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INFLUENCE OF SEASON AND LOCATION ON PULMONARY RESPONSE TO CALIFORNIA'S SAN JOAQUIN VALLEY AIRBORNE PARTICULATE MATTER

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Season and location have documented impacts on particulate matter (PM)-induced morbidity and mortality. Seasonal and regional influences on the physical and chemical properties of $PM_{2.5}$ (also known as fine/ultrafine PM) contribute to differences in exposure burden and adverse respiratory health outcomes experienced in California's San Joaquin Valley (SJV), which ranks among the worst in the nation for PM pollution. Current regulations are driven by the association between mass concentrations and adverse health outcomes. However, this association is difficult to reproduce in toxicological studies and suggests a role for other parameters, such as chemical composition, involved in PM-induced adverse pulmonary health effects. Pulmonary toxicity of summer/winter and rural/urban SJV PM was evaluated given the unique geography, metereology and sources of the region. Healthy juvenile male mice inhaled summer/winter and urban/rural concentrated ambient PM (CAP) or ambient PM for 6 h/d for 10 d, and pulmonary inflammatory responses were measured 48 h postexposure. Exposure concentrations ranged from 10 to 20 $\mu g/m^3$ for ambient air control mice and from 86 to 284 $\mu g/m^3$. Mice exposed to rural but not urban CAP, displayed significant neutrophil influx that was more than 50-fold greater than control levels, which ranged from 21 to 60 neutrophils/ml for all experiments. Pulmonary neutrophilic inflammation was measured despite lower CAP concentrations in the rural compared to the urban location and in the absence of cytotoxicity, oxidative stress, or elevations in cytokine and chemokines expression. Further, the inflammatory responses induced by rural winter CAP were associated with the highest levels of organic carbon (OC) and nitrates (NO_3^-). Evidence indicates that regional/seasonal influences on PM chemical composition rather than PM mass may be associated with increased PM-induced adverse health effects.

Many of the most productive agricultural counties in the nation are located in California's San Joaquin Valley (SJV), which is approximately 250 miles long, running through the middle of the state. While the SJV leads in agricultural value with the production of grapes, almonds, milk, oranges, and cattle, unfortunately, it also leads as one of the most polluted regions in the state and the country. The SJV is designated as a nonattainment area

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for airborne particulate matter (PM) pollution, and several counties in this region experience ambient PM concentrations that frequently exceed the average hourly National Ambient Air Quality Standard (NAAQS) of 35 $\mu g/m^3$ of fine/ultrafine PM (also referred to as $PM_{2.5}$; diameter $D_p < 2.5$ mm). Exposure to elevated levels of PM poses documented health risks to the 3 million residents of SJV, as evidenced by higher frequency of emergency-room visits and hospital admissions and increased morbidity and mortality due to cardiopulmonary complications, as reported for this region by Lipsett et al. (1997) and for other regions (Krewski et al. 2005; Meng et al. 2010; Ostro et al. 2006).

PM pollution generated and retained in the bowl-shaped SJV originates from a variety of sources, including resident vehicles, vehicles traveling up and down the state on Highway 99 and Interstate 5, local farming and industry, and transport from the San Francisco Bay Area and Sacramento, which accounts for approximately 11% of the regional air pollution according to the SJV Air Pollution Control District. In addition, the SJV region is impacted by unique regional and seasonal patterns (wintertime stagnation events with valley fog and hot, dry summers with long sunny days), as well as the seasonal planting and harvesting of crops. PM-induced health effects have been evaluated in toxicological studies in many regions of the United States, as documented in human subjects (Dominic et al. 2005; Ghio et al. 2000) and in laboratory species (Clarke et al. 2000; Saldiva et al. 2002). Many PM-induced toxicity studies reported a role for season and location in PM toxicity (Becker et al. 2005; Hetland et al. 2005). When analyzed on a national scale, seasonal impacts on respiratory and cardiovascular diseases were highest during winter and may be reflective of seasonal and regional differences in emissions and atmospheric chemistry and therefore chemical composition (Bell et al. 2007; Seagrave et al. 2006). Despite established associations between PM exposure and increased cardiopulmonary morbidity and mortality within California (Ostro et al. 2006; 2007), the toxicity of inhaled SJV PM as a function of season and location has not been extensively studied (Meng et al. 2010; Smith et al. 2003). Differential PM-induced toxicity in other regions of the United States are likely attributable to seasonal and regional variability in PM concentrations (higher in winter) and chemical composition (influences of temperature and humidity) (Ham and Kleeman 2011; Lewis et al. 2010). A systematic evaluation of seasonal/regional contributions to PM-induced toxicity supports more targeted PM regulation to provide improved protection of public health.

Four field studies were performed in Fresno County, located in the center of the SJV, to evaluate regional/seasonal health impacts of inhaled SJV PM in a mouse model. Exposure atmospheres were created using a versatile aerosol concentration enrichment system (VACES), a system designed to support in vivo toxicity studies when coupled to an exposure chamber system (Kim et al. 2000; 2001a; 2001b). This approach allows for the evaluation of PM-induced adverse health effects at elevated but still realistic PM concentrations known as concentrated ambient particles (CAP). Two study locations, urban Fresno and rural Westside, CA, were chosen to highlight PM physical and chemical characteristics arising from seasonally and regionally specific sources, geography, and meteorology that impact the region. In the present study, the induction of pro-inflammatory, cytotoxic responses and pro-oxidant stress within the lungs of mice were examined following repeated, short-term exposure to SJV CAP in the summer and winter at Fresno and Westside. Biological responses to CAP were evaluated in the context of associations with CAP physical (mass, particle number, and surface area) and chemical composition.

MATERIALS AND METHODS

Animals

Twelve- to 14-wk-old male C57BL/6 mice weighing between 25 and 30 g were purchased from Charles River Laboratories, Inc. (Raleigh, NC), and shipped to the University

of California, Davis. Experimental and sham controls were transported in a climate-controlled van to the field site (Fresno or Westside, CA). At each field site, described in detail by Ham and Kleeman (2011), mice were placed in a climate-controlled mobile trailer unit where they were housed in plastic cages with TEK-Chip pelleted paper bedding (Harlan Teklad, Madison, WI), had access to food and water ad libitum, and experienced a 12-h light/dark cycle. Mice were housed in these cages for the duration of the study except during CAP inhalation, when they were transferred to exposure chambers. Prior to CAP exposures, mice were acclimated to these exposure chambers for 3 d for 8 h/d. Mice were weighed and randomly divided into ambient air and CAP exposure groups and were placed into exposure chambers for 6 h/d for 10 d over a 2-wk period (5 d exposed, 2 d unexposed, 5 d exposed) in urban Fresno and rural Westside during summer or winter. At necropsy, mice were subdivided into two separate groups with one group designated for lung lavage/protein analyses and one group designated for histology. Throughout the project, animals were handled in accordance with standards established by the U.S. Animal Welfare Acts as set forth in the National Institutes of Health Guidelines (Institute of Laboratory Animal Resources 1996).

Concentrated Ambient Particle (CAP) Exposure

Inhalable fine/ultrafine (F/UF, also known as $PM_{2.5}$) CAP were provided by a versatile aerosol concentration enrichment system (VACES) designed and evaluated in laboratory and field settings by Kim et al. (2000; 2001a; 2001b) and used extensively in our laboratory (Smith et al. 2003). Briefly, 220 L/min of ambient air is drawn in and passed through a preimpactor that removes particles with an aerodynamic diameter larger than 2.5 μm, which is representative of the regulatory NAAQS for $PM_{2.5}$. The air is drawn through a saturation–condensation system that grows the particles to 2- to 3-μm droplets that are then concentrated by a virtual impactor operated with a minor flow rate of 10 L/min to give a theoretical magnification of 22. Excess water vapor is removed as particles rapidly pass through silica-lined diffusion dryers, and the particles are returned to their original size prior to delivery to inhalation chambers. Field evaluations by Kim et al. (2001b) showed that the concentration enrichment process did not differentially affect particle size distribution or concentrations of chemical components, including volatile species, present in ambient PM. Laboratory investigations of the volatile, highly water-soluble species confirmed these field evaluations (Jung et al. 2010). Further, the VACES enhanced ultrafine PM without substantial changes to particle number or morphology. For these studies, CAP inhalation experiments were conducted during the morning and early afternoon, approximately 9:00 a.m. to 3:00 p.m., to capture the development and dissipation of $PM_{2.5}$ emissions from the morning rush hr event and nearby agricultural activities beginning around 6:00 a.m. Flow to individual exposure chambers was measured and balanced to ensure consistent exposure, and mice were systematically rotated within and between the chambers.

Exposure Atmosphere Characterization

Ambient and CAP mass and chemical composition were determined through direct filter analysis. Gas-phase pollutant measurements were obtained using archived hourly data collected by the California Air Resources Board (CARB) using the iADAM Air Quality Data Statistics online database (http://www.arb.ca.gov/adam) for stationary monitors located in urban Fresno (First Street Station) and a representative rural location near Westside (Parlier Station). Ambient and CAP exposure was characterized using PM collected adjacent to the VACES inlet (ambient PM) and from quartz filters placed in series with the CAP inhalation chambers to determine size and chemical composition distribution using previously reported methods (Herner et al. 2005; 2006). Briefly, six micro-orifice uniform deposit cascade impactors (MOUDIs, MOUDI model 110,

TABLE 1. CAP Characterization: Mass, Number, and Surface Area

Location/ Season	Mass Concentration (µg/m3)		Number Concentration (#/cm^3)		Surface Area Concentration (nm^2/cm^3)		Particle Concentration Factor
	Ambient	*CAP*	*Ambient*	*CAP*	*Ambient*	*CAP*	
Fresno Summer	20	284	7290	104038	4.00E+08	6.00E+09	14
Fresno Winter	17	156	1120	10070	9.00E+07	8.00E+08	9
Westside Summer	10	126	9104	120016	6.00E+07	8.00E+08	13
Westside Winter	15	86	2628	14956	3.00E+08	1.00E+09	6

Note. Real-time PM mass, number, and surface area concentrations were obtained at 2-min intervals with a scanning mobility particle sizer (SMPS) and aerodynamic particle sizer (APS) for each site/season CAP experiment: Fresno summer, Fresno winter, Westside summer, and Westside winter. Particle concentration factors are based on PM mass.

MSP Corp.), and three reference ambient air samplers (RAAS, RAAS2.5–400, Andersen Instruments) were operated in parallel to obtain sufficient mass for chemical analysis and to provide duplicate measurements for quality control checks. An Air and Industrial Hygiene Laboratory (AIHL) cyclone separator was used upstream of each MOUDI to remove coarse particles that might otherwise bounce off collection substrates. Each leg of the RAAS filter samplers was configured with the same cyclone at the same flow rate so that the sum of MOUDI impaction stages could be compared to the colocated filter measurement. Colocated ambient PM samples were compared through a regression analysis to directly assess precision and gain some indirect insight into accuracy (since expected sampling biases are different for filter collection vs. impaction). Three MOUDIs were loaded with Teflon (R2PJ047, Pall Corp.) filters for gravimetric, water-soluble ions, and trace species analysis. Three additional MOUDIs were operated with aluminum foil substrate and quartz fiber filters for gravimetric and carbonaceous analysis. Gravimetric measurement on all filters was completed using a CAHN-33 microbalance (resolution of ±1 µg). Real-time PM mass, number, and surface area concentrations were obtained at 2-min intervals with a scanning mobility particle sizer (SMPS, TSI model Classifier 3080, DMA 3081, CPC 3025) and aerodynamic particle sizer (APS, TSI model 3321) and are depicted in Table 1. Substrates were analyzed for levels of chemical species known to contribute highly to PM mass, be source tracers, or possess potential toxicity. Teflon substrates were used to determine levels of 8 major water-soluble ions—chloride (Cl^-), nitrates (NO_3^{2-}), sulfates (SO_4^{2-}), sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^+), and calcium (Ca^{2+})—using ion chromatography, and levels of 23 trace elements (Table 2) using inductively coupled plasma–mass spectrometry (ICP-MS). Assessment of elemental carbon (EC) and organic carbon (OC) was performed on Teflon and foil substrates using a thermo-optical method following the temperature protocol specified by the National Institute of Occupational Safety and Health (NIOSH) (Herner et al. 2005). Quality control was maintained by summing the concentrations of each species on each MOUDI stage (six filters) to calculate an integrated MOUDI $PM_{1.8}$ concentration ($\sum$ (MOUDI)) and comparing this to the RAAS $PM_{1.8}$ concentration of each species (one filter). Only those measurements with $0.5 \leq \sum(\text{MOUDI})/\text{RAAS} \leq 1.5$ were used in the current study. Chemical composition data was divided by total CAP mass and is expressed as µg/µg CAP or ng/µg CAP for major and trace species, respectively.

TABLE 2. CAP Characterization: Major and Trace Chemical Components

	Fresno		Westside	
	Summer	Winter	Summer	Winter
Total CAP Mass ($\mu g/m^3$)	284	156	126	86
Major Species (μg / μg CAP)				
Organic Carbon (OC)	0.206	0.163	0.171	0.334
Elemental Carbon (EC)	0.047	0.026	0.028	0.048
Nitrate (NO_3^-)	0.054	0.315	0.044	0.455
Sulfate (SO_4^{2-})	0.120	0.057	0.102	0.060
Ammonium (NH_4^+)	0.031	0.108	0.037	0.153
Chloride (Cl^-)	0.006	0.003	0.008	0.013
Trace Species (ng /μg CAP)				
Lithium (Li)	0.003	nd	nd	nd
Sodium (Na)	3.854	0.378	1.429	0.322
Magnesium (Mg)	1.055	0.120	0.275	0.074
Aluminum (Al)	8.035	3.536	7.468	3.176
Phosphorus (P)	0.004	0.001	0.003	0.002
Sulfur (S)	79.899	46.686	43.067	29.635
Potassium (K)	0.950	0.628	0.180	0.153
Calcium (Ca)	1.392	0.429	0.400	0.205
Titanium (Ti)	0.112	0.046	0.052	0.026
Manganese (Mn)	0.030	0.039	0.020	0.011
Iron (Fe)	0.613	1.291	0.358	0.153
Copper (Cu)	0.108	0.066	0.095	0.008
Zinc (Zn)	0.113	0.112	0.096	0.044
Gallium (Ga)	0.003	0.002	0.001	0.000
Germanium (Ge)	0.003	0.003	0.003	0.002
Arsenic (As)	0.005	0.013	0.001	0.007
Selenium (Se)	0.019	0.006	0.005	0.007
Bromine (Br)	0.335	0.146	0.185	0.083
Rubidium (Rb)	nd	nd	nd	nd
Strontium (Sr)	0.007	0.002	0.007	0.002
Tin (Sn)	0.145	0.045	0.148	0.042
Antimony (Sb)	0.069	0.055	nd	0.002
Barium (Ba)	0.066	0.047	0.022	0.009

Note. Average concentrations for several major PM components measured over the 10-d study duration for each site/season CAP experiment: Fresno summer, Fresno winter, Westside summer, and Westside winter. Data are normalized to the total CAP mass and are expressed as $\mu g/\mu g$ CAP or ng/μg CAP for major or trace components, respectively. Extraction and analytical methods are outlined in the text.

PM samples were tested for the presence of endotoxin using according to the manufacturer's instruction (*Limulus* amebocyte lysate [LAL] assay, catalogue number KQCL-1000, Chromogenic, Lonza, MD). All samples were assayed without dilution with the exception of those from the Fresno summer exposure due to background interference. The assay limit of detection is 0.0005 EU/ml. Values were normalized to PM mass and expressed as EU/μg PM.

Bronchoalveolar Lavage (BAL)

One set of mice was designated for bronchoalveolar lavage (BAL) and lung tissue collection for protein analysis. The trachea was cannulated, the lungs were lavaged with 2 ml total flushed thrice into and out of the lung, and collected BAL was centrifuged at 1200 × g for 10 min at 4°C. The cell pellet was resuspended to determine total cell count and viability. Cell viability was determined by measurement of 0.4% trypan blue exclusion from cells using a hemocytometer (Sigma, St. Louis, MO). Cytospins were prepared using a Shandon Cytospin (Thermo Shandon, Inc., Pittsburg, PA) using 100 μl of the cell suspension for each slide. Slides were dried at room temperature prior to fixing and staining with DiffQuick (International Reagent Corp, Kobe, Japan). BAL cell profiles were determined using light microscopy (1000 cells/sample).

Biochemical Analyses of BAL

Cell-free supernatant was analyzed for cell damage; total protein levels (Quick Start Bradford Protein Assay, catalogue number 500-0201, Biorad, Hercules, CA) and lactate dehydrogenase (LDH) activity (LDH Cytotoxicity Assay Kit, catalogue number 10008882, Cayman Chemical, Ann Arbor, MI) were determined according to the manufacturer's recommended protocols.

Protein Expression in Lung Tissue

Protein from the right caudal lobe was extracted using Qiagen TissueLyser (Valencia, CA) to ensure consistent homogenization for all lung tissue samples. Samples were centrifuged at 4500 × g for 15–20 min, and supernatant was collected and stored overnight at –80°C.

Total protein was determined using the Lowry et al. (1951) method with a commercial kit and bovine serum albumin (BSA) standards (DC Protein Assay Kit I, catalogue number 500-011, BioRad, Hercules, CA). Protein levels of key pro- and anti-inflammatory cytokines and chemokines were analyzed using a customized multiplex enzyme-linked immunosorbent assay (ELISA) with selected cytokines and chemokines (Bio-Plex Pro Assays, catalogue number MF000KCAF0Y, BioRad, Hercules, CA). Targets evaluated included simultaneous quantitative analysis of protein expression of interleukin (IL)-1α, -1β, -6, -10, -12(p70), and -17; granulocyte–macrophage colony-stimulating factor (GM-CSF); granulocyte colony-stimulating factor (G-CSF); interferon gamma (IFN-γ); keratinocyte-derived chemoattractant (KC); monocyte chemoattractant protein (MCP-1); monocyte inflammatory protein (MIP-1α); and tumor necrosis factor (TNF)-α. Oxidant stress was assessed in lung tissue through analysis of stress-response protein heme oxygenase-1 (HO-1) by commercial ELISA kit (Mouse Heme Oxygenase-1 EIA Kit, catalogue number MK125, Takara Bio, Inc., Shiga, Japan).

Lung Histology

A second set of mice was designated solely for histology. For these mice, the trachea was cannulated and lung lobes were fixed with 4% paraformaldehyde. Lungs were processed for analysis via light microscopy of 5-μm paraffin-embedded sections. Lung sections were prepared to visualize the third, mid-level, and distal airway generations in cross section of the left lung lobe. Lung sections were stained with hemotoxylin and eosin (H&E) for histological and pathological assessment and alcian blue/periodic acid Schiff's stain (AB/PAS) for evaluation of mucosubtances within the main axial path of the airways. Sections were labeled immunohistochemically with myeloperoxidase (MPO), an abundant enzyme in neutrophil granulocytes (Bradley et al. 1982), according to the manufacturer's instruction (Thermo Fisher Scientific, Fremont, CA).

Statistics

JMP statistical software was used to calculate descriptive statistics and data analysis (JMP, SAS Institute, Inc., Cary NC). Data in tables and figures are expressed as mean values ± standard error of the mean ($n = 5$–6 per experimental group). Using location/season/exposure as a single factor, a multivariate analysis of variance (MANOVA) was used on log-transformed data to examine the effect of CAP exposure compared to ambient air. Briefly, MANOVA results in an overall estimation of effect on the set of dependent variables that protect against an inflated type 1 error. Pairwise effects of season/location were evaluated using one-way analysis of variance (ANOVA) with Tukey's post hoc analysis to protect against type 1 error. Differences were considered statistically significant when p values were less than .05.

RESULTS

Exposure Atmosphere Characterization

CAP physical characteristics and chemical composition for both sites/seasons are presented in Tables 1 and 2, respectively. Figure 1 illustrates differences in major chemical species between studies. At both Westside and Fresno, the delivered CAP concentration was approximately 13– to 14-fold higher than the ambient concentration during summer and approximately 6– to 9-fold higher than the ambient concentration in winter based on mass concentration (Table 1). The difference in enrichment is likely due to seasonal differences in temperature and humidity. Even though CAP mass concentrations were highest for the Fresno summer and lowest for the Westside winter exposures (Figure 2), all of the CAP concentrations measured were significantly above the 24-h average NAAQS for $PM_{2.5}$. CAP particle number concentration was highest during the summer at both locations compared to the winter. CAP surface area particle concentrations were highest during the Fresno summer and Westside winter exposures, while the Fresno winter and Westside summer were relatively

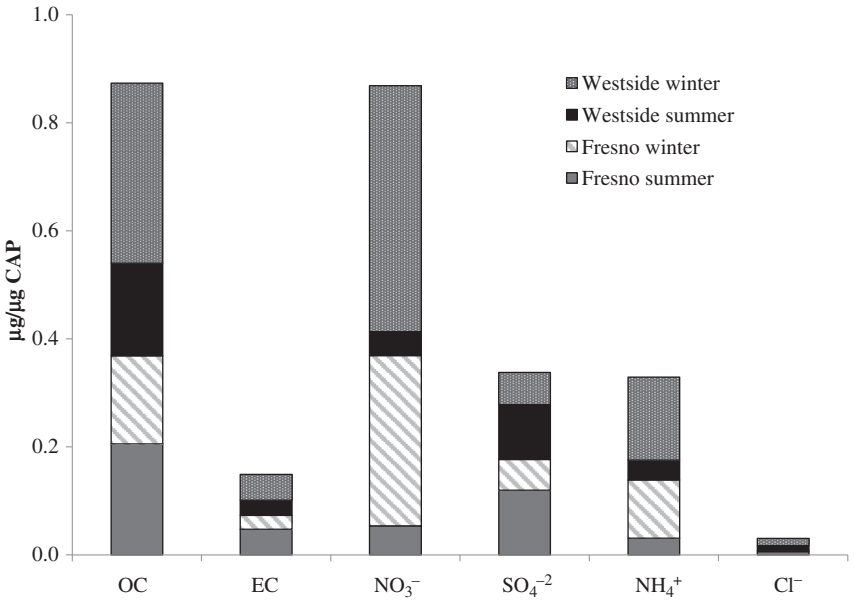

FIGURE 1. CAP characterization: major chemical components. Graphical representation of major chemical components for each site/season experiment normalized to the total CAP mass and expressed as µg/µg CAP. Major components include organic carbon (OC), elemental carbon (EC), nitrates (NO_3^-), sulfates (SO_4^{2-}), and chloride (Cl^-).

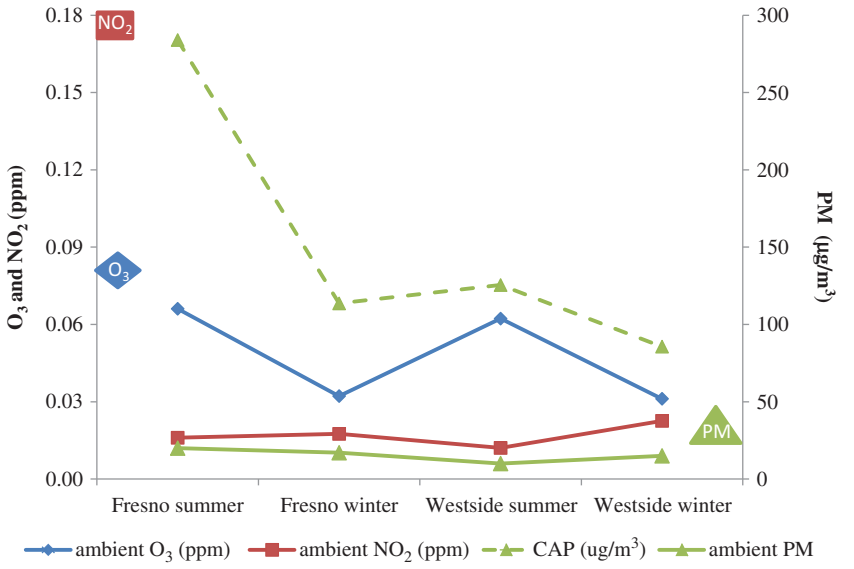

FIGURE 2. Exposure atmosphere characterization. For all exposures, mice inhaled concentrated ambient particles (CAP) as delivered by the versatile aerosol concentration enrichment system (VACES). CAP concentrations are presented graphically by triangles connected by a dashed line. Ambient levels of PM, ozone (O_3), and nitrogen dioxide (NO_2) are represented by triangles, diamonds, and squares, respectively, and are connected by a solid line. The VACES does not concentrate gases and therefore during each site/season CAP experiment control and CAP mice were exposed to equivalent levels of O_3 and NO_2. During all field studies only CAP concentrations were above regulated levels, while O_3, NO_2, and ambient PM were below NAAQS, which are denoted by the larger equivalent shape for each pollutant (color figure available online).

lower and equivalent to each other. As illustrated in Table 2 and Figure 1, site/season differences in CAP chemical composition were observed. When the concentration of major species was normalized to CAP mass, OC and, to a lesser extent, EC levels were highest during the Westside winter experiment compared to the other experiments. Similarly, levels of NH_4^+

and NO_3^- were highest during the Westside winter experiment. In Fresno, OC and EC levels were highest in summer compared to winter but still lower than observed in Westside. As expected based on seasonal patterns, NH_4^+ and NO_3^- were elevated at least threefold during Fresno winter compared to Fresno summer. In contrast, for both locations, SO_4^{2-} and Cl^- were higher during summer compared to winter. The majority of trace species (ng/μg CAP) were higher in summer compared to winter with the exception of arsenic, which was highest in winter for both locations. Zinc, iron, sodium calcium, barium, and sulfur were higher in urban Fresno compared to rural Westside.

In Figure 2, gas-phase pollutants, O_3 and NO_2, are presented as 2-wk averages for each site/season based on CARB monitoring data. As expected, ozone levels were approximately twofold higher in summer compared to winter, while nitrogen oxide levels were relatively consistent across all studies. According to California's ambient air quality standards, the maximum 8-h average concentration for O_3 is 0.07 ppm and the maximum 1-h average for NO_2 is 0.18 ppm (CARB 2010). Hourly measurements reported on the CARB website (see Methods) indicate that for the majority of each exposure period, O_3 and NO_2 levels were below regulated levels with the exception of select hours in Fresno. Endotoxin measurements were within the range of the LAL assay and were below 0.1 EU/μg for all samples (data not shown).

Cellular/Biochemical Analysis of Lavage Fluid

Trends toward an elevation in the total cells recruited to the lungs in CAP-exposed animals compared to ambient air-exposed animals for both seasonal Fresno and the Westside winter exposures did not reach statistical significance (Figure 3A). Exposure to CAP significantly elevated lung neutrophils compared to exposure to ambient air in Westside winter and summer, with the most robust difference present in winter (Figure 3B). No statistically significant differences were observed in the amount of total protein present (Figure 4A) or LDH activity levels (Figure 4B) in the BAL supernatant between mice exposed to CAP and ambient air for any of the four field experiments, even though there was a there was a trend toward significantly elevated LDH activity levels in Fresno summer CAP-exposed mice compared to ambient air exposed mice. In Figures 3 and 4, CAP mass concentrations are plotted over BAL inflammatory indices to provide a visual demonstration of the absence of a relationship between adverse health effects and PM mass concentrations.

Biochemical Analysis of Lung Tissue

No marked difference in lung tissue HO-1, an oxidative stress marker, protein levels were found for the 4 experiments (Figure 4C). Figures 5–7 depict protein expression of key proinflammatory mediators in ambient

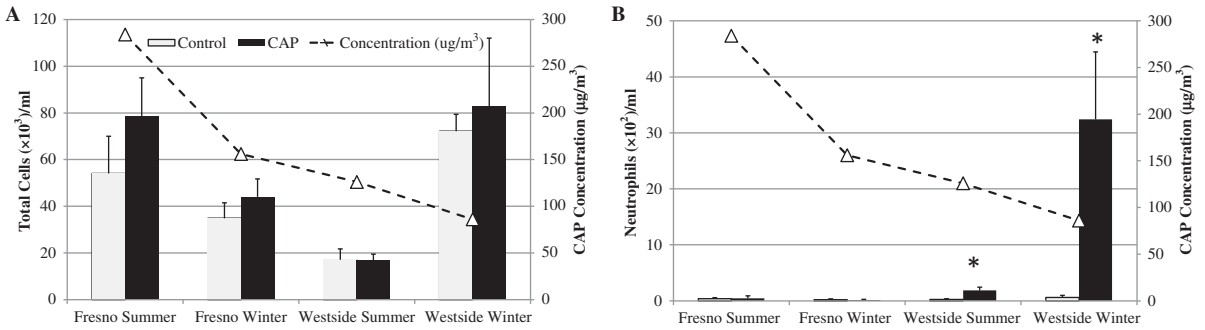

FIGURE 3. BAL cell profile. Total leukocytes (A) and neutrophils (B) recovered via bronchoalveolar lavage 48 h post exposure from mice exposed to ambient air (white bars) or CAPs (black bars), expressed as mean ± SEM ($n = 6$). The triangles connected by a dashed line graph the mean mass concentration of CAP in the chambers during each study. Asterisk indicates significant difference from ambient air control ($p < .05$).

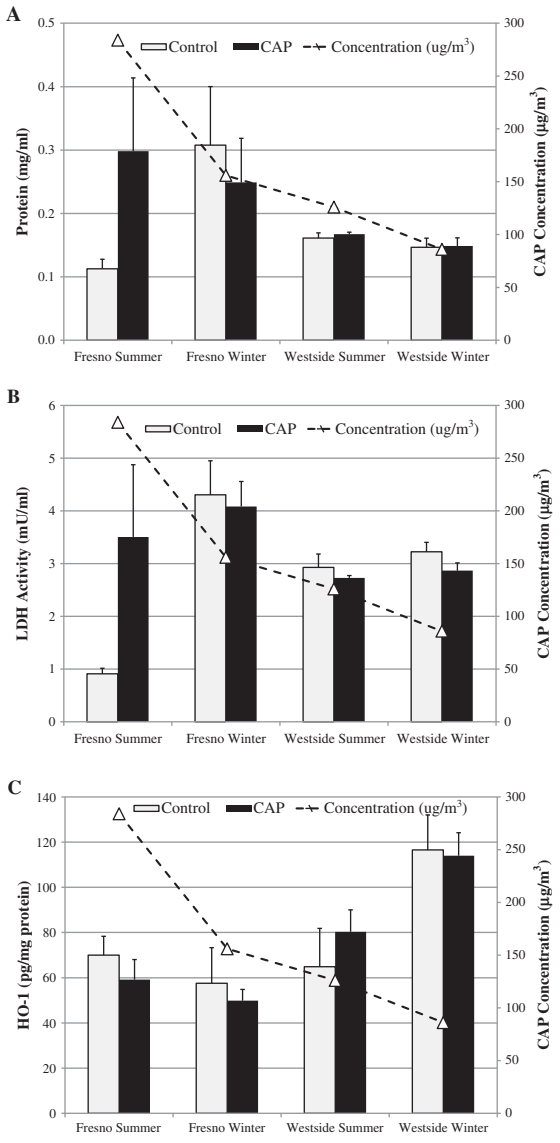

FIGURE 4. BAL and lung tissue assays. Protein (A) and lactate dehydrogenase (LDH) expression (B) in BAL fluid and heme oxygenase-1 (HO-1) expression (C) in lung tissue recovered at 48 h post exposure from mice exposed to ambient air (white bars) or CAPs (black bars), expressed as mean ± SEM ($n = 6$). The triangles connected by a dashed line graph the mean mass concentration of CAP in chambers during each study.

air and CAP exposed groups. After being exposed to Fresno summer CAP, animals displayed significantly reduced protein levels of proinflammatory chemokines KC and MCP-1 and proinflammatory cytokine IFN-γ (Figures 5A and 6A) compared to animals exposed to ambient air. Despite significant elevations in neutrophil recruitment observed in the Westside studies, no significant differences in inflammatory mediators were measured in Westside CAP-exposed mice compared to control mice. For cytokines IL-10, IL-17, and G-CSF, expression was below detection (data not shown).

Lung Histology

Histological assessment of lung sections did not demonstrate significant pathologic changes of the airways or parenchyma following exposure to ambient air or CAP. The subtle presence of neutrophils was noted in the lungs of mice exposed to both ambient air and CAP. Immunohistochemical staining with myeloperoxidase (MPO) indicated that the neutrophils were more prevalent in the lungs of mice exposed to CAP, primarily in airway walls and in capillaries near bronchiole–alveolar duct junctions (Figure 8). Neutrophils were most prominent in mice following CAP exposure during the winter season at Westside. Although mice exposed to CAP demonstrated an increased frequency for AB/PAS stained mucosubstances in the most proximal airways of the lungs (Figure 9), especially the Westside winter exposure group, quantitative histological analysis did not achieve statistically significant differences. Mucosubstances were found in or near the second-generation intrapulmonary airways of CAP-exposed animals and were typically located closest to the accompanying arterial and pulmonary veins. In a few instances, the presence of these positive cells was observed to extend to the third-airway generations.

DISCUSSION

National trends show a strong epidemiological association between exposure to elevated PM concentrations and increased frequency of morbidity and mortality (Dominici et al. 2005; Samet and Krewski 2007; Samet et al. 2000). Lipsett et al. (1997) and others have reported similar trends for several counties within California, including some within the SJV (Ostro et al. 2006; 2007). Numerous human

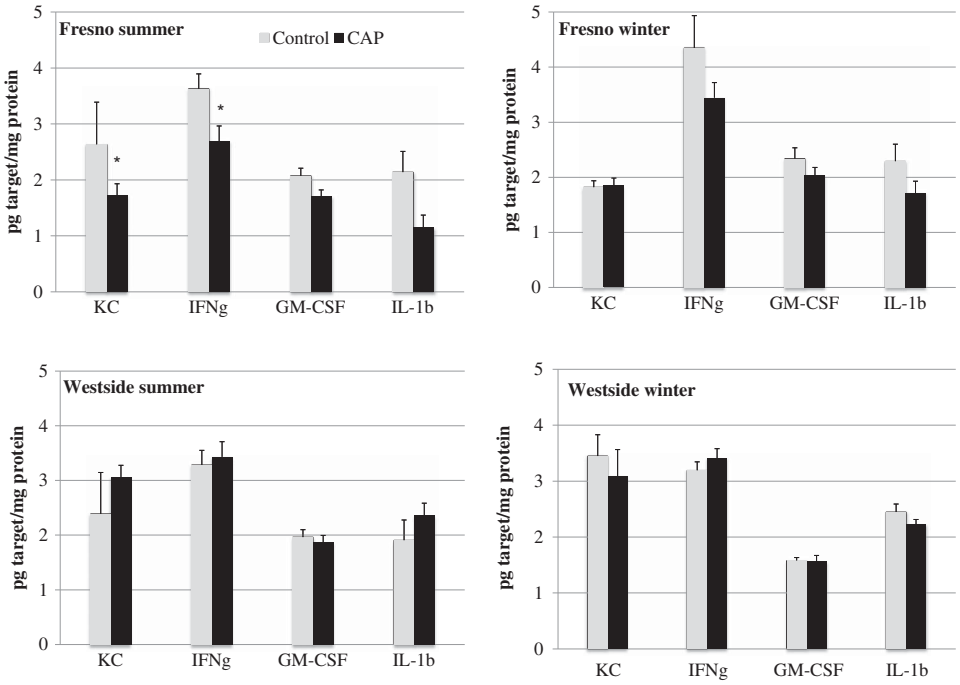

FIGURE 5. Proinflammatory cytokine and chemokine expression in lung tissue. Expression of KC, IFN-γ, GM-CSF, and interleukin (IL)-1β in right caudal lung tissue recovered 48 h post exposure from mice exposed to ambient air (white bars) or CAPs (black bars) during Fresno summer and winter and Westside summer and winter, expressed as mean ± SEM ($n = 6$). Asterisk indicates significant difference from ambient air control ($p < .05$).

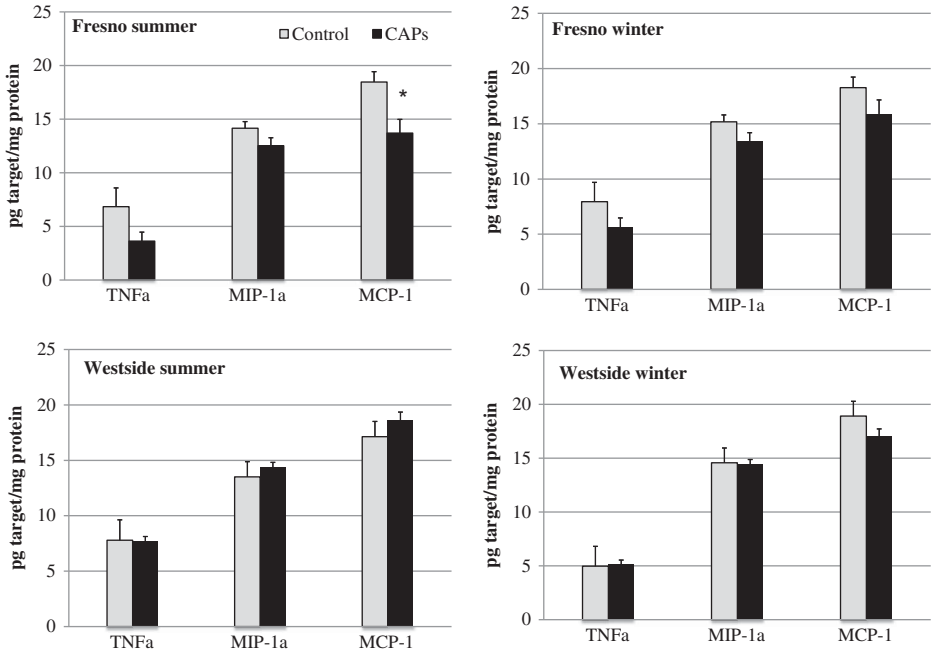

FIGURE 6. Proinflammatory cytokine and chemokine expression in lung tissue. Expression of TNF-α, MIP-1α, MCP-1 in right caudal lung tissue recovered 48 h post exposure from mice exposed to ambient air (white bars) or CAPs (black bars) during Fresno summer and winter and Westside summer and winter, expressed as mean ± SEM ($n = 6$). Asterisk indicates significant difference from ambient air control ($p < .05$).

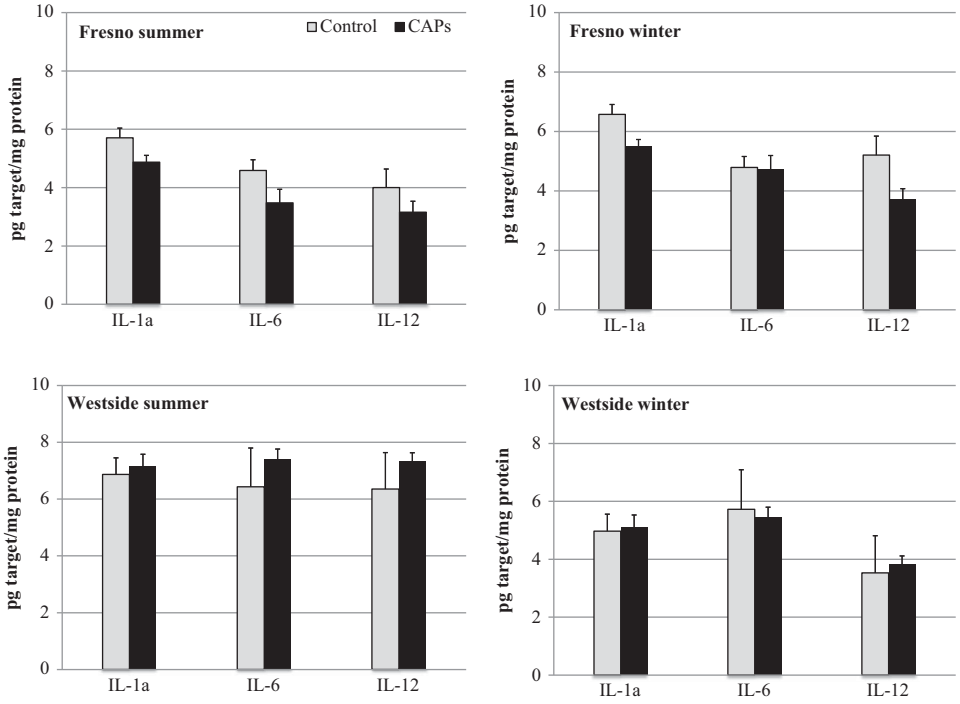

FIGURE 7. Proinflammatory cytokine and chemokine expression in lung tissue. Expression of interleukin (IL)-1α, IL-6, and IL-12 in right caudal lung tissue recovered 48 h post exposure from mice exposed to ambient air (white bars) or CAPs (black bars) during Fresno summer and winter and Westside summer and winter, expressed as mean ± SEM ($n = 6$).

and animal toxicological studies indicated that PM exposure induced adverse health effects and provide evidence that PM-induced toxicity is dependent upon season and location (Bell et al. 2008; Hetland et al. 2005; Peng et al. 2005; Samet et al. 2007; Seagrave et al. 2006). However, few studies evaluated SJV PM-induced toxicity in the context of distinct seasonal and regional influences on PM physical and chemical properties. This evaluation is important because the heavily populated SJV is classified as both a federal and state nonattainment area for PM and little is known about the PM characteristics driving adverse health effects.

This set of field studies evaluated the potency of inhaled summer/winter and urban/rural SJV PM in the context of seasonal and regional contributions to PM-induced toxicity. Acute pulmonary inflammation is consistently used as a measure of PM-induced response and is reported as a sensitive endpoint for other CAP studies in humans and laboratory species (Ghio et al 2000; Smith et al. 2003). More specifically, neutrophilic inflammation observed in numerous PM toxicity studies is currently regarded as a central mechanism for disease development and exacerbation within the respiratory tract (Pope and Dockery 2006) and represents a relevant biological endpoint for toxicity comparisions. Another mechanistic hypothesis for disease development and exacerbation is the development of oxidative stress, a result of neutrophilic oxidative bursts or due to the association of redox active components on the PM surface (Becker et al. 2005). Therefore, inflammatory, cytotoxic, and oxidant stress endpoints following repeated, short-term SJV CAP inhalation in a healthy mouse model were characterized. Results indicate that inhaled PM-induced toxicity differs between sites and seasons and that these differences do not appear to be associated with PM mass concentration. In the present study, PM-induced toxicity appears to be associated with relatively higher exposure to OC and NO_3^- present in Westside winter CAP compared to other site/season experiments despite

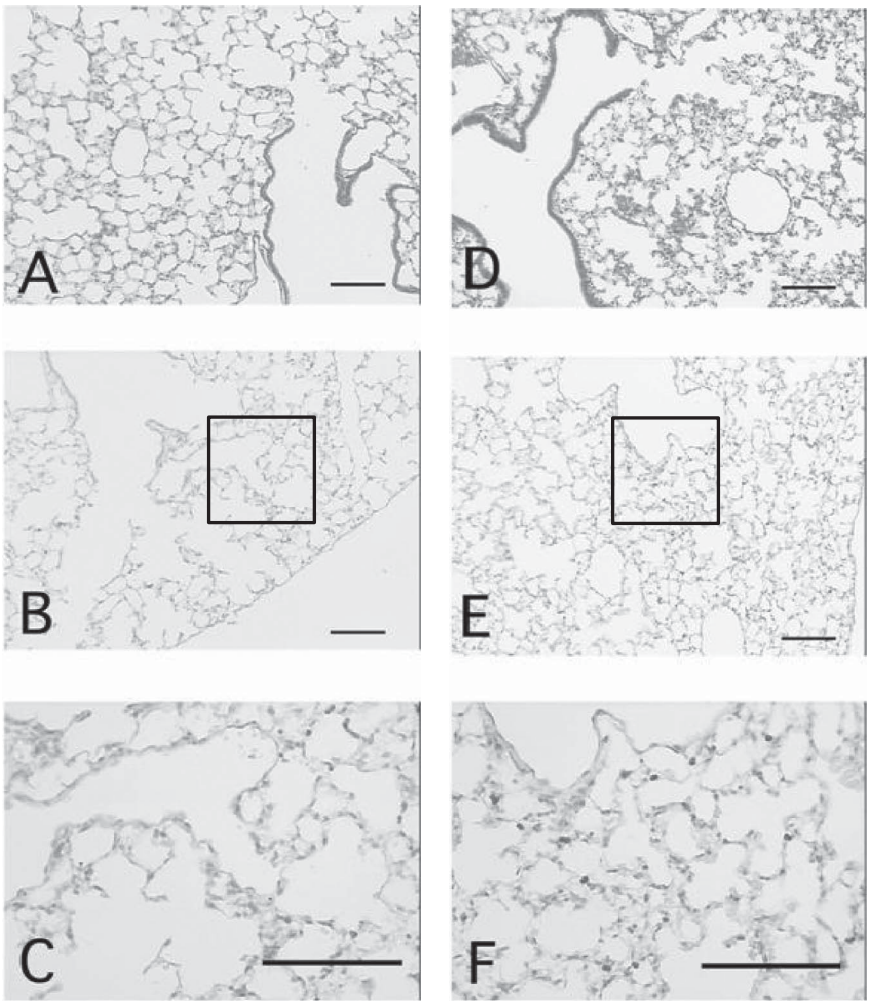

FIGURE 8. Lung histology: inflammation. Representative lung sections depicting cellular infiltration of the lungs of mice exposed to ambient air (A–C) and CAPS (D–F) during the Westside Winter experiment. (A) and (D) demonstrate subtle cellular infiltration at low magnification. (B) and (E) demonstrate diffuse presence of neutrophils labeled with myeloperoxidase, with boxed areas shown at a higher magnification in (C) and (F), respectively. Magnification bar is equivalent to 100 μm.

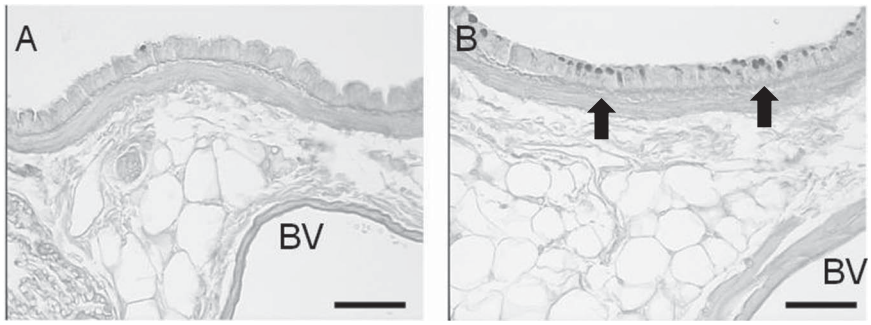

FIGURE 9. Lung histology: mucosubstances. Representative lung sections stained with alcian blue/periodic acid Schiff stain (AB/PAS), which is identified with black arrows, from mice exposed to ambient air (A) or CAPS (B) during the Westside winter experiment. There was an increased frequency of epithelial cells staining positive for presence of mucosubstances within proximal airways of mice exposed to CAPS (B). BV, blood vessel. Magnification bar is equivalent to 50 μm.

exposure to the lowest CAP concentration. These results suggest that PM-induced toxicity may be dependent on site/season impacts on PM chemical composition rather than PM mass concentration.

In this set of field studies, rural Westside PM elicited a more robust inflammatory response in the lungs of mice compared to urban PM following repeated short-term inhalation for a total of 10 d. More specifically, rural Westside summer and winter CAP, at mass concentrations ranging from 86 to 126 $\mu g/m^3$, elicited in the lungs of healthy mice neutrophilic inflammation that was not observed in the lungs of Westside control mice exposed to ambient air. In contrast, mice exposed to summer/winter urban Fresno CAP did not exhibit an inflammatory response despite exposure to relatively higher PM mass concentrations ranging from 156 to 284 $\mu g/m^3$ compared to other site/season experiments. The significant elevation in neutrophil recruitment observed following inhalation of rural CAP during summer and winter seasons was independent of changes in total cells recruited to the lungs or proinflammatory cytokine and chemokine expression in lung tissue. Most notably, inflammatory responses were the most robust in the group of mice exposed to the lowest CAP concentration during the Westside winter study compared to the summer study. Cellular inflammation was observed in the presence of subtle increases in mucosubstances within the airway epithelium of Westside winter CAP-exposed mice. Data suggest that season and location influence PM-induced toxicity in a manner that does not appear to relate to PM mass concentrations.

Epidemiological studies routinely use PM mass concentrations collected from stationary monitors to evaluate adverse health impacts through hospital morbidity and mortality records. However, a consistent association between exposure to elevated PM mass concentration and toxicity has been difficult to reproduce in toxicological studies where measured endpoints are often more acute than chronic health impacts. In this set of field studies, no significant associations were found between inflammatory responses and PM physical parameters, mass, number, or surface area when these parameters were averaged weekly (5 d) or over the entire study duration (10 d) for all 4 studies. For all studies CAP concentrations in wk 1 and 2 were similar with the exception of Fresno summer, when wk 2 average CAP concentrations were numerically higher than wk 1 (data not shown). The presence of inflammation under relatively low CAP concentration days in rural Westside compared with urban Fresno supports previous findings that low CAP concentrations induce a more robust pulmonary inflammatory response than high CAP concentrations (Kodavanti et al. 2005) and that these responses are likely driven by CAP chemical composition (Saldiva et al. 2002).

A major hypothesis of this study was that site/season impacts on particle composition might be associated with biological responses in healthy mice, based on seminal studies indicating that marked changes in ambient conditions significantly affect health outcomes (Pope 1989; Pope et al 2007). A recent time-series analysis demonstrated a stronger association between daily mortality and concentrations of elemental carbon (EC), organic carbon (OC), nitrates (NO_3^-), and other metals and weaker associations with $PM_{2.5}$ mass in CA (Ostro et al. 2007). In the present study, an enhanced contribution from OC and NO_3^- observed during Westside winter was associated with significant increases pulmonary inflammation compared to ambient air controls that was not observed in other site/seasons. It is important to note that normalization of chemical composition to CAP mass was a critical step in elucidating these associations. This is in agreement with previous studies from our laboratory showing that fall and winter SJV PM induce significant neutrophilic inflammation in the presence of elevated PM mass, OC, and NO_3^- (Smith et al. 2003). Levels of trace chemical species, including notable transition metals zinc and iron, were not significantly associated with inflammatory responses observed. Arsenic was the only trace metal to exhibit a distinct seasonal pattern, higher in winter compared to summer for each location, but was not associated

with inflammatory response patterns. Overall, these findings contribute to the greater body of evidence that CAP-induced inflammation is influenced by PM chemical composition more so than PM mass (Kodavanti et al. 2000; 2005; Saldiva et al. 2002; Harkema et al. 2004; Cassee et al. 2005).

In this study, site-specific and seasonal sources, source proximity, geography, and meteorological patterns likely explain observed differences in ambient particle concentration, number, and chemical composition during summer and winter seasons in urban and rural locations (Ham and Kleeman 2011). These parameters contribute to heterogeneity in toxicological studies that has been attributed to chemical composition (Gilmour et al. 2007; Happo et al. 2004; 2010; Jalava et al. 2004; Seagrave et al. 2006; Steerenberg et al. 2006). During these four field studies, chemical composition varied according to expected patterns of emission sources and meteorology influenced by season and location within the SJV according to reports from Kleeman et al. (2008) and others (Chow et al. 1992; 1993; 2008; Ham et al. 2010; Watson et al. 2002). Ham and Kleeman (2011) sampled concurrently to the toxicological studies reported here and used a molecular marker chemical mass balance (MM-CMB) source apportionment method to evaluate source contributions for each site/season experiment. Source apportionment results indicate that OC is generated from residential wood combustion, diesel engines, and meat cooking, although the source origin of a majority of the organic PM fraction could not be identified. This is likely indicative of the influence of atmospheric processing to primary organic aerosol (POA) and secondary organic aerosol (SOA) that occurs during the study hours. The rural location has higher POA and SOA compared to the urban location, which may represent the influence of additional atmospheric aging time that occurs as PM is transported from urban locations. These patterns can be attributed to differences in source type and strength and degree of atmospheric processing occurring in the presence of seasonal trends, including wintertime stagnation events and hot, dry summers. Seasonal atmospheric processes influence degrees of chemical volatility and partitioning of chemical species between the gas or particle phases, which may lead to differences in adsorbed components such as various polycyclic aromatic hydrocarbons (PAH), a main component of OC. Increased atmospheric OC in the winter compared to the summer may represent a difference in the degree of source contributions, with a greater contribution from residential wood combustion for home heating (Chen et al. 2007). Increased potency of rural compared to urban CAP may result from a difference in the degree of atmospheric processing. Taken together, these factors may explain the increased potency of rural winter CAP compared to CAP in the other site/season experiments.

The approach used in this study has several unique advantages to evaluate regional and seasonal impacts on SJV PM-induced toxicity. First, the VACES allows for direct evaluation of the impacts of exposure to elevated PM concentrations as experienced in the real world. The exposure system allows for these evaluations in the presence of ambient levels of gas-phase pollutants, such as ozone (O_3) and nitrogen oxides (NO_x). CAP exposures were clearly elevated to above the current PM NAAQS, in contrast to gas-phase pollutants, O_3 and NO_2, which were generally below the NAAQS and did not appear to influence biological responses in this study. In addition, male C57/BL6 mice were selected for these studies due to extensive use as described in the literature and in environmental and inhalation studies. Male mice were used to avoid potential influences of hormonal fluctuations on measured responses. Sham controls were handled and transported in an identical fashion to exposed groups to allow for direct comparisons between ambient and CAP-induced health effects for each experiment.

Although the present study suggests a role for PM chemical composition in adverse health effects, other potential explanations for these findings are worth noting. Lack of an association between PM mass and biological responses may be a result of the averaging of

exposure concentrations over the longer exposure duration compared to previous studies from our laboratory, where associations were found between 3-d averages and biological responses (Smith et al. 2003). Although each CAP experiment is different, repeated studies during equivalent seasons at Fresno and Westside may provide further confirmation of association with key chemical components. Correlations between health parameters and specific chemical components were derived from the combination of multiple experiments in the same location/season to generate significant correlations that were not evident in single experiments (Cassee et al. 2005; Kodavanti et al. 2005; Saldiva et al. 2002).

Additional factors to contemplate from the results of this study include the importance of endpoints, timing, and adaptability of the respiratory tract following repeated CAP exposure. Braga et al. (2001) and others reported that detectable PM-induced biological effects can be measured at 48 h post exposure to CAP (Steinvil et al. 2009; Ostro et al. 2007). However, the absence of a pulmonary response despite the highest urban exposure concentrations in the urban setting emphasizes the importance of optimizing endpoint timing to detect inflammatory responses following exposure to different PM concentrations or compositions. Daily repeated exposure to CAP may have influenced the responsiveness of the lungs to additional insults and possibly adaptability as suggested in PM, O_3, and endotoxin inhalation studies (Elder et al. 2000; Pereira et al. 2007; Plopper et al. 1994; van Bree et al. 2002). Ozone inhalation studies suggest that consecutive exposures (12 h/5 d) result in an almost complete disappearance of inflammatory responses that are observed after a single day of exposure (van Bree et al. 2002). Further, as demonstrated by van Bree et al. (2002), the time period after attenuation is followed by a gradual recovery and an almost complete return to original susceptibility to ozone. Rodents exposed continuously (20 h) but not intermittently (5 h/4 d to approximately 100 $\mu g/m^3$ urban PM had altered levels of lung peroxidation (Pereira et al. 2007). Similarly, rats preexposed to low doses of endotoxin were less responsive to a high dose challenge compared to naive rats, suggesting an adaptive response (Elder et al. 2000). Therefore, in the healthy model used here, the necessary biological response to avoid considerable damage that results from consistent presence of phagocytes, such as macrophages and neutrophils, may result in a decreased responsiveness to subsequent exposures (Soehnlein and Lindbom 2010). The importance of the potential adaptive response induced by repeated PM exposure is unknown. However, it is possible that the attenuation of the pulmonary inflammatory response, evident here in the absence of Fresno PM-induced neutrophil influx and reductions in cytokines and chemokines, may impact the normal physiological response needed to prevent further damage (van Bree et al. 2002).

In conclusion, rural, but not urban, CAP induced inflammatory responses not associated with particle mass concentration in the lungs of healthy mice. Data presented here report associations between the greatest biological responses and levels of OC and NO_3^- observed in the winter rural experiment. These findings suggest that the enhanced potency of rural PM may be due to atmospheric aging and particle transport from urban centers. Clear relationships between health endpoints and specific PM physical and chemical properties will improve the understanding of health risks. Reported national associations between adverse health effects and ambient PM mass have driven current regulation; however, results presented here confirm the need for further assessment of the toxicity of SJV PM due to the unique nature of this region of the United States. This study supports the notion that PM pollution is both an urban and a rural problem and that the SJV represents an ideal location for further research at the urban–rural interface. Future research to investigate the specific role and mechanisms associated with toxicity of carbonaceous and nitrate PM components is needed. Further, optimization of measurement timing following daily, repeated inhalation of ambient PM needs to be investigated.

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