

Pulmonary Health Effects Following Repeated Exposure and Cessation to Wintertime Particulate Matter from California and China

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Abstract

Background: Epidemiological studies show a strong association between fine particulate matter (PM 2.5) air pollution and adverse pulmonary effects. While PM concentration can vary by time and location, PM toxicity has been most recently linked to both physicochemical composition and exposure scenario. To study the relevance of particle characteristics to toxicity, winter PM 2.5 samples were obtained from three geographically similar regions (Sacramento, California, USA; Jinan, Shandong, China; and Taiyuan, Shanxi, China), with typically high atmospheric PM 2.5 emissions. PM extract samples (PM CA, PM SD, and PM SX, respectively) were administered by oropharyngeal aspiration (OPA) to different groups of BALB/C mice, at equal mass concentrations [0 (water vehicle control only) or 20 µg/50 µL], on five different occasions over a two-week period, for a cumulative PM dose of 0 or 100 µg/mouse. Mice were necropsied on Days 1, 2 and 4 after the final exposure, and pulmonary effects were evaluated by bronchoalveolar lavage (BAL), histopathology, quantitative polymerase chain reaction tests, and enzyme-linked immunosorbent assays.

Results: Unique differences were noted in the chemical composition for each geographic region with PM SX containing the highest concentration of sulfates (organic and inorganic). A systematic examination of the time lag effects of repeated PM exposure demonstrated unique differences. In mice administered PM SX versus the control, BAL neutrophilia, alveolitis, and bronchiolitis were observed on Days 1 and 4. By Day 4, PM SX-exposed mice also exhibited increased gene expression for multiple inflammatory cytokines/chemokines (interleukin 1 beta, tumor necrosis factor alpha, chemokine C-X-C motif ligands-3 and -5), and increased levels of monocyte chemoattractant protein-1 relative to control-, PM CA-, or PM SD-exposed mice.

Conclusions: Direct comparison of the toxic effects of three geographically different PM samples on an equal mass basis demonstrate unique pathobiology with increasing lag time post-exposure. Higher sulfate levels in PM SX versus PM CA and PM SD may contribute to the greater inflammatory responses noted that progressed over time.

1. Background

Fine particulate matter (particles with an aerodynamic diameter < 2.5 µm; PM_{2.5}) air pollution is a growing universal health concern causing 4.2 million premature deaths worldwide in 2016. Physicochemical characteristics of PM can be highly variable due to different emission sources, weather conditions, and interactions with other solid/liquid and organic/inorganic particles suspended in the atmosphere [1].

Particles originating from different sources, locations and times can exhibit unique levels of toxicity associated with cardiorespiratory effects, such as asthma and COPD [2]. Particle size can also greatly influence health outcomes, although coarse PM (particles with a diameter between 10 and 2.5 µm; PM_{10-2.5}) is known to irritate the eyes and nose, ambient PM_{2.5} has greater potential for harmful health effects due to its ability to penetrate beyond the air passages of the upper respiratory tract and deposit in the distal lung and alveoli where gas exchange occurs [3, 4]. With clearance from the respiratory tract via mucociliary transport, lymphatic drainage, or systemic blood circulation, PM_{2.5} can also bind to cells and tissues to affect extra-pulmonary organs [5, 6].

To compare the biological effects of PM_{2.5} from different geographic locations, PM samples were collected from cities in California (Sacramento) and China (Jinan and Taiyuan). These cities were chosen due to their heavy urbanization, industrial and agricultural economies, moderately dry and sunny winters, and long histories of relatively high PM_{2.5} levels, especially during the winter season when meteorological inversions are more frequent and longer in duration [7, 8]. California's Central Valley typically has high levels of air pollution due to its geologic bowl shape which leads to the retention and accumulation of PM from vehicular exhaust, local farming, and industrial businesses [9]. Previous studies have shown that lung disease-related emergency department visits increase with PM_{2.5} concentrations [10, 11]. Comparison of the 2014 active asthma prevalence in Sacramento County and the state of California among individuals ages 18–64 showed a higher prevalence in the former (12%) versus the latter (7.6%) when active asthma is defined as having been diagnosed by a professional and afflicted by an asthma attack within the preceding year [12]. The capital cities of Jinan and Taiyuan in the Chinese provinces of Shandong and Shanxi, respectively, represent regions of strong economic growth dominated by abundant production and combustion of coal.

Shandong Province is one of the most prosperous and heavily polluted provinces in the country, with significant coal mining, oil refineries, metallurgical and industrial mechanical sectors [13]. In 2011, Jinan has experience an annual average PM_{2.5} concentration of 149 µg/m³, one of the highest reported cities in the world [14]. Similarly, Shanxi Province is China's largest coal producing region, and Taiyuan is one of the most polluted cities in China. From 2009 to 2010, the annual average PM_{2.5} concentration in Taiyuan was 220 µg/m³, more than 20 times the World Health Organization's 10 µg/m³ air quality guideline for mean annual PM_{2.5} emissions [8, 15, 16, 17].

Whereas short exposures to high PM concentrations have been thought to trigger acute reactions, such as acute myocardial infarction and asthma exacerbation [18, 19], longer and more consistent PM exposures have been associated with chronic diseases such as chronic obstructive pulmonary disease and lung cancer [20]. To elucidate the effects of repeated exposure to source-oriented PM in the present study, we compared the inflammatory responses produced by different regional PM_{2.5} extracts administered via oropharyngeal aspiration over multiple days. Although comparative analyses of pulmonary inflammatory responses to PM from California and China have been reported [21, 22], the present study is the first to assess the differential time-lag effects of repeated exposure to PM from Sacramento, Jinan, and Taiyuan. Results from the present study are discussed in relation to previous findings [21, 22] to further illustrate how different exposure paradigms influence observed responses.

2. Materials And Methods

Methods in the present study have been reported previously [21, 22] but are restated herein for reader accessibility.

2.1. Ambient PM_{2.5} Collection

Ambient winter PM_{2.5} collection occurred in Sacramento, California; Jinan, Shandong; and Taiyuan, Shanxi. The California sampling site was located in downtown Sacramento, at the northeast corner of T and 13th streets, on the rooftop of a two-story building (N38°34', W121°29'). Downtown Jinan's collection site was located on the rooftop of a three-story primary school, Wang She Ren (N36°40', E117°09'). The sampling site in downtown Taiyuan was located on the rooftop of the five-story College of Environmental Science and Resources building at Shanxi University (N37°47', E112°34'). All three sampling locations are proximal to major freeways and surrounded by a combination of residential, commercial, and industrial sites.

In Sacramento, a high-volume sampler system (Tisch Environmental Inc., TE-6070V2.5-HVS) was used to collect PM_{2.5} for seven days. The system had a PM_{2.5} size-selective head (Tisch Environmental Inc., TE-6001) that ran at a flow rate of 40 cubic feet per minute (cfm), and was fitted with Teflon-coated borosilicate glass microfiber filters (Pall Corporation, TX40H120WW). The filters were pre-cleaned by repeated sonication in a mixture of Milli-Q water, dichloromethane, and hexane prior to sampling [23].

In Jinan and Taiyuan, PM_{2.5} was collected for one day using a high-volume particle collector (Thermo Anderson, HVAIR100) operated at a flow rate of 40 cfm. The shorter collection times in China were due to higher density and heavier loading of PM relative to the sampling site in California. The sampler was fitted with a PM_{2.5} size-selective opening and 90 mm diameter quartz microfiber filters (Whatman, WHA1851090). Twenty-four hours before collection, the quartz filters were pre-heated at 450°C for the removal of any potential endotoxin from the filters.

2.2. PM Sample Extraction

Following sample collection in Sacramento and Jinan, the filters were post-weighed to calculate the collected PM mass (post weight – pre weight = PM mass), placed in Milli-Q water, and sonicated for 1 hour to obtain PM extracts. The sonicated PM extracts were passed through 0.2 µm pore size syringe filters, and the collected solution (approximately 100 mL) was lyophilized and stored at -80°C. A more detailed description of PM extraction methods is provided by Bein and Wexler [23].

Filters from Taiyuan were post-weighed to calculate the collected PM mass, cut into segments for placement in a 250 mL conical flask with 30 mL Milli-Q water, and sonicated for 30 minutes (3 cycles of 10 minutes each) to produce a PM extract. The extract was filtered through six layers of sterile gauze, lyophilized to powder, and stored at -80°C.

Later, the frozen, lyophilized PM extracts from Sacramento, Jinan, and Taiyuan were weighed, resuspended in Milli-Q water, and sonicated for 20 minutes to derive PM extract samples (PM_{CA}, PM_{SD}, and PM_{SX}, respectively) of the same concentration (1 µg/ µL; Figure 1). All PM samples were frozen at -20°C until needed.

2.3. Chemical Characterization of Stock Extract Samples via High-Resolution Aerosol Mass Spectrometry (HR-AMS)

Chemical characterization was performed at the University of California, Davis. Frozen stock PM extracts were thawed to 4°C, sonicated for 20 minutes for thorough PM dispersal, and diluted by Milli-Q water to 0.1 µg/µL. Subsequently, 1 mL of each diluted stock sample was individually passed through a constant output aerosol generator with an inert argon carrier gas, and a silica gel diffuser where the sample became atomized. HR-AMS was used to determine the chemical composition of each extract sample. Chemical species such as nitrate, sulfate, chloride, ammonium, and organic compounds were quantified [24]. The organic compounds were fragmented and further analyzed to determine their elemental composition (as regards hydrogen, carbon, oxygen, and nitrogen) and average degree of oxidation as detailed by Aiken *et al.* [25], Ghio *et al.* [26], Sun *et al.* [21], and Zhang *et al.* [22].

2.4. Animals and Exposure

The UC Davis Institutional Animal Care and Use Committee approved of all animal procedures and housing practices. Sixty-three male, 6-week old BALB/C mice were purchased (Envigo, Hayward, CA) and randomly assigned to one of four groups exposed to 1) vehicle control (Milli-Q water; n=9), 2) PM_{CA} (n=18), 3) PM_{SD} (n=18); or 4) PM_{SX} (n=18). All mice were acclimated for 2 weeks prior to the start of the exposure period, maintained on a 12-hour light/dark cycle, and housed three per cage with sterile laboratory bedding and *ad libitum* access to food and water.

Immediately before exposure, the stock extracts of PM_{CA}, PM_{SD}, and PM_{SX} were defrosted, sonicated for 20 minutes and diluted by Milli-Q water to a concentration of 20 µg/50 µL. Single oropharyngeal aspiration (OPA) exposures occurred five times, once per day on 1, 4, 7, 10, and 14 days (Figure 2). During exposure, the mice were sedated by inhaling of isoflurane with oxygen at a 3:1 ratio [9]. Each mouse was given 50 µL of Milli-Q water, PM_{CA}, PM_{SD}, or PM_{SX} via OPA such that the exposure volume was consistent across all groups as was each single PM mass (20 µg) for all PM-exposed groups. A single dose of this nature has been used previously [21] to examine PM effects. With high ambient PM levels noted in each of the sampled cities (in particular China), a total PM dose of 100 µg administered over a two-week period could approach levels experienced by inhalation of ambient air over this same period of time.

2.5. Bronchoalveolar Lavage Fluid (BALF)

Necropsies and biological sample collections were completed on Days 1, 2, and 4 after the final OPA exposure (15, 16 and 18 days, respectively). On each of these days, mice were randomly selected from each group (n=3 controls, and 6/PM group), weighed, and euthanized for necropsy with a 0.2 mL intraperitoneal injection of Beuthanasia-D pentobarbital solution (65 mg/kg body weight; Nembutal, Cardinal Health, Sacramento, CA). Each mouse was cannulated intratracheally, and the left mainstem airway was clamped while the right lung was lavaged with two aliquots (0.6 mL/aliquot) of Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich, St Louis, MO). The collected BALF was centrifuged at 2000 'g at 4°C for 15 minutes to pellet the cells. BALF supernatant was decanted, frozen in liquid nitrogen, and stored at -80°C. Cell pellets were resuspended in 500 mL PBS for determination of total cell numbers and viability using a hemocytometer and 0.4% Trypan Blue solution for viability detection (Sigma-Aldrich).

BALF cytocentrifuge slides were prepared using a Shandon Cytospin (Thermo Shandon, Inc., Pittsburgh, PA). The slides were then stained with DippKwik Differential Stain (American MasterTech, Lodi, CA) to determine the proportions of BAL macrophages, neutrophils, eosinophils, and lymphocytes in cell counts (500 cells/animal) performed using Brightfield microscopy. The experimental protocol is shown in Figure 3.

2.6. Lung Collection

The left lungs were inflated-fixed at 30 cm of pressure for one hour, stored in 4% paraformaldehyde for 48 hours, and subsequently transferred into 70% ethanol for later tissue processing for histopathology. The right lung lobes of each mouse were placed in two cryovials—one vial for the cranial and middle lobes, and the other for the caudal and accessory lobes. All vials were stored at -80°C until further use.

2.7. Semi-Quantitative Lung Histopathology

Following placement of the left lung in 70% ethanol, four transverse slices (levels) were prepared as a method to uniformly sample histological changes throughout the entire lobe. These tissue slices were dehydrated, embedded in paraffin, sectioned at a thickness of 5 µm, placed on glass slides, stained with Harris hematoxylin and eosin (H&E; American MasterTech, Lodi, CA), and cover slipped.

Four transverse slices were stained for each mouse (n=9/control or 18/PM group). All slides were examined independently by two blinded observers (WY and SCV) for the presence of inflammation, cellular infiltrates, and cellular/tissue remodeling (i.e. desquamation and squamous metaplasia of airway epithelial cells, septal wall thickening) in alveolar ducts and airways using a semi-quantitative scoring scale. Previously published rubrics [27] were used to rank the severity (absent to marked; 0-3) and extent (0: no changes, 1: less than one-third of the slide, 2: one-half of the slide, 3: two-thirds of the slide) of the observed remodeling and inflammation. For each mouse, the final score for a given parameter (e.g. alveolitis) was the averaged product of the severity and extent scores for the four transverse slices. Use of the products resulted in scores ranging from 0-9 thus increasing the probability of finding significant ($p < 0.05$) differences between groups [28]. Detailed semi-quantitative scoring guidelines are shown in supplemental Table S1.

2.8. Gene Expression Analysis

Ribonucleic acid (RNA) was isolated from the right caudal and accessory lung lobes using TRI Reagent (Sigma-Aldrich) and Quick-RNA Miniprep kit (Zymo Research, Irvine, CA). RNA was converted to copy deoxyribonucleic acid (cDNA) using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystem's, Indianapolis, IN). Gene-specific mouse primers (0.2 µM; IDT, Coralville, IA), cDNA (2 µL/reaction), and SYBR Green (Applied Biosystem) DNA-binding stain were used for quantitative polymerase chain reaction (qPCR) measurements. Expression of genes for inflammatory cytokines, interleukin-1 beta (*IL-1 β*) and tumor necrosis factor alpha (*TNF- α*), and neutrophil chemokines, chemokine (C-X-C Motif) ligands-3 and -5 (*CXCL-3* and *CXCL-5*) were examined. Expression was assessed using the $\Delta\Delta$ -Ct method and standardized to the expression of elongation factor 1-alpha 1 (*EEF1a1*) housekeeping genes [29]. Mouse gene primers were designed using Primer3 primer design software [30]. Primers used in this study are detailed in Supplemental Table S2.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs (Biologend, San Diego, CA) were performed on the homogenized cranial and middle lobes of the right lung to analyze concentrations of specific proteins including TNF- α ; monocyte chemoattractant protein-1 (MCP-1), a chemoattractant responsible for monocyte and macrophage recruitment; CXCL-1, seen in inflammation or wound healing; and IL-1 β and IL-6, both mediators of inflammatory responses. The cranial and middle lobes from control- and PM-exposed animals and standards from the R&D Systems ELISA kits (1000 µg/mL to 7.8 µg/mL) were prepared and examined in duplicate in 96-well plates using a SpectroMax plate reader (Molecular Devices, Sunnyvale, CA). Duplicate readings were averaged. All concentrations were normalized to total lung protein and reported in pg of specific protein per mg of lung tissue.

2.10. Statistical analysis

No data points were excluded prior to statistical analysis. All statistical tests were performed using GraphPad PRISM 8.0 software. A value of $p < 0.05$ was considered statistically significant. For each measured endpoint, Shapiro-Wilk tests were first used to detect normality, a one-way analysis of variance (ANOVA) and *post hoc* Tukey's test were performed to determine differences due to treatment. All data in the present document are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Chemical Composition of PM Extracts

Organic compounds comprised the largest fraction of the total mass in each of the extract samples accounting for 54%, 57%, and 45% in PM_{CA} , PM_{SD} , and PM_{SX} , respectively (Fig. 4A-C). Within the organic fraction, carbon and oxygen accounted for at least 87% of the mass (Fig. 4D-F). Of the other compounds measured by HRAMS, nitrate and sulfate fractions were highly variable while those of chloride and ammonium were relatively similar among the California and China PM extracts. Nitrate was more abundant in California PM compared to China PM, while sulfate was most prevalent in PM_{SX} versus the other two PM extracts (Fig. 4A-C). The sulfate and nitrate measured in all three PM samples were principally inorganic, identified by the presence of anions in both the ammonium sulfate and ammonium nitrate. However, based on ion balance analysis, a significant fraction of the sulfate in PM_{SX} was found to be organic.

Figure 4. Chemical composition of wintertime PM extracts from Sacramento, California; Jinan, Shandong; and Taiyuan, Shanxi. Characterization was performed using high-resolution time-of-flight aerosol mass spectrometry. Charts (A-C) in the top row illustrate bulk composition, including organic compounds. Charts (D-F) in the bottom row show elemental composition including the degree of oxidization.

3.2. Cell Differentials in BALF

Recovery time did not appear to play a role in the responses observed in BALF as there were no statistically significant differences between necropsy days (Fig. 5). No significant ($p < 0.05$) exposure-related differences were noted for total cell counts of mice exposed to vehicle control versus PM_{CA} , PM_{SD} , or PM_{SX} irrespective of the recovery time post-OPA (Fig. 5A). In contrast, significantly increased neutrophil numbers were observed on post-OPA Days 1 and 4 in all PM-exposed groups relative to controls ($p < 0.05$ for all comparisons), and on Day 4 in PM_{SX} -exposed mice relative to their PM_{CA} - and PM_{SD} -exposed counterparts ($p = 0.0326$ and $p = 0.0196$, respectively; Fig. 5B). Interestingly, no statistically significant treatment-related effects were observed on Day 2 (Fig. 5B), as neutrophil numbers in all PM-exposed groups dropped temporarily to near-control levels.

Figure 5. Oropharyngeal aspiration of regional particulate matter (PM) extracts produced acute and subacute neutrophilia in mice up to four days post exposure. Animals were exposed on five separate days over a two-week period to Milli-Q water (control; $n = 9$), or a PM extract (20 $\mu\text{g}/50\mu\text{L}$) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi (PM_{CA} , PM_{SD} , or PM_{SX} , respectively). Each mouse received a 50 μL aspirate on the exposure days (total PM dose = 0 or 100 $\mu\text{g}/\text{mouse}$). Mice from each group were then necropsied on Day 1, 2 or 4 after the last exposure. Graphs show counts of total bronchoalveolar lavage fluid (BALF) cells (A) and neutrophils (B) collected at different post-exposure time-points. Total cell and neutrophil data were analyzed separately via one-way ANOVA tests and are presented as the mean \pm standard error of the mean. * indicates a significant ($p < 0.05$) difference from control. Brackets indicate significant ($p < 0.05$) differences between PM-exposed groups.

3.3. Histological analysis

There were no significant ($p < 0.05$) differences in bronchiolar inflammation scores on post-OPA Day 1 (Fig. 6A). However, on Day 2, PM_{SX} -exposed mice exhibited significantly ($p = 0.0179$) more bronchiolar inflammation than controls (Fig. 6A). By Day 4, both groups given Chinese PM extracts exhibited more severe bronchiolitis than controls ($p = 0.0399$ for PM_{SD} ; $p = 0.0027$ for PM_{SX} ; Fig. 6A), with neutrophilic influxes into the peribronchiolar and alveolar regions of the lungs that were not observed in other groups (Figs. 7A-D). Mice exposed to PM_{SX} were found to have visible black particles (likely due to coal combustion) in macrophages throughout the alveolar regions of the lungs.

In contrast to the bronchioles, perivascular and subpleural regions, the alveoli appeared to be most affected by PM. On post-OPA Days 1 and 2, significantly greater alveolitis was observed in groups administered PM_{SD} ($p = 0.0206$ and 0.0361, respectively) or PM_{SX} ($p = 0.0057$ and 0.0172, respectively) versus vehicle control (Fig. 6B). Over these two days, no significant intra-group differences in alveolitis were observed within the PM_{SD} or PM_{SX} groups. By Day 4, all three PM-exposed groups exhibited higher scores for alveolitis than controls (Fig. 6B; $p = 0.0012$, $p = 0.0043$, and $p = 0.0001$ for PM_{CA} , PM_{SD} , and PM_{SX} , respectively). PM_{SD} - and PM_{SX} -exposed mice had average scores for alveolitis of 1.8 and 2.4, respectively, with notable changes in alveolar wall thickening, and numerous free macrophages distributed throughout the parenchyma (Fig. 8A-D), while control mice had an average score of 0.7, with thin-walled alveolar septa and relatively few luminal macrophages (Figs. 6B and 8). Although inter-group differences were not statistically significant among the PM-exposed mice, those administered PM_{SX} appeared to demonstrate the greatest effects (Figs. 8A-D) with cellular debris and numerous foamy, particle-laden macrophages aggregated in the alveolar airspaces (Figs. 8D).

Figure 6. Extracts of Chinese particulate matter (PM) were potent stimulators of lung inflammation in mice. Graphs show semi-quantitative histopathology scores from the bronchiolar (A) and alveolar (B) lung regions of mice culled on 1, 2 or 4 days after the last exposure. Mice were oropharyngeally exposed on five separate days over a two-week period to an aspirate of MilliQ water (control; $n=9$), or a PM extract (20 $\mu\text{g}/50\mu\text{L}$) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi. Each mouse received a 50- μL aspirate on the exposure days (total PM dose = 0 or 100 $\mu\text{g}/\text{mouse}$). Final scores were averaged from the products of the severity and extent scores in each region of the lungs. For each time-point, an ANOVA was applied to determine effects due to the aspirate. Data are presented as the mean \pm standard error of the mean. * indicates a significant ($p < 0.05$) difference from control. $N = 18/\text{group}$ (6/group/day).

Figure 7. Bronchiolar inflammation in mice from particulate matter (PM) extracts from Taiyuan, China. Panels are light micrographs of hematoxylin- and eosin-stained lung tissues collected on Day 4 after the last of five separate 50 μL oropharyngeal aspiration exposures to a MilliQ water control (A; $n = 9$) or a 20 $\mu\text{g}/50\mu\text{L}$ PM extract ($n = 18/\text{group}$) from Sacramento, CA (B); Jinan, Shandong (C); or Taiyuan, Shanxi (D). Total PM dose was 0 or 100 $\mu\text{g}/\text{mouse}$ over 14 days. Arrows indicate neutrophils and arrowheads indicate macrophages. AW = airway, scale bar is 20 μm .

Figure 8. Alveolitis in mice exposed to particulate matter (PM) extracts from California and China. Panels are light micrographs of hematoxylin- and eosin-stained lung tissues collected on Day 4 after the last of five separate 50 μL oropharyngeal aspiration exposures to a MilliQ water control (A; $n = 9$) or a

20 µg/50 µL PM extract ($n = 18/\text{group}$) from Sacramento, CA (B); Jinan, Shandong (C); or Taiyuan, Shanxi (D). Total PM dose was 0 or 100 µg/mouse over 14 days. Arrows pinpoint alveolar inflammation. Scale bar is 20 µm.

3.4. qPCR Analysis of Gene Expression

PM-induced recruitment of neutrophils into the lungs can be directed by numerous chemotactic mediators including IL-1 β , TNF- α , CXCL-3 and CXCL-5 [31]. Results demonstrated expression of IL-1 β and TNF- α genes were significantly increased in PM_{SX}- versus control-exposed mice on Days 2 ($p = 0.0261$ and $p = 0.0415$, respectively) and 4 ($p < 0.0001$ and $p = 0.0062$, respectively; Figs. 9A-B). Similar increases were observed on Day 4, IL-1 β in PM_{CA}-exposed mice relative to controls ($p = 0.0049$) and in PM_{SX}-exposed mice relative to their PM_{SD}-exposed counterparts ($p = 0.0152$; Figs. 9A-B). Gene expression of CXCL-3 and CXCL-5 were also significantly elevated in PM- versus control-exposed mice, with PM_{SX} producing increases in one or both chemokines at all post-OPA time-points ($p < 0.05$); PM_{CA} producing increases in CXCL-3 ($p = 0.0351$) and CXCL-5 ($p < 0.0001$) on Day 4 alone; and PM_{SD} producing an increase in CXCL-5 ($p = 0.0233$) on Day 4 alone (Figs. 10A-B).

Figure 9. Extracts of particulate matter (PM) from Taiyuan, China increased gene expression for the inflammatory cytokines, Interleukin (IL)-1 β , Tumor Necrosis Factor (TNF- α). Graphs show results from quantitative polymerase chain reaction assays performed on mRNA obtained from the lungs of mice culled on 1, 2, or 4 day(s) after the last 50 µL oropharyngeal aspiration (OPA) exposure to MilliQ water (vehicle control; $n = 9$), or a PM extract (20 µg/50 µL) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi ($n = 18/\text{group}$). OPA occurred on five separate days over a two-week period yielding a total PM dose of 0 or 100 µg/mouse. Gene levels were analyzed for each animal and averaged for each treatment group. Gene expression is shown relative to the housekeeping gene, EEF1a1. Data are shown as the mean ± SEM. One-way ANOVA and Tukey tests were performed at a significance level of $p < 0.05$. * indicates a significant ($p < 0.05$) difference from vehicle control; brackets indicate significant ($p < 0.05$) differences between non-control groups.

Figure 10. Extracts of particulate matter (PM) from California and China increased gene expression for neutrophil mediation of monocyte chemotaxis. Graphs show results from quantitative polymerase chain reaction assays performed on mRNA obtained from the lungs of mice culled on 1, 2, or 4 day(s) after the last 50 µL oropharyngeal aspiration (OPA) exposure to MilliQ water (vehicle control; $n = 9$), or a PM extract (20 µg/50 µL) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi ($n = 18/\text{group}$). OPA occurred on five separate days over a two-week period yielding a total PM dose = 0 or 100 µg/mouse. Expression of CXCL-3 and CXCL5 genes (A and B, respectively) was measured for each animal, averaged for each treatment group, and assessed relative to the housekeeping gene, EEF1a1. One-way ANOVA and Tukey tests were performed at a significance level of $p < 0.05$. Resulting data are shown as the mean ± SEM. * indicates a significant ($p < 0.05$) difference from sham control; brackets indicate significant ($p < 0.05$) differences between non-control groups.

3.5. Quantification of Cytokines by ELISA

Although six cytokines were examined, concentrations of three proteins (TNF- α , CXCL-1, and IL-6) were too low to quantify reliably with limits of detection at 5.16, 66.521, 8.301 ng/mL, respectively. Of the two remaining cytokines, IL-1 β (data not shown) and MCP-1 (Fig. 11), only protein levels of the latter varied significantly among the PM- and control-exposed groups, with PM_{SX} producing increases relative to control on Days 2 and 4 ($p = 0.0306$ and $p < 0.0001$, respectively); and PM_{CA} and PM_{SD} producing similar increases on Day 18 alone ($p = 0.0037$ and $p = 0.0437$, respectively).

Figure 11. Mice exhibited subacute increases in Monocyte Chemoattractant Protein (MCP)-1 levels following exposure to particulate matter (PM) extracts from California or China. The bar graph shows the result from enzyme-linked immunosorbent assays performed on lung tissues of mice culled on 1, 2 or 4 day(s) after the last 50 µL oropharyngeal aspiration (OPA) exposure to MilliQ water (vehicle control; $n = 9$), or a PM extract (20 µg/50µL) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi ($n = 18/\text{group}$). OPA occurred on five separate days over a two-week period yielding a total PM dose = 0 or 100 µg/mouse. Separate ANOVAs were performed for each time-point to determine inter-group differences due to exposure. * indicates a significant ($p < 0.05$) difference from control; brackets indicate significant ($p < 0.05$) differences between non-control groups.

4. Discussion

Global air pollution in the form of PM emissions is of major concern. The associated effects can be seasonal with sustained levels of elevated ambient particles over time, especially during the winter season. PM exposures can arise from a multitude of sources and range from short-term to prolonged and/or intermittent. Various sizes of PM can affect children, the elderly, and those with pre-existing health issues. However, long-term exposure to PM_{2.5} is most associated with morbidity and premature mortality [32], and has been described as one of the leading risk factors for premature mortality contributing to 800,000 premature deaths each year [1].

Several reviews have concluded that chemical PM components can play an important role in post-exposure health effects [33, 34]. Therefore, to enact the proper exposure controls (e.g. protective equipment, concentration limits), it is critical to determine which PM component(s), or combinations thereof, are most harmful to human health [35]. In the present study, lung tissues semi-quantitatively examined, demonstrated different responses to the various PM extract samples. This could be due to differing chemical compositions influenced by regional emission sources such as vehicular exhaust, local farming operations, as well as industrial businesses in the Sacramento region, or coal production in the Shandong and Shanxi provinces [8, 9].

A previous study [21] compared inflammatory effects of acute exposure to PM_{CA} and PM_{SX}, but exposure occurred on only one day. OPA was performed once, with each mouse given a total of 50 µL of Milli-Q water or one of the PM extracts (1 µg/µL), and necropsies were completed 24 hours thereafter. Results showed that PM_{CA} produced significantly ($p < 0.05$) higher levels of BALF neutrophils and alveolar inflammation scores than control and PM_{SX}. However, in the present study, we included another China PM sample (PM_{SD}); performed repeated exposures over two weeks using 20 µg of PM in 50 µL suspensions of aspirates on each of the five OPA days to avoid inflammation from high administered fluid volumes; and examined effects at three post-OPA time-points (Days 1, 2, and 4) to model sub-acute health implications for human populations. Results of BAL (Fig. 5B), histopathology (Figs. 6–8), gene expression (Figs. 9–10),

and ELISA (Fig. 11) analyses in the present study showed that all the tested PM extracts caused inflammatory responses at Day 4, but PM_{SX} often provoked statistically significant changes (relative to control) at earlier time-points (Figs. 5B, 6, and 9–11). These results were different from those reported by Sun et al. [21], likely due to longer exposure and recovery periods to PM.

We report herein that repeated PM_{SX} exposure produced higher BALF neutrophil numbers than PM_{CA} and PM_{SD} on post-OPA Day 4 (Fig. 5B), and higher ($p < 0.05$) bronchiolar and alveolar inflammation scores relative to controls on more days than PM_{CA} and PM_{SD} (Fig. 6A-B). Repeated exposure to PM can lead to an increase in neutrophils found in BAL because neutrophil influx is causally associated to lung injury [31, 36], with the relative number of neutrophils entering the lungs typically reflective of the severity of the biological response. Neutrophils are the first non-resident cells to be recruited to the site of inflammation. For this study, neutrophil influx occurs by egress of these cells from the capillaries of the airways, and transport through the epithelium into the airway lumen and alveoli [37]. This was confirmed in our study by the histological evidence of neutrophils in the pulmonary interstitium and among epithelial cells (Fig. 7).

Compared to control-, PM_{CA} - and PM_{SD} -exposed mouse groups, mice exposed to PM_{SX} also exhibited significantly increased mRNA for TNF- α and IL-1 β , cytokines involved in pulmonary inflammatory reactions [38], at Days 2 and 4 (Figs. 9A-B); and CXCL-3 and CXCL-5 neutrophil chemokines [39, 40] at Day 4 (Figs. 10A-B). MCP-1 protein was increased in all PM-exposed groups relative to controls at Day 4, but PM_{SX} produced the longest-lasting response that was also stronger than that for PM_{SD} (Fig. 11). MCP-1, is a chemoattractant responsible for monocyte and macrophage recruitment. However, growing evidence shows MCP-1 may also be involved in attracting neutrophils [41]. Therefore, increased MCP-1 protein levels could have contributed to the BALF neutrophilia observed in PM-exposed mice (Figs. 5 and 11).

Although statistically significant differences were only occasionally observed between PM_{SX} - and other PM-exposed groups, PM_{SX} exposure most frequently produced differences relative to control. Cumulatively, BALF neutrophil and lung histopathology results suggested the chemical composition of PM_{SX} may be contributing to its seemingly greater toxicity relative to PM_{CA} and PM_{SD} on an equal PM mass basis. Although we were unable to definitively identify the chemical constituent(s) that produced the observed biological effects, we believe organosulfates and polycyclic aromatic hydrocarbons (PAHs) likely contributed to the differential induction effects observed.

The three PM extracts tested in the present study had similar chemical compositions, with $\leq 10\%$ difference in the fractions of organic compounds, chloride, and ammonium. However, a major difference was observed when comparing sulfate levels, which measured at 2%, 14%, and 26% in PM_{CA} , PM_{SD} , and PM_{SX} , respectively (Figs. 4A-C). Several studies indicated sulfate-associated particles (i.e., fossil fuel combustion products) are among the most toxic in terms of effects on annual cardiopulmonary and cardiovascular diseases as well as lung cancer mortality [33, 42]; and sulfate has been associated with increased percentages of BALF neutrophils [33].

Particulate air pollution—characterized by high secondary aerosol concentrations including organosulfates—has been a serious environmental problem during recent winters in China [43, 44]. Therefore, higher fractions of organosulfates are plausible in PM_{SX} and PM_{SD} relative to PM_{CA} . While in the present study sulfates were not speciated, and inorganic sulfate is likely quite innocuous, future exposure studies should quantify organic and inorganic sulfates to better understand the health risks they pose.

In addition to organosulfates, based on the source, PM may also be composed of polycyclic aromatic hydrocarbons (PAHs). PAHs have received considerable attention due to their potential toxic, carcinogenic, and mutagenic effects, and coal combustion is known to produce PAHs [45]. Coal consumption in China in 2012 reached 2.75 billion tons of standard coal, approximately one-half of the global coal consumption, and Shanxi province is one of the largest coal production centers in China [46]. A study by Zhang et al. [47] identified multiple PAHs in PM_{SX} , with benzo(b)fluoranthene (B[b]F), benzo(e)pyrene (B[e]P) and benzo(a)pyrene (B[a]P) predominant. Therefore, the greater toxicity of PM_{SX} relative to PM_{CA} and PM_{SD} might suggest PM_{SX} contains a greater PAHs concentration and/or more potent PAHs than the other two PM extracts (although unconfirmed). Future studies might wish to quantify PAHs present in all extracts to further explore the potential mechanisms by which PAHs provoke inflammatory responses.

Although animals in the present study were not exposed by inhalation, OPA is the most effective method to simulate inhalation of equal mass dose to compare PM extracts from diverse geographic locations. Findings from the present study provide further evidence for those mechanisms by which PM extracts from different regions of the world promote time-lag effects. Further global research is necessary to better understand how the chemical composition of PM correlates to cumulative and prolonged adverse health effects. Future studies could add more post-OPA time points, compare pulmonary health effects of repeated exposure to extracts of $\text{PM}_{2.5}$ from other regions of the world, and/or test the impact of increasing PM-sulfur content on the degree of inflammation. These future studies would significantly add to the existing knowledge to correlate PM composition to pulmonary responses, and inform future regulations on source-specific emissions to better protect and benefit human health.

5. Conclusions

In summary, the results of this study suggest when evaluating PM_{CA} , PM_{SD} and PM_{SX} samples on an equal mass basis, elevated sulfate-associated chemical composition may be an important factor in PM toxicity. Following repeated exposure over a two-week period, mice exposed to PM_{SX} demonstrated the greatest inflammatory response found in the increases of pulmonary neutrophil numbers, pro-inflammatory cytokines and chemokines levels that is thought to be due to its highest sulfate than other PMs. Furthermore, exposure to all three PM samples produced greater toxicity at post-OPA day 4, compared to day lag 1 and 2, consistent with universal time-lag effects of PM health effects. Significantly increased expression of IL-1 β , TNF- α , CXCL-3 and CXCL-5, as well as the protein levels MCP-1 was noted with exposure to PM_{SX} in contrast to exposure to PM_{SD} and PM_{CA} ($p < 0.05$). Through these findings, there is a strong evidence to show how particulate matter from different regions promote time-lag effects, but are strongly influenced by chemical composition to produce greater adverse

health effects. These findings provide further evidence to highlight the need to develop source-specific regulations to support greater protection of human health.

Declarations

Ethics approval and consent to participate

The UC Davis Institutional Animal Care and Use Committee approved of all animal procedures and housing practices.

Consent for publication

The need for consent to publish is not applicable.

Availability of supporting data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

Both authors WY and SCV contributed equally to this work. WY, SCV and KEP developed the concept and study design and contributed to writing the paper. WY and SCV analyzed and interpreted the data, WY drafted the manuscript. CCF performed the animal exposure and necropsy. KJB, WL, LC and HW designed and produced the particles, QZ and DEY characterized the chemical composition of particles, CFV provided qPCR technique assistance, WY and SCV performed histopathological analysis. CWW prepared the figures. All the authors critically reviewed and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures

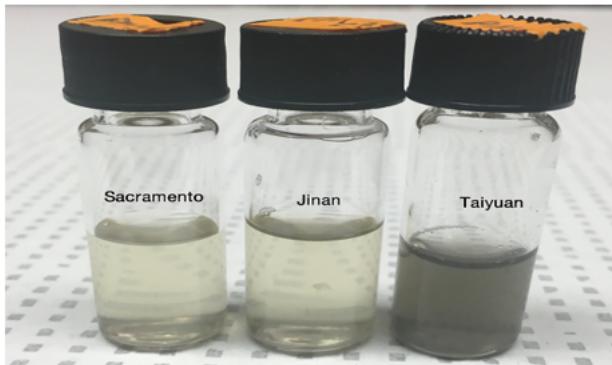


Figure 1

PM samples at equal mass concentrations. Image shows stock extracts of PM from Sacramento, California (left); Jinan, Shandong (middle); and Taiyuan, Shanxi (right) at a concentration of 1 µg/µL (1 mg/mL) suspended in nanopure water.

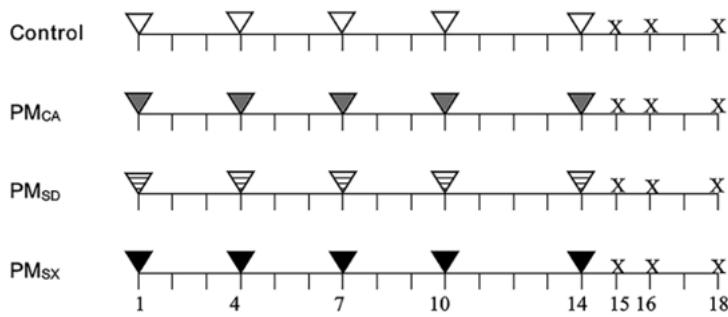


Figure 2

Exposure and biological sample collection timeline. The experimental design schedule for four different mouse treatment groups are shown for each oropharyngeal aspiration and necropsy. The aspirates were 50 µL Milli-Q water (control; white triangles), or a 20 µg/50 µL PM extract sample from Sacramento, California (gray triangles); Jinan, Shandong (striped triangles); or Taiyuan, Shanxi (black triangles). Each triangle in the figure represents a single exposure on 1, 4, 7, 10, or 14 days. Each "X" represents the necropsy and collection biological samples on post-OPA Day 1, 2 or 4 (15, 16, or 18 days). A total of 3 controls and 6 PM-exposed mice were taken for each necropsy day.

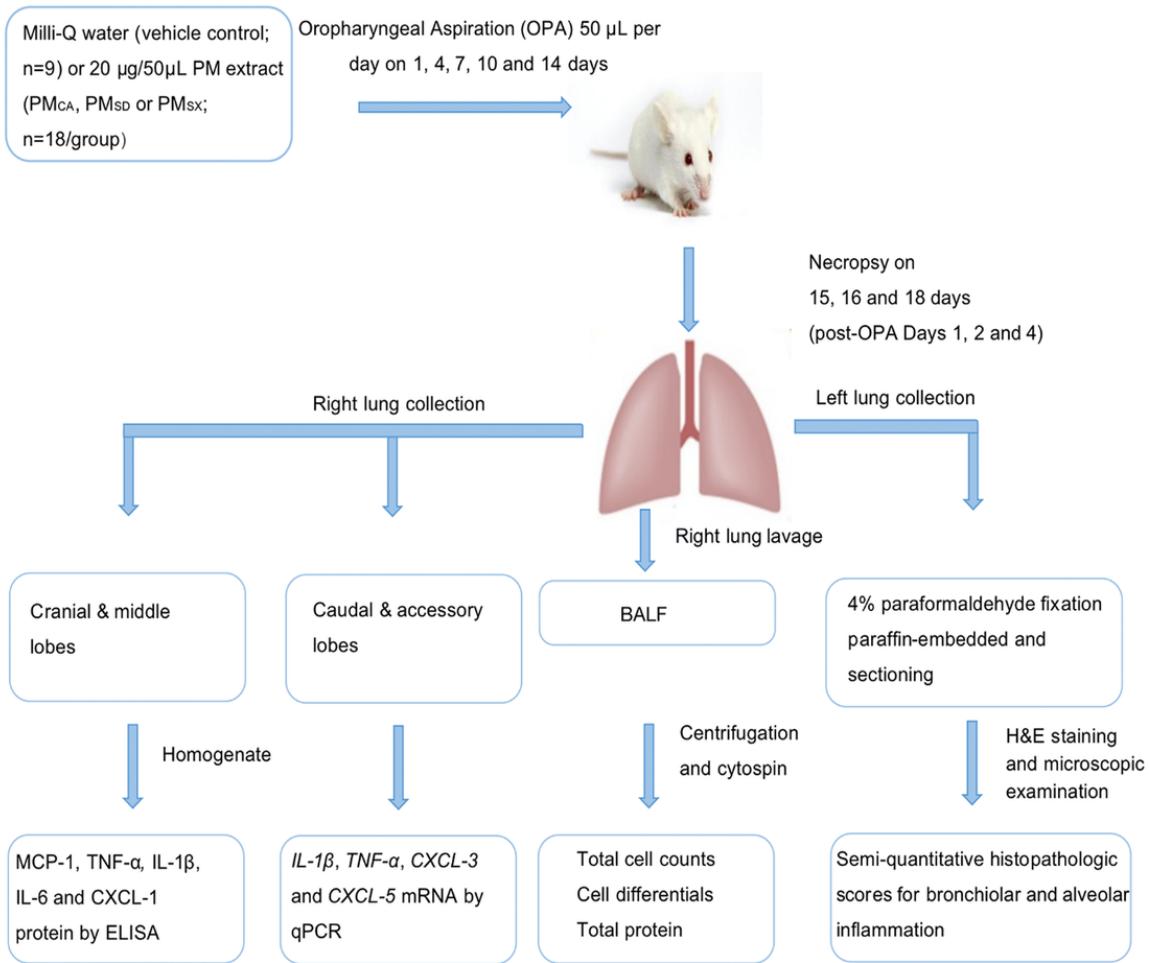


Figure 3

Experimental protocol. Four different treatment groups are shown along with their respective oropharyngeal aspiration exposures and post-necropsy processing.

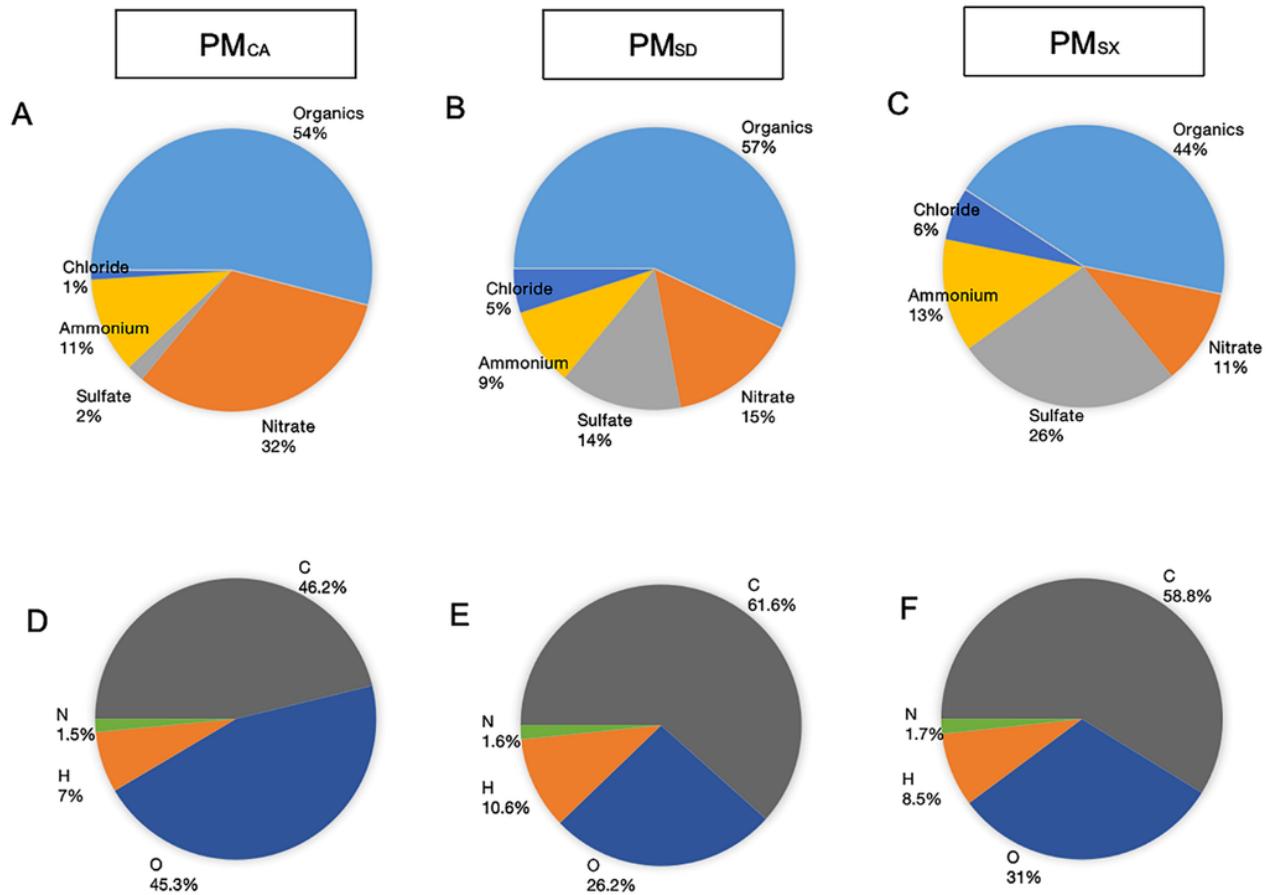


Figure 4

Chemical composition of wintertime PM extracts from Sacramento, California; Jinan, Shandong; and Taiyuan, Shanxi. Characterization was performed using high-resolution time-of-flight aerosol mass spectrometry. Charts (A-C) in the top row illustrate bulk composition, including organic compounds. Charts (D-F) in the bottom row show elemental composition including the degree of oxidization.

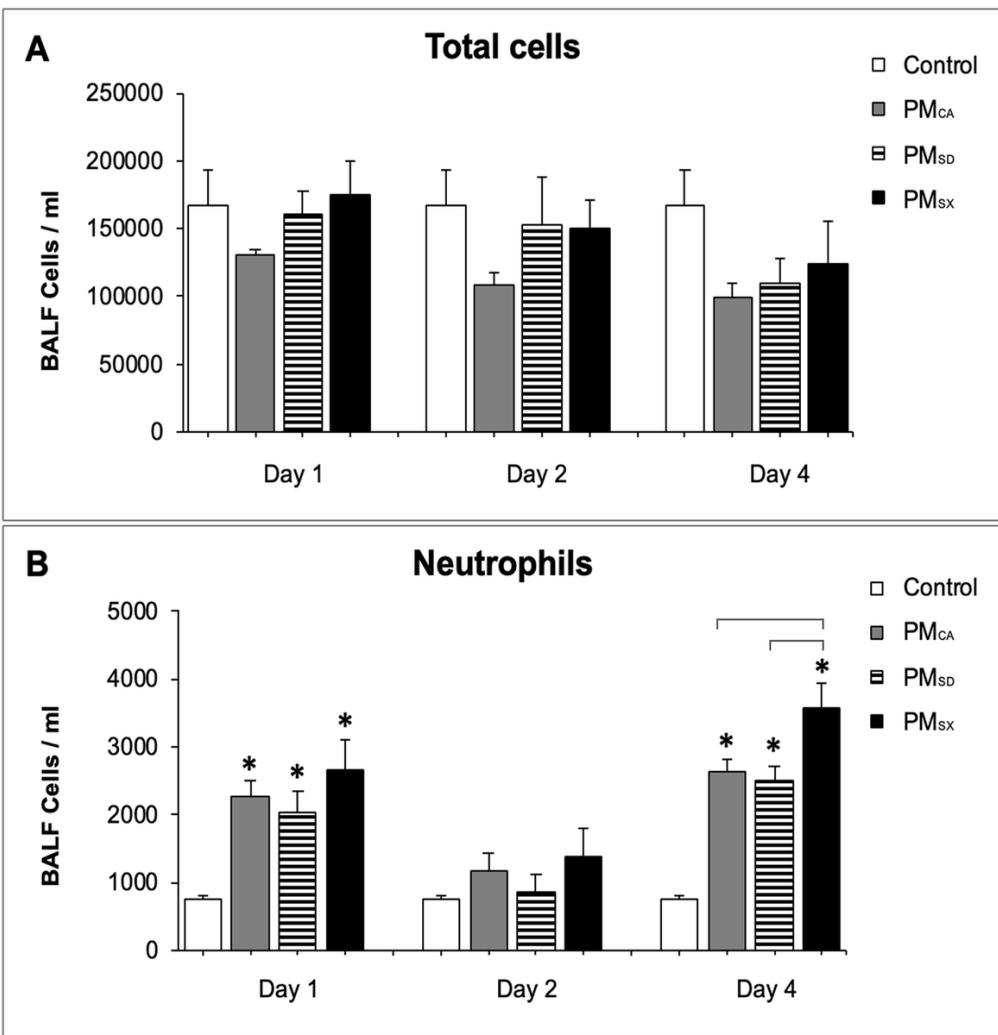


Figure 5

Oropharyngeal aspiration of regional particulate matter (PM) extracts produced acute and subacute neutrophilia in mice up to four days post exposure. Animals were exposed on five separate days over a two-week period to Milli-Q water (control; n=9), or a PM extract (20 µg/50µL) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi (PMCA, PMSD, or PMSX, respectively). Each mouse received a 50 µL aspirate on the exposure days (total PM dose = 0 or 100 µg/mouse). Mice from each group were then necropsied on Day 1, 2 or 4 after the last exposure. Graphs show counts of total bronchoalveolar lavage fluid (BALF) cells (A) and neutrophils (B) collected at different post-exposure time-points. Total cell and neutrophil data were analyzed separately via one-way ANOVA tests and are presented as the mean ± standard error of the mean. * indicates a significant ($p < 0.05$) difference from control. Brackets indicate significant ($p < 0.05$) differences between PM-exposed groups.

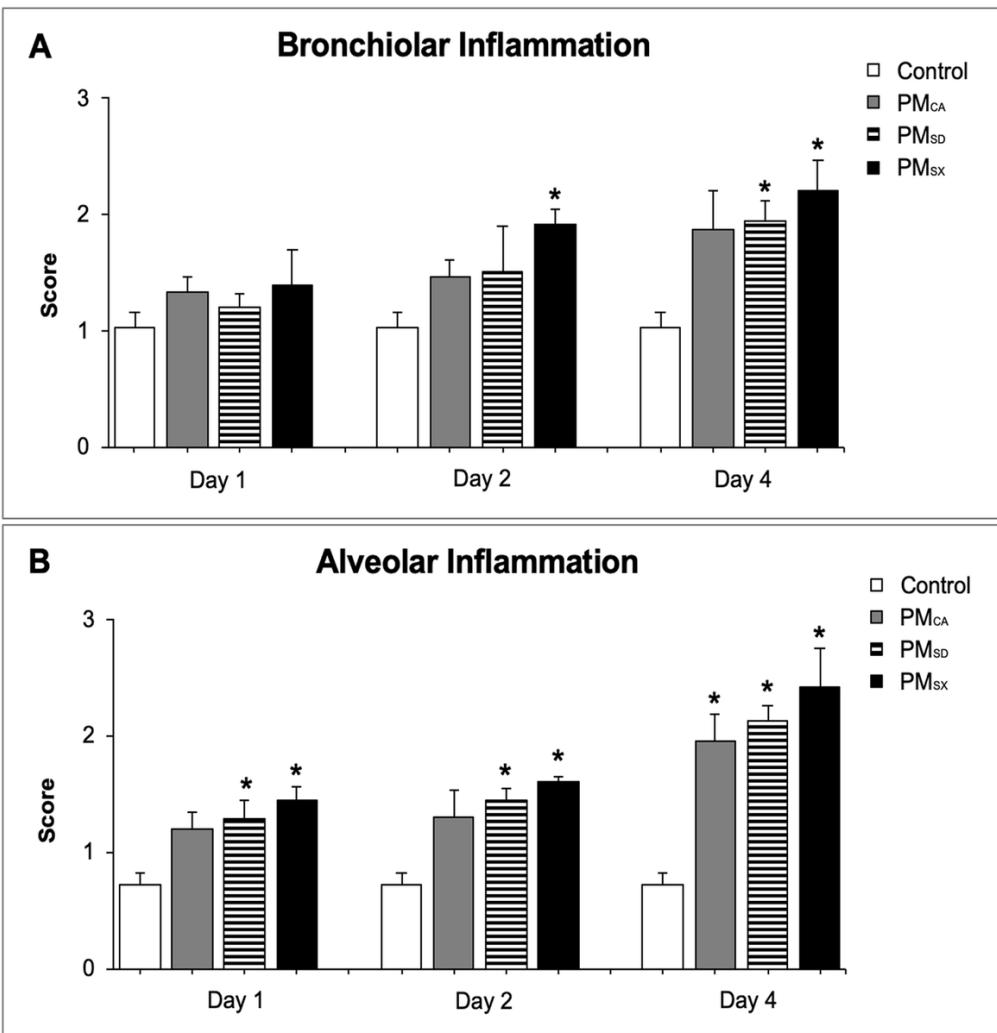


Figure 6

Extracts of Chinese particulate matter (PM) were potent stimulators of lung inflammation in mice. Graphs show semi-quantitative histopathology scores from the bronchiolar (A) and alveolar (B) lung regions of mice culled on 1, 2 or 4 days after the last exposure. Mice were oropharyngeally exposed on five separate days over a two-week period to an aspirate of Milli Q water (control; n=9), or a PM extract (20 µg/50 µL) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi. Each mouse received a 50-µL aspirate on the exposure days (total PM dose = 0 or 100 µg/mouse). Final scores were averaged from the products of the severity and extent scores in each region of the lungs. For each time-point, an ANOVA was applied to determine effects due to the aspirate. Data are presented as the mean ± standard error of the mean. * indicates a significant ($p < 0.05$) difference from control. N = 18/group (6/group/day).

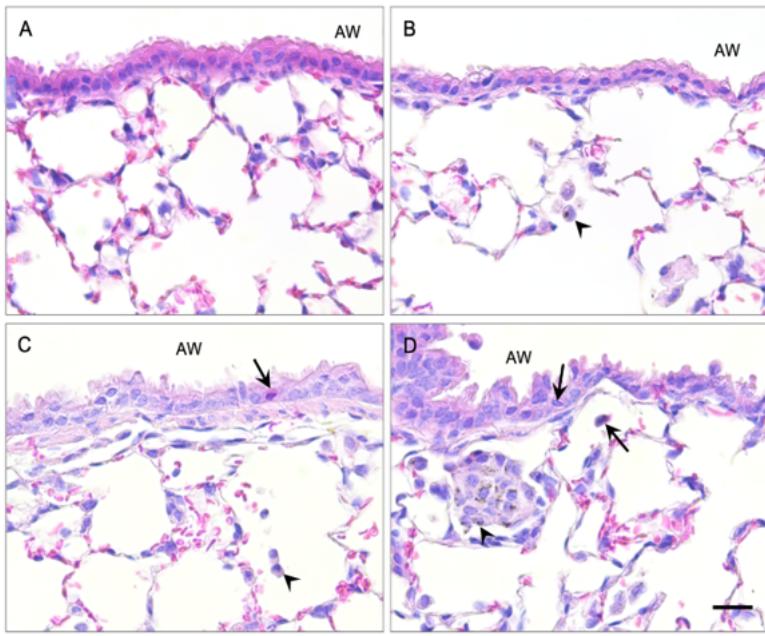


Figure 7

Bronchiolar inflammation in mice from particulate matter (PM) extracts from Taiyuan, China. Panels are light micrographs of hematoxylin- and eosin-stained lung tissues collected on Day 4 after the last of five separate 50 µL oropharyngeal aspiration exposures to a Milli Q water control (A; n=9) or a 20 µg/50µL PM extract (n=18/group) from Sacramento, CA (B); Jinan, Shandong (C); or Taiyuan, Shanxi (D). Total PM dose was 0 or 100 µg/mouse over 14 days. Arrows indicate neutrophils and arrowheads indicate macrophages. AW= airway, scale bar is 20 µm.

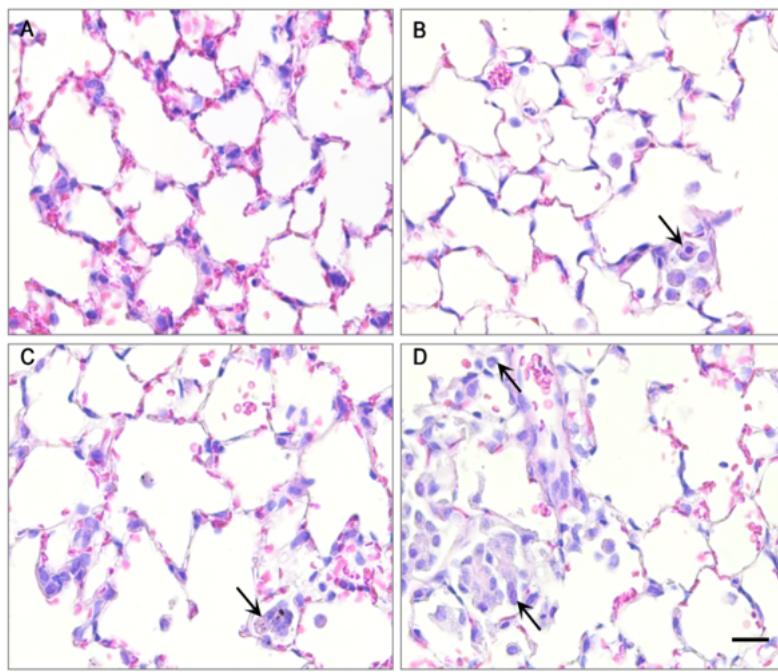


Figure 8

Alveolitis in mice exposed to particulate matter (PM) extracts from California and China. Panels are light micrographs of hematoxylin- and eosin-stained lung tissues collected on Day 4 after the last of five separate 50 µL oropharyngeal aspiration exposures to a Milli Q water control (A; n=9) or a 20 µg/50µL PM extract (n=18/group) from Sacramento, CA (B); Jinan, Shandong (C); or Taiyuan, Shanxi (D). Total PM dose was 0 or 100 µg/mouse over 14 days. Arrows pinpoint alveolar inflammation. Scale bar is 20 µm.

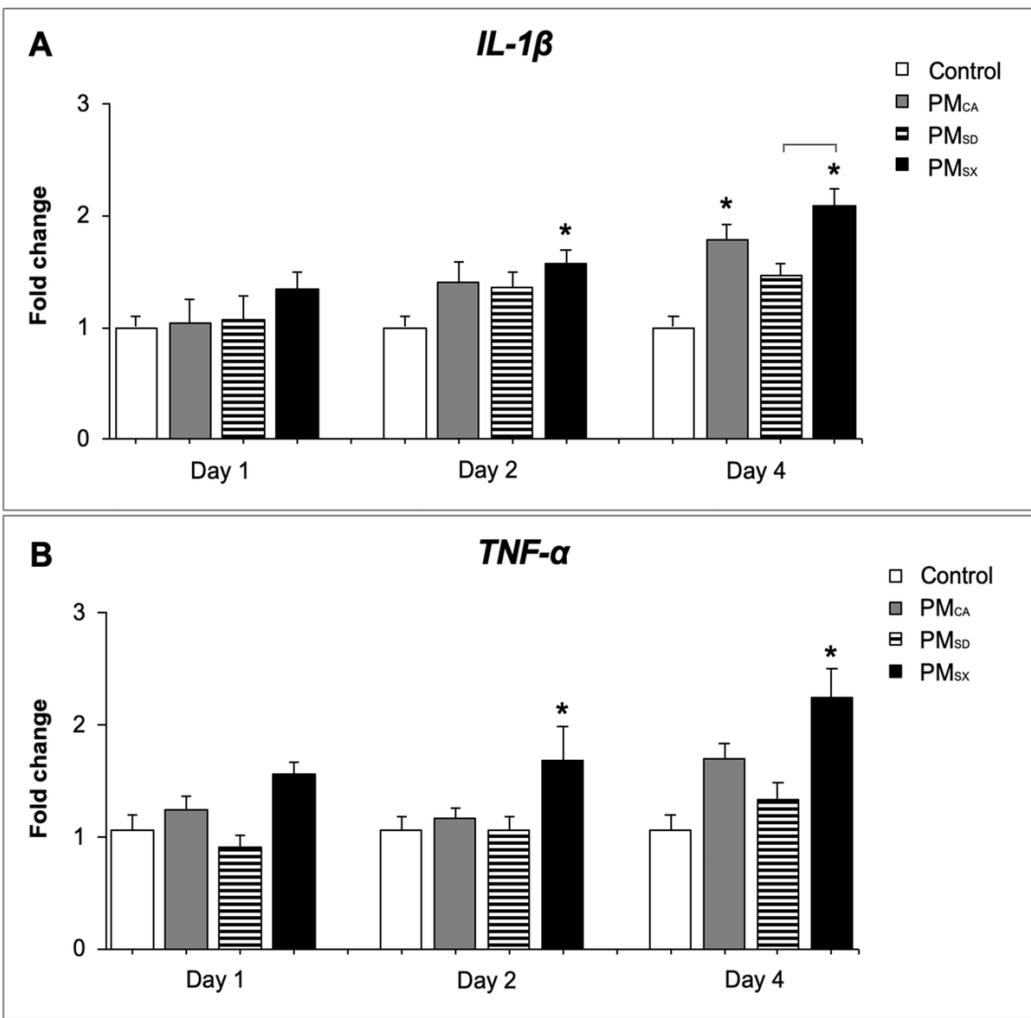


Figure 9

Extracts of particulate matter (PM) from Taiyuan, China increased gene expression for the inflammatory cytokines, Interleukin (IL)-1 β , Tumor Necrosis Factor (TNF- α). Graphs show results from quantitative polymerase chain reaction assays performed on mRNA obtained from the lungs of mice culled on 1, 2, or 4 day(s) after the last 50 μ L oropharyngeal aspiration (OPA) exposure to Milli Q water (vehicle control; n=9), or a PM extract (20 μ g/50 μ L) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi (n=18/group). OPA occurred on five separate days over a two-week period yielding a total PM dose of 0 or 100 μ g/mouse. Gene levels were analyzed for each animal and averaged for each treatment group. Gene expression is shown relative to the housekeeping gene, EEF1a1. Data are shown as the mean \pm SEM. One-way ANOVA and Tukey tests were performed at a significance level of p < 0.05. * indicates a significant (p < 0.05) difference from vehicle control; brackets indicate significant (p < 0.05) differences between non-control groups.

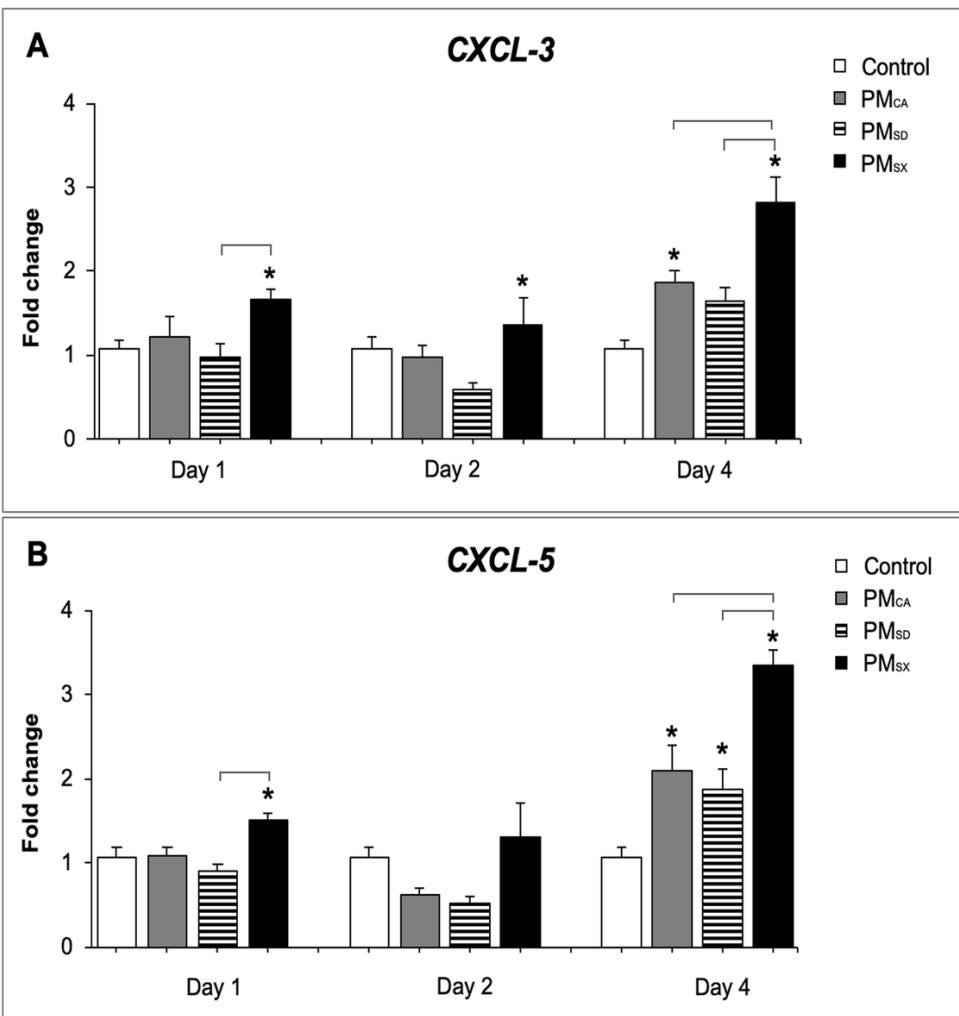


Figure 10

Extracts of particulate matter (PM) from California and China increased gene expression for neutrophil mediation of monocyte chemotaxis. Graphs show results from quantitative polymerase chain reaction assays performed on mRNA obtained from the lungs of mice culled on 1, 2, or 4 day(s) after the last 50 μ L oropharyngeal aspiration (OPA) exposure to Milli Q water (vehicle control; n=9), or a PM extract (20 μ g/50 μ L) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi (n=18/group). OPA occurred on five separate days over a two-week period yielding a total PM dose = 0 or 100 μ g/mouse. Expression of CXCL-3 and CXCL 5 genes (A and B, respectively) was measured for each animal, averaged for each treatment group, and assessed relative to the housekeeping gene, EEf1a1. One-way ANOVA and Tukey tests were performed at a significance level of p < 0.05. Resulting data are shown as the mean \pm SEM. * indicates a significant (p < 0.05) difference from sham control; brackets indicate significant (p < 0.05) differences between non-control groups.

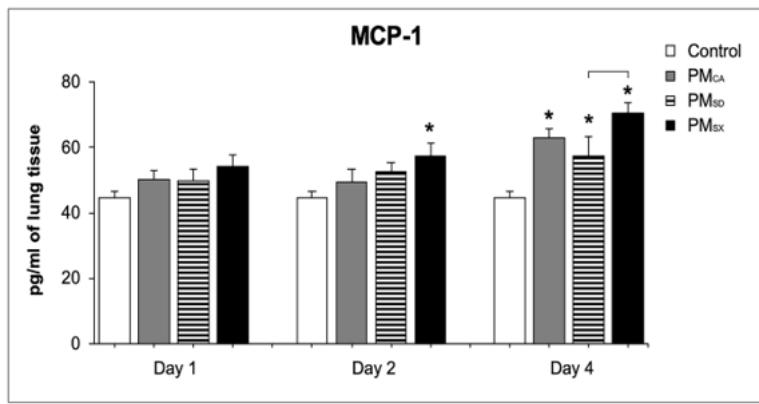


Figure 11

Mice exhibited subacute increases in Monocyte Chemoattractant Protein (MCP)-1 levels following exposure to particulate matter (PM) extracts from California or China. The bar graph shows the result from enzyme-linked immunosorbent assays performed on lung tissues of mice culled on 1, 2 or 4 day(s) after the last

50 μ L oropharyngeal aspiration (OPA) exposure to Milli Q water (vehicle control; n=9), or a PM extract (20 μ g/50 μ L) from Sacramento, CA; Jinan, Shandong; or Taiyuan, Shanxi (n=18/group). OPA occurred on five separate days over a two-week period yielding a total PM dose = 0 or 100 μ g/mouse. Separate ANOVAs were performed for each time-point to determine inter-group differences due to exposure. * indicates a significant ($p < 0.05$) difference from control; brackets indicate significant ($p < 0.05$) differences between non-control groups.

Supplementary Files

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