25 μg and 100 μg also showed that coarse PM more potently induced pulmonary inflammation than that of fine or ultrafine PM [30]. Happo et al. [31] also found that coarse PM and $PM_{2.5-1}$ had substantially higher inflammatory potency than PM_1. Another similar study that compared size-fractioned PM toxicity in samples collected near and far from urban highways showed that coarse PM produced more significant pulmonary inflammation than that of fine and ultrafine PM on a comparative mass basis [25]. Our results (e.g., increased neutrophilia and elevation of IL-6 and TNF-α in BALF) were similar to those reported in the above-cited
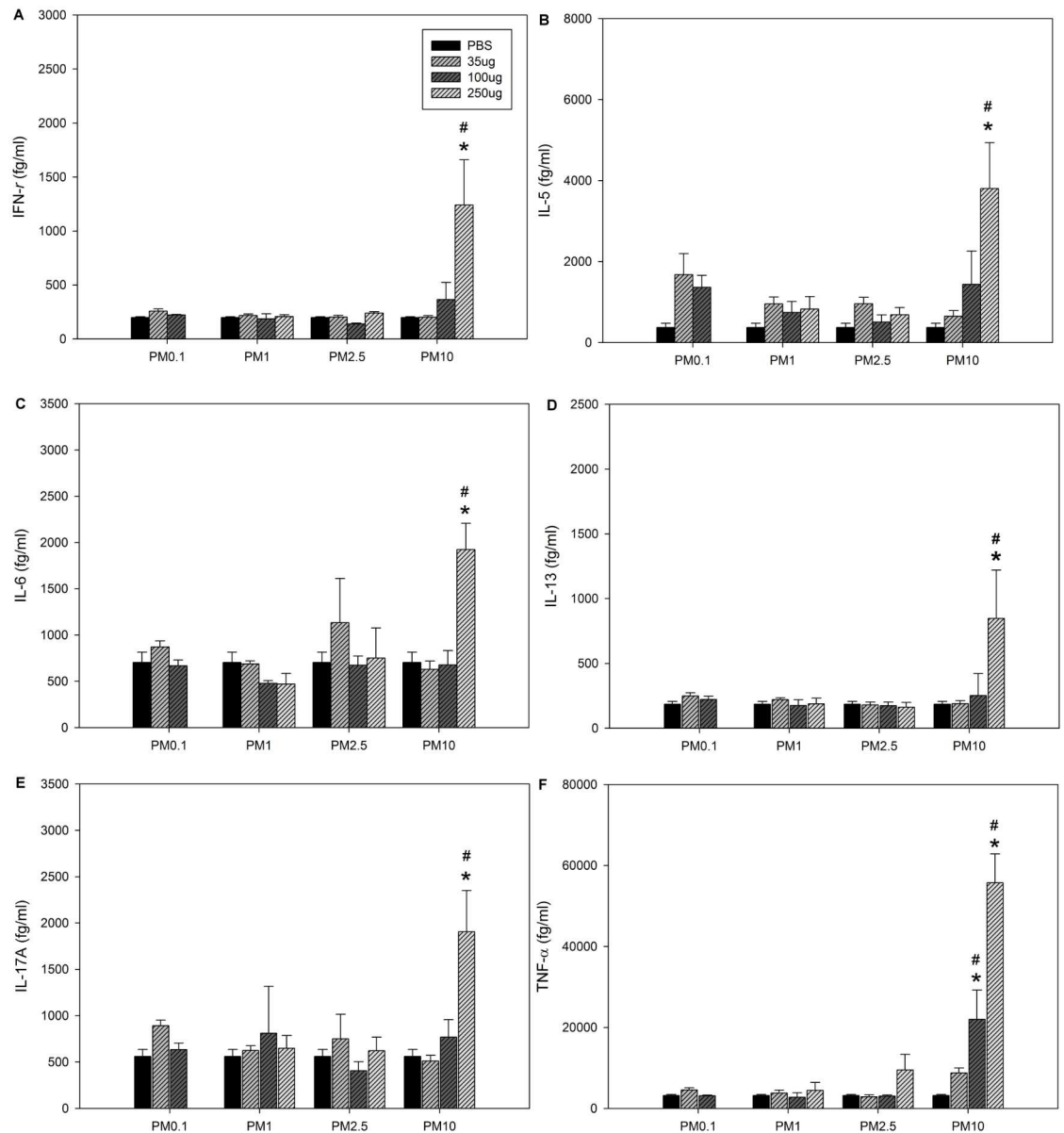
Fig 3. Cytokine levels in BALF following the 2nd exposure to PM. A: IFN-γ. B: IL-5. C: IL-6. D: IL-13. E: IL-17A. F: TNF-α. The mice were exposed to 100 μL aliquots of solutions by intratracheal instillation on days 0 and 7 as described in the *Materials and Methods* section. PBS served as a vehicle control. The data are presented as the mean ± SEM (n = 5 per treatment group). *$p < 0.05$ compared to the PBS control group; #$p < 0.05$ compared to the other groups at the same dose.

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studies and suggest that PM_{10} exerts a greater inflammatory effect than that of $PM_{2.5}$ on a mass basis. The findings that PM_{10} induced the greatest degree of airway inflammation may be attributed to enriched metal constituents in PM_{10} [32], more biogenic materials with the highest endotoxin contents [31], or chemical contents adhered to PM_{10} [33]. However, many types of combined effects may take place due to complex urban PM.

Our results demonstrated that an allergic immune response, including the recruitment of eosinophils (approximately 4% of total inflammatory cells) and the up-regulation of Th2 cell-related cytokine release, such as IL-5 and IL-13, was observed in the BALF of the mice that
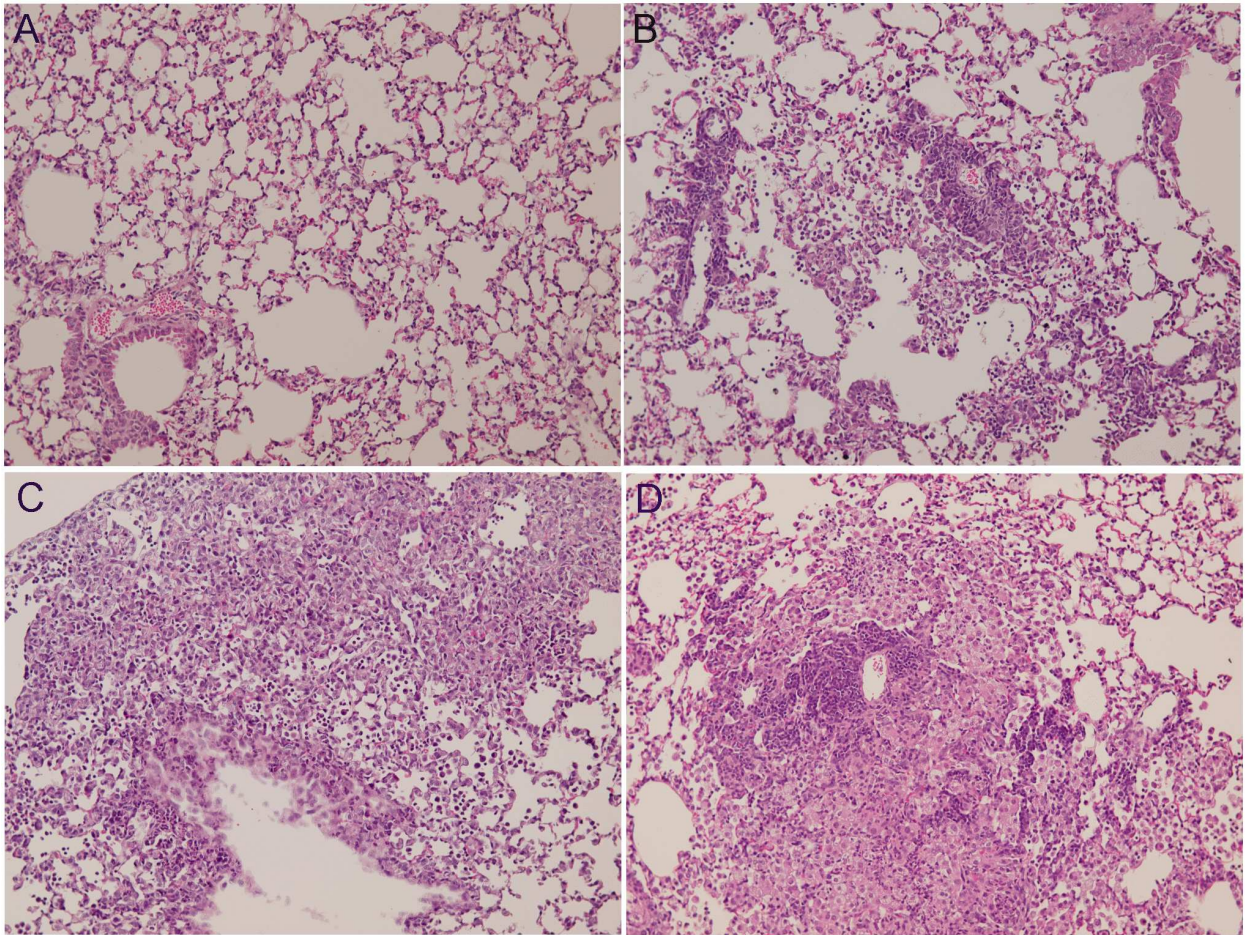
Fig 4. Representative histology of lung tissue (200X) 3 days after the second intratracheal instillation of PM. Lungs were excised and fixed with formaldehyde, sectioned in 5-μm-thick slices, and the slides were stained with H&E. (A) Vehicle (VEH) control tissue. (B) Tissue exposed to high-dose $PM_{2.5}$ showing focal aggregation of lymphocytes and foamy histiocytes in the alveolar space. (C) Tissue exposed to medium-dose PM_{10} showing focal infiltration of lymphocytes and foamy histiocytes into the alveolar space. (D) Tissue exposed to high-dose PM_{10} showing focal infiltration of neutrophils, lymphoplasma cells and foamy histiocytes.

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were exposed to PM_{10}. Although typical models of allergic airway inflammation generally demonstrate much higher percentages of airway eosinophilia, the mice were exposed to PM only in the sensitization phase without further allergen challenge. Additionally, typical allergens (OVA) or adjuvants (aluminum hydroxide) were not used in our experiment. It has previously been shown that a single oropharyngeal exposure to ambient PM (mean hydrodynamic diameter: 0.4 μm) collected from Baltimore city air induced eosinophilic infiltration (<2% of inflammatory cell) in the BALF of mice. In the same study, ambient PM was also shown to activate myeloid dendritic cells (mDCs) and further drive Th2 cytokine responses in naïve T cells [34]. The repeated intratracheal instillation of healthy mice with PM samples from urban Baltimore (aerodynamic diameter <0.85 μm, 0.5 mg in 50 μL PBS) induced airway hyper-responsiveness, small but significant elevations in eosinophil counts in BALF, and increased Th2 expression of IL-17A, IL-5, and IL-13 cytokines in lung cells but no increase in total serum IgE levels [26]. Immature dendritic cells (DCs) are highly effective at taking up foreign bodies and directing the primary immune response [35]. An *in vitro* study showed that exposure to ambient PM enhanced DC activation and that co-cultures of ambient PM-stimulated DCs with

Table 4. Coefficients for the correlations between the constituents in the PM samples and the measured inflammatory responses.

	Inflammatory biomarker						
	Neutro	**Eosino**	**IFN-γ**	**TNF-α**	**IL-5**	**IL-6**	**IL-17A**
Na⁺	0.663**	0.809**	0.398**	0.544**	0.309*	0.217	0.331**
NH₄⁺	0.348**	0.276*	0.143	-0.035	0.026	-0.172	0.099
K⁺	0.163	0.218	0.181	-0.153	0.198	-0.119	0.178
Mg²⁺	0.562**	0.73**	0.424**	0.429**	0.427**	0.264*	0.366**
Ca²⁺	0.54**	0.672**	0.404**	0.467**	0.423**	0.244	0.335**
Cl⁻	0.569**	0.743**	0.417**	0.448**	0.433**	0.269*	0.372**
NO₃⁻	0.446**	0.666**	0.314**	0.342**	0.285*	0.224	0.291*
SO₄²⁻	0.394**	0.309*	0.145	-0.042	0.037	-0.176	0.104
OC	0.144	0.313*	0.094	-0.154	0.191	-0.081	0.142
EC	0.102	0.259*	0.225	-0.113	0.25	-0.04	0.218
Endo	0.796**	0.6**	0.211	0.426**	-0.001	-0.057	0.109

The values are represented as Spearman's correlation coefficients.

**p<0.01

*p<0.05

Neutro, percentage of neutrophil; Eosino, percentage of eosinophil; Endo, endotoxin.

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alloreactive naïve CD4+ T cells produced Th2-like patterns of cytokine production [36]. A recent in vitro study reported that a co-culture of human myeloid DCs with PM can stimulate autologous human memory CD4 T cells (Tms) to secrete IFN-γ and IL-13 and drive the expansion and differentiation of a mixed population of Th1, Th2 and Th17 effector memory cells [37]. Although the exposure doses, sizes and compositions of the PM samples were different, our observations together with previous studies indicate that exposure to ambient PM can induce allergic airway inflammation.

The inflammatory pathways involved in the development of asthma are complex, and eosinophils play a central role in the pathogenesis of asthma. Eosinophils increase vascular permeability, epithelial damage, mucus hypersecretion, and smooth muscle constriction through their release of granule-associated basic proteins, lipid mediators and reactive oxygen species (ROS) [38]. IL-5 is an important mediator of eosinophil proliferation, differentiation, maturation, migration, and survival in response to environmental stimuli [39]. IL-13 activates macrophages and eosinophils to accelerate inflammation and stimulates B-cells to produce IgE antibodies. IL-13 also contributes to airway hyper-responsiveness and airway remodeling through epithelial cell hypersecretion, subepithelial fibroblast proliferation and smooth muscle hypertrophy in the airway [40]. IL-17A may induce epithelial structural changes and smooth muscle contraction; the presence of IL-17A in BALF has been associated with asthma severity in clinical studies. An association between IL-17A and neutrophilic airway inflammation has also been established in murine asthma models. Collectively, these results suggest that IL-17A plays a central role in the pathophysiologies of certain asthma phenotypes [41].

PMs are composed of carbon cores and a number of soluble and insoluble components, including acids, organic chemicals, metals, endotoxin, pollen or fungi debris on their surface. Thus, the underlying mechanisms by which PM exerts biological effects are complex. Various allergens, such as fungal spores and pollen, are more efficiently carried to the airways when they are constituents of PM [42,43]. PM exposure may impact many cell types at different levels of immune regulation. A Th2-dominant immune response plays a central role in allergic airway inflammation; however, innate immunity, such as that via dendritic cell and epithelial

cell responses, was also important in disease expression [44]. PM has been shown to stimulate innate immune responses through airway epithelial cells and dendritic cells [45]. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is expressed in a variety of tissues. The activation of the AhR may regulate gene expressions involving detoxication enzymes (CYP1A1 and CYP1B1), as well as inflammatory (TNF-α and IL-6) and T-cell differentiation (Foxp3, IL-17 and GATA3) genes [46]. Polycyclic aromatic hydrocarbons (PAHs) contained in the PMs, common AhR agonists, have been shown to enhance Th17 cell differentiation and IL-17 secretion through the aryl hydrocarbon receptor, indicating that PM can directly modulate T cell function [47]. An *in vitro* study also showed that exposure to ambient PM enhanced DC activation and that co-cultures of ambient PM-stimulated DCs with alloreactive naïve CD4+ T cells produced Th2-like patterns of cytokine production [25,31]. The innate immune cells contain pattern recognition receptors (PRRs), including toll-like receptors (TLRs), nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), among others [48]. These receptors detect exogenous or endogenous toxic substances by priming and activating the inflammasome, a cytosolic protein complex that can trigger the maturation of pro-inflammatory chemokines and cytokines and assist the host in regulating the balance between tissue repair and inflammation [49]. PM_{10} has been shown to activate an NLRP3 inflammasome/IL-1 receptor axis involving IL-1β, CCL-20 and GM-CSF production, which causes dendritic cell activation and neutrophilia [50]. Additionally, the production of oxidative stress (either the direct induction of reactive oxygen species attributed to organic and metal components of the PM or the secondary induction of local cellular responses after PM exposure) induced the activation of the mitogen-activated protein (MAP) kinase cascade and NF-κB transcription factors, which control the activity of genes involved in cellular activation and inflammation and appear to be fundamental to the PM-induced immunoregulatory effect [51].

As the size of particles decreases, the total surface area increases. Thus, the eosinophilic inflammatory effect of ultrafine particles was reported to be greater than that of fine particles [24]. In our study, PM_{10} induced a more prominent eosinophilic inflammatory response than that of $PM_{2.5}$ or $PM_{0.1}$. Our findings suggest that the compositions of PM may also result in differences. We found that PM_{10} had a higher endotoxin level than that of fine and ultrafine PM and that the endotoxin level was associated with neutrophilic inflammation. Our findings were consistent with those of previous PM studies [25,31]. The underlying mechanism of endotoxin-induced inflammation involves the detection of endotoxin by a complex of CD14, Toll-like receptor 4 (TLR4) and MD-2. The recognition of endotoxin leads to the activation of NF-κB family transcription factors and mitogen-activated protein kinases pathways of innate immune cells, which directs the expression of proinflammatory cytokines, chemokines and adhesion molecules [52,53]. However, the causal relationship between endotoxin and eosinophilic airway inflammation and Th2 immune response are more complex. According to the 'hygiene hypothesis', the decreasing incidence of infections is associated with an increasing incidence of allergic disease [54]. However, epidemiological evidence has indicated that endotoxin exposure is a risk factor for increased asthma prevalence [55,56], and animal studies of the immunomodulatory effects of endotoxin have yielded different and sometimes conflicting results, as reviewed by Zhu et al [57]. Several investigators demonstrated that low-dose endotoxin with OVA induced Th2 immune responses, whereas high-dose endotoxin with OVA induced Th1 inflammation [58]. Our current results showed that endotoxin level was positively associated with elevated percentages of neutrophils and eosinophils as well as increased expression of TNF-α in BALF. It is possible that the dose, route and timing of endotoxin exposure may determine subsequent inflammatory responses.

In the current study, we found that select PM constituents showed statistical correlations with inflammatory responses. However, PM constituents from the same sources are also complex, and they may interact with each other. Therefore, it is difficult to determine the causal relationship between any single component that is significantly associated with inflammation; as such, investigations into the relationships that exist between particle sources and health effects should be carried out [59].

Taken together, our results indicate that ambient PM can induce Th1/Th2/Th17 inflammatory responses through different additive or synergistic mechanisms driven both by individual compounds and combinations of compounds.

Conclusions

Due to wide variations in the sources and compositions of PM as well as the different PM exposure protocols used in the previous animal studies, there is a lack of consistent findings regarding the physiological effects of PM in the airway. In the current study, we found that exposure to PM increased inflammatory responses, including augmented neutrophil infiltration and increased TNF-α and IFN-γ production, as well as allergic immune responses, including augmented eosinophil infiltration and increased production of Th2-related cytokines (IL-5 and IL-13), in the BALF of mice that were repeatedly exposed to PM_{10} in the absence of an additional OVA. Our results further indicate that ambient PM exposure may increase asthma morbidity.

Supporting information

S1 File. Inflammatory cell profiles in BALF.
(XLS)

S2 File. Cytokine levels in BALF.
(XLS)

Acknowledgments

Author Contributions

Conceptualization: KLH CCKC TJC.

Data curation: KLH SYL YHL CCKC TJC.

Formal analysis: KLH SYL YHL CCKC TJC.

Funding acquisition: CCKC TJC.

Investigation: KLH SYL CCKC TJC.

Methodology: KLH SYL CCKC TJC.

Project administration: KLH SYL CCKC TJC.

Resources: KLH SYL YHL CCKC TJC.

Software: KLH v CCKC.

Supervision: CCKC TJC.

Validation: KLH SYL CCKC TJC.

Visualization: KLH SYL TJC.

Writing – original draft: KLH SYL.

Writing – review & editing: KLH TJC.

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