Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circulation Research 2010, 107:200-203: originally published online July 1, 2010
doi: 10.1161/CIRCRESAHA.110.222679
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Rationale: Acute and chronic exposures to airborne particulate matter (PM) have been linked in epidemiological studies to a wide spectrum of cardiovascular disorders that are characterized by a dysfunctional endothelium. The pathophysiological mechanisms underlying these associations are unclear.

Objective: To examine whether exposure to fine PM with an aerodynamic diameter of <2.5 μm ($PM_{2.5}$) affects the circulating levels of endothelial progenitor cell (EPC) populations, systemic inflammation and coagulation.

Methods and Results: Phenotypically distinct EPC populations were quantified by flow cytometry in young (18 to 25 years) adult humans exposed to episodic increases in $PM_{2.5}$ along the Wasatch Mountain Front in Utah. In addition, Sca-1^+/Flk-1^+ cells were measured in the peripheral blood of mice exposed to concentrated particles from ambient air in Louisville, Ky. In both studies, PM exposure was negatively correlated with circulating EPC levels. In humans, statistically significant associations between $PM_{2.5}$ exposure and the plasma levels of platelet–monocyte aggregates, high-density lipoprotein, and nonalbumin protein were also observed. Episodic increases in $PM_{2.5}$ did not change plasma levels of C-reactive protein, interleukin-1β, interleukin-6, fibrinogen, or serum amyloid A.

Conclusions: Episodic exposure to $PM_{2.5}$ induces reversible vascular injury, reflected in part by depletion of circulating EPC levels, and increases in platelet activation and the plasma level of high-density lipoprotein. These changes were also accompanied by an increase in nonalbumin protein and may be related to mechanisms by which exposure to particulate air pollution increases the risk of cardiovascular disease and adverse cardiovascular events. (*Circ Res*. 2010;107:200-203.)

Key Words: endothelial progenitor cell ■ airborne particulate matter ■ pollution ■ endothelial repair

Acute and chronic exposure to elevated levels of fine airborne particulate matter (PM) is associated with an increase in the incidence of adverse cardiovascular events,[1,2] atherogenesis, cardiovascular disease (CVD) risk, and cardiovascular mortality. In urban environments, fine PM (PM with aerodynamic diameter of <2.5 μm [$PM_{2.5}$]) is generated mostly by fossil fuel combustion in automobiles or by industrial processes. Although several mechanisms have been proposed to account for the link between PM exposure and CVD risk, endothelial dysfunction has emerged as a key feature of PM toxicity. Inhalation of concentrated $PM_{2.5}$ induces acute conduit artery vasoconstriction in humans and chronic deficits in endothelium-mediated vasodilation in mice.[1,2]

The adult endothelium is a differentiated cell layer that provides a nonthrombotic interface between parenchymal cells and peripheral blood. Defects in its function arise because of the upregulated expression of proinflammatory and prothrombotic molecules or from defective, endogenous repair capacity. Evidence from multiple studies suggests that the endothelium is continually repaired by progenitor cells mobilized from specific niches such as the bone marrow. These cells express both endothelial and stem cell markers, and their circulating levels in blood are reflective of CVD risk and burden.[3,4] The present study was designed to examine how exposure to $PM_{2.5}$ affects endothelial progenitor cell (EPC) populations and whether this was associated with changes in systemic inflammation, coagulation, or plasma lipids.

Methods

For the human study, 16 (8 male and 8 female), young (18 to 25 years of age), nonsmoking, healthy (no existing acute or chronic disease) adults of normal weight (body mass index, 19 to 25) with no reported exposure to second-hand smoke were recruited in Provo, Utah. In the Utah Valley of the Wasatch Front, winter temperature inversion episodes elevate PM levels as emissions become trapped in a stagnant air mass near the valley floor. These episodes occur under somewhat predictable conditions that include a combination of snow cover, high barometric pressure, and low or falling clearing index.[5] Arrangements were made with the research participants to have their blood drawn 4 times between January and early March of 2009

Original received April 21, 2010; revision received June 17, 2010; accepted June 17, 2010. In May 2010, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.6 days.

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This manuscript was sent to Ali J. Marian, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.110.222679**

during a period of high pollution ($PM_{2.5}$ >40 $\mu g/m^3$), a period of moderate pollution ($PM_{2.5}$, ≈20 to 40 $\mu g/m^3$), and 2 periods of low pollution ($PM_{2.5}$ <10 $\mu g/m^3$) (Figure 1A). In the murine study, 28 C57BL/6 mice were exposed to either filtered air or $PM_{2.5}$, concentrated ≈8-fold from ambient downtown Louisville air for 6 hours per day for 9 consecutive days (see the Online Data Supplement, available at http://circres.ahajournals.org) at 2 different times.

Results

Ambient levels of $PM_{2.5}$ recorded in January to early March of 2009 by 2 Utah Valley monitoring sites are shown in Figure 1A. A detailed characterization of changes in PM composition during winter inversion has been recently published.[6] A substantial air pollution episode with peak $PM_{2.5}$ concentrations occurred around January 22nd, with a more moderate episode approximately 10 to 14 days later and a return to baseline levels thereafter. Blood samples were obtained 4 times from each study participant to provide measurements at both high and low PM levels for each individual. EPC populations were identified by a 7-color cytometry procedure. The data were regressed on $PM_{2.5}$ (average of 24 hours before blood draw) controlling for subject-specific fixed effects, using heteroskedasticity-consistent covariance matrix estimators.

The most abundant progenitor cell population ($CD31^+/CD34^+$) was negatively correlated with ambient $PM_{2.5}$ levels (Table 1). In addition, CD45±CD133 cell populations also demonstrated negative associations with varying statistical strength. The strongest statistical correlation was observed for $CD34^+/CD31^+/CD45^+/CD133^+$ cells (Figure 1B). Some data sets showed differences in variability. For example, in Figure 1B, at $PM_{2.5}$ concentrations of ≈35 $\mu g/m^3$, there is much less variability in some data. These results suggest the need to estimate standard errors and probability values based on heteroskedasticity-consistent covariance matrix estimators. In most cases, these estimators resulted in slightly larger standard errors and corresponding probability values. Nevertheless, similar regression results were observed when these specific measurements were deleted from the regression analysis.

In addition to EPC levels, changes in systemic inflammation, coagulation, and plasma lipids were measured. As listed in Table 1, acute exposure to $PM_{2.5}$ was significantly correlated with an increase in platelet ($CD41a^+$)–monocyte ($CD45^+$) aggregates and high-density lipoprotein (HDL) cholesterol levels. No associations with serum amyloid A and C-reactive protein (Table 1) or low-density lipoprotein cholesterol, triglycerides, fibrinogen, stromal cell-derived factor-1, interleukin-6, interleukin-1β, vascular endothelial growth factor, platelet factor-4 (data not shown) were observed. $PM_{2.5}$ levels were, however, most strongly associated with nonalbumin plasma protein (NAP) levels (Table 1). Absolute levels, or levels of these proteins expressed as a percentage of total plasma protein, were both highly associated with $PM_{2.5}$ (Table 1). Unlike associations of PM with platelet–monocyte aggregates and HDL, which were of marginal significance, there was no anomalous heteroskedasticity observed in the NAP data. Given the incredible strength of the statistical association ($P<0.0001$) and given the remarkable consistency across all subjects (Table 1, Figure 1C), the association between NAP and $PM_{2.5}$ is unlikely to be an artifact of multiple hypothesis testing. Finally, there were no changes in markers of liver or skeletal muscle injury (data not shown), indicating that the effects observed are not symptoms of general tissue injury.

Because several confounding factors can influence the levels of circulating EPCs in humans, we examined PM-induced changes in mice. These mice were exposed to filtered air or concentrated ambient particles (CAPs) from downtown Louisville air for 9 days, and blood levels of $Sca\text{-}1^+/Flk\text{-}1^+$ cells were measured. As shown in Figure 2, CAPs exposure resulted in a ≈30–50% decrease in $Sca\text{-}1^+/Flk\text{-}1^+$ cells during the 2 exposure periods. CAPs exposure was also associated with signifi-

Non-standard Abbreviations and Acronyms

CAPs	concentrated ambient air particles
CVD	cardiovascular disease
EPC	endothelial progenitor cell
HDL	high-density lipoprotein
NAP	nonalbumin protein
PM	particulate matter

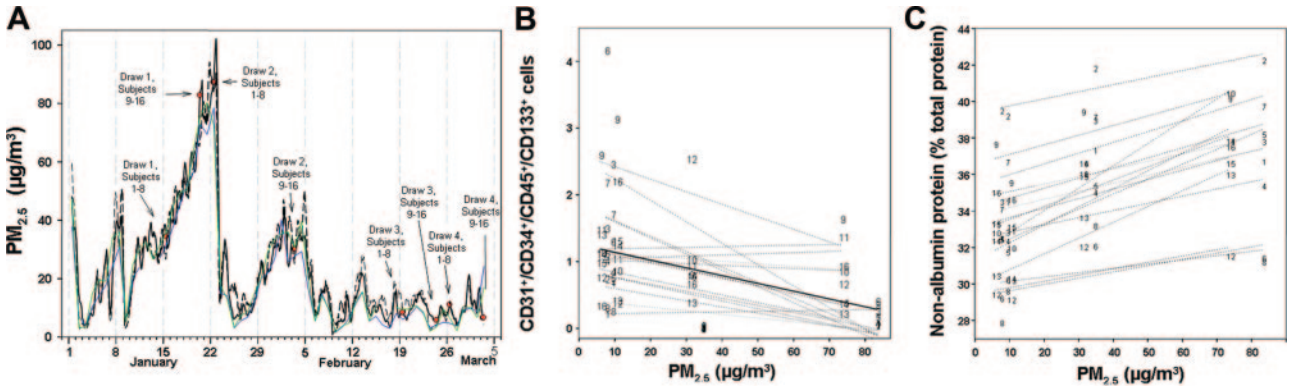

Figure 1. $PM_{2.5}$ levels are inversely correlated with human EPC number. A, Twelve-hour lagged-moving average $PM_{2.5}$ levels **(solid and dashed black lines)** and the daily 24-hour $PM_{2.5}$ concentrations **(blue and green lines)** recorded at 2 monitoring sites in the Utah Valley between January 1 and March 5, 2009. **Red dots** represent the times of blood draws. **B,** Regression analysis of the relationship between $CD31^+/CD34^+/CD45^+/CD133^+$ cells (normalized to volume) and previous 24-hour $PM_{2.5}$ level. **C,** Regression analysis of the relationship between nonalbumin protein (as a percentage of total plasma protein) and average previous 24-hour $PM_{2.5}$ levels. Individual data points are labeled with the subject numbers, and individual-level regression is represented by **dotted lines**. **Solid line** represents regression analysis from the pooled fixed-effects regression model.

Table 1. Summary Statistics and Regression Coefficients for Study Variables Regressed on $PM_{2.5}$ From Models Controlling For Subject-Specific Fixed Variables

Parameter	Regression Coefficient	SE	*P*
$CD34^+/CD31^+$ cells	−5.691	1.764	0.002
$CD34^+/CD31^+/CD45^-$ cells	−4.275	1.569	0.009
$CD34^+/CD31^+/CD45^-/CD133^-$ cells	−4.244	1.559	0.009
$CD34^+/CD31^+/CD45^+$ cells	−1.416	0.431	0.002
$CD34^+/CD31^+/CD45^+/CD133^-$ cells	−1.074	0.392	0.009
$CD34^+/CD31^+/CD45^+/CD133^+$ cells	−0.342	0.090	0.0004
Platelet–monocyte aggregates	1.387	0.683	0.048
HDL-C	1.943	0.740	0.012
CRP	−0.020	0.025	0.430
Serum amyloid A	−14.82	31.32	0.638
Total plasma protein	0.383	0.163	0.023
Plasma albumin	0.033	0.104	0.751
Nonalbumin protein	0.349	0.069	<0.0001
Nonalbumin protein/total plasma protein	2.848	0.379	<0.0001

The data were regressed on $PM_{2.5}$ (average of 24 hours before blood draw $\times 50$ $\mu g/m^3$). CRP, C-reactive protein; HDL-C, HDL cholesterol.

Table 2. Blood and Plasma Parameters in C57BL/6 Mice Exposed to Air or Concentrated Ambient Particulate Matter ($PM_{2.5}$) in July 2009

Variable	Air	$PM_{2.5}$
HCt (decimal fraction)	0.44.±0.01	0.44±0.00
Buffy coat (decimal fraction)	0.01±0.00	0.01±0.00
Cholesterol (mg/dL)	56.8±1.3	62.4±1.1*
HDL-C (mg/dL)	41.1±1.2	44.7±1.0*
LDL-C (mg/dL)	9.8±0.6	10.5±0.6
Triglycerides (mg/dL)	45.6±3.3	48.2±4.0
TP (g/dL)	4.07±0.12	4.26±0.07
ALB (g/dL)	2.99±0.07	3.07±0.06
ALT (U/L)	20±2	28±5
AST (U/L)	49±5	58±4
Creatinine (mg/dL)	0.25±0.01	0.25±0.01
Nonalbumin protein (g/dL)	1.08±0.08	1.19±0.04

Values are means±SEM. HCt, hematocrit; HDL-C, HDL cholesterol; LDL-C, low-density lipoprotein cholesterol; TP, total protein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase. *$P<0.05$ from control by 1-way ANOVA and Bonferroni post test (n=4).

cant increases in total cholesterol and HDL but no changes in NAP (Table 2). No significant changes in the number of bone marrow–derived EPCs were observed after 1 week in culture (Online Figure VI). Collectively, these findings suggest that exposure to particulate air pollution in mice decreases EPC levels.

Discussion

The major finding of this study is that exposure to high $PM_{2.5}$ levels induces reversible vascular injury, as evidenced by a suppression of circulating EPC levels in both humans and mice. In humans, this was also accompanied by an increase in platelet activation and elevated levels of plasma HDL and NAP. No significant changes in markers of systemic inflammation or tissue injury were observed. These results support the notion that suppression of EPC levels in peripheral blood may be an important feature and, perhaps, a significant mechanism of PM-induced cardiovascular injury. Moreover, EPC level may be a sensitive, albeit nonspecific, biomarker of endothelial injury caused by PM exposure.

Although the mechanisms by which PM exposure decreases the circulating EPC levels remain unclear, concurrent increases in thrombosis and HDL indicate that the loss of EPCs from peripheral blood may be attributable to endothelial injury. Consistent with this idea, the increase in NAP is likely reflective of an increase in globulin levels. After albumin, globulins are the second-most abundant proteins in the plasma, and an increase in their levels may be reflective of a mild systemic immune response.

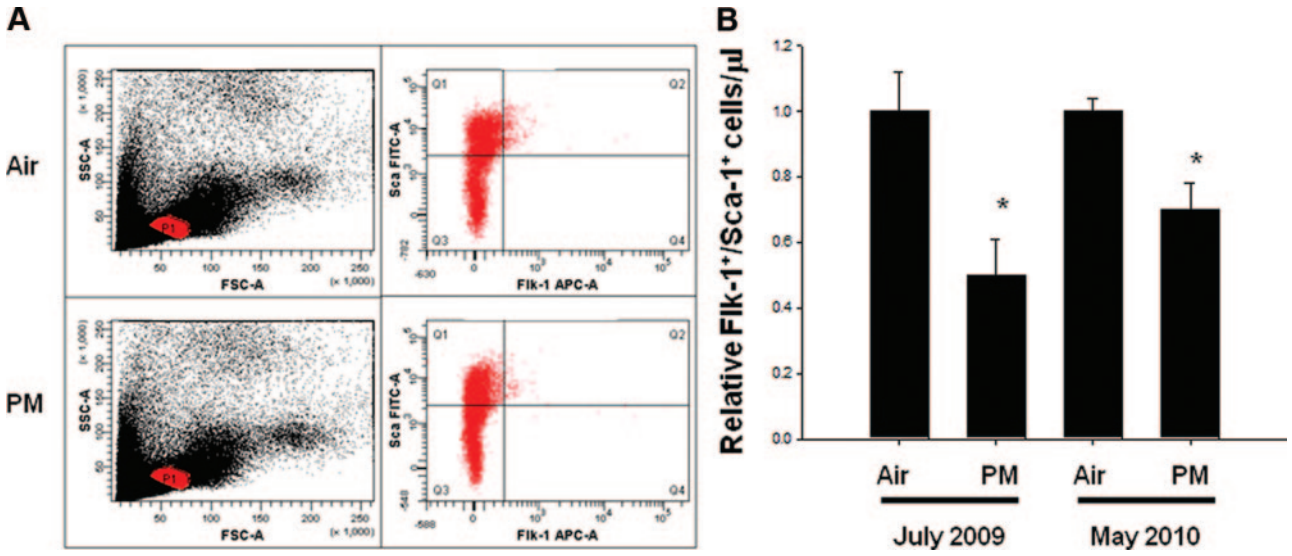

Figure 2. $PM_{2.5}$ exposure decreases EPC levels in mice. Flow cytometric analysis of peripheral blood obtained from mice exposed to filtered air or concentrated air particulates. **A,** Sca-1^+/Flk-1^+ cells **(right graphs)** were quantified in a gated lymphocyte population identified in an SSC vs FSC dot plot **(left graphs)**. **B,** EPC levels per microliter of blood in mice exposed to air or $PM_{2.5}$ during the indicated exposure periods (n=4, July 2009; n=8, May 2010; * $P<0.05$).

Vascular dysfunction and endothelial injury are well-described effects of PM exposure.[1,2] Our observation that PM decreased EPC levels in humans and mice exposed to similar doses (25 μg/kg for per 24 hours per day and 54 μg/kg for 6 hours per day, respectively), and despite potential compositional differences between Provo and Louisville air, suggests that suppression of EPC levels is a robust response to PM exposure. Previous studies show that chronic exposure to tobacco smoke, which contains high levels of $PM_{2.5}$ and other pollutants, is also associated with low EPC levels.[7] Thus, exposure to air particulates and/or their copollutants from several sources may have the general property of reducing circulating EPC levels. Further studies are required to identify specific PM components that might be related to EPC suppression.

The $CD34^+$ cell population, which was significantly correlated with $PM_{2.5}$ levels, has been shown previously to be associated with CVD risk.[4] Moreover, stronger association with $CD133^+$ than $CD133^-$ cells suggests that PM exposure affects the immature, early EPC population. This population shows a 5-fold reduction in patients with coronary artery disease and is predictive of adverse cardiovascular events in patients with preexisting CVD.[4] Significantly, the levels of nonmonocytic EPC population ($CD45^-$) cells were also suppressed on PM exposure. Therefore, depletion of multiple EPC populations could contribute to CVD risk imposed by $PM_{2.5}$ exposure by inducing deficits in endothelial repair and angiogenesis. However, we could not study functional changes because the effects of PM on EPC levels in humans were reversible. Moreover, ex vivo assays to assess EPC function or proliferation require prolonged (7- to 21-day) culture, during which time the PM-induced changes are likely to be lost. Nevertheless, reversible suppression of EPC levels suggests that exposure to PM induces a transient mismatch between EPC utilization and recruitment. Given that this gap is robustly associated with several cardiovascular diseases,[3,4] it appears likely that depletion of circulating EPCs, along with changes in blood coagulation, lipids, and nonalbumin proteins is reflective of vascular injury induced by PM, even in the absence of overt cardiovascular disease.

Acknowledgments

We thank Dr David Ingram (Indiana University School of Medicine) and members of his laboratory for their help and advice in EPC analysis.

Sources of Funding

This work was supported, in part, by grants from the Environmental Protection Agency (833336701), National Center for Research Resources (RR024489), National Institute of Environmental Health Sciences (ES11860), and contract W81XWH-10-1-0398 from U.S. Army Medical Research and Material Command (USAMRMC) and TATRC. C.A.P. is partially supported by the Mary Lou Fulton Professorship.

Disclosures

None.

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Novelty and Significance

What Is Known?

- Several epidemiological studies show that acute exposure to elevated levels of fine airborne particulate matter is associated with an increase in the risk of adverse cardiovascular events.
- Controlled laboratory exposure to particulate matter has been reported to induce acute conduit artery vasoconstriction in humans and chronic deficits in endothelium-mediated vasodilation in mice.
- Endothelial progenitor cells (EPCs) in peripheral blood contribute to postembryonic endothelial repair and regeneration and a decrease in circulating EPC levels is reflective of cardiovascular disease risk and burden.

What New Information Does This Article Contribute?

- An increase in airborne particulate matter attributable to winter temperature inversion-episode in Utah Valley of the Wasatch Front was associated with a reversible decrease in circulating levels of EPC in a cohort of young (18 to 25 years) healthy adults.
- The increase in particulate matter was also accompanied by an increase in plasma levels of platelet–monocyte aggregates, high-density lipoprotein, and nonalbumin protein. No changes in C-reactive protein, interleukin-1β, interleukin-6, fibrinogen, or serum amyloid A were observed.
- Circulating levels of EPC were also decreased in mice exposed to concentrated airborne particles from downtown Louisville, Ky.

Particulate air pollution contributes to cardiovascular dysfunction and mortality, but the mechanisms for this remain unclear. Here, we show that episodic exposure to high levels of particulate matter decreased circulating EPCs in young adults and that this effect was reversible. These effects were accompanied by an increase in markers of thrombosis but no change in systemic inflammation. Exposure to concentrated PM also decreased circulating EPCs in mice. Consistent data between humans and mice at 2 locales suggests that depletion of circulating EPCs is a characteristic feature of PM exposure and may be one mechanism by which PM contributes to cardiovascular disease.

Supplemental Material

Episodic Exposure to Fine Particulate Air Pollution Decreases Circulating Levels of Endothelial Progenitor Cells

Timothy E. O'Toole[1*], Jason Hellmann[1], Laura Wheat[1], Petra Haberzettl[1], Jongmin Lee[1], Daniel J. Conklin[1], Aruni Bhatnagar[1], and C. Arden Pope III.[2]

Supplemental Detailed Methods

A) human subject enrollment and baseline characteristics

A total of 16 healthy young adults in the Provo, Utah area were enrolled in the study. An introduction to the study and a questionnaire was administered to potential participants to determine eligibility. Exclusion criteria included: 1) not 18 – 30 years of age, 2) active smoker, 3) unwilling to participate and/or sign the consent form; 2) body weight of less than 110 pounds; 3) health problems that would preclude participation, including lack of two healthy arms, any known chronic pulmonary or cardiac disease, current cold, flu, or other infectious illness, chronic renal failure, Parkinsonism, alcohol abuse, mental illness, bleeding disorders, pregnancy, past or current history of hepatitis, AIDS or HIV; 4) currently lives, works, or attends school with exposure to environmental tobacco smoke. Summary characteristics of this cohort and baseline levels of study variables are listed in Online Table I. All research protocols and consent forms were approved by the Institutional Review Board for human subjects at Brigham Young University. One strength of this panel-study design with fixed-effect regression analysis is that the effect estimates are probably not due to confounding factors such as age, gender, exposure to cigarette smoke, or underlying chronic disease, as individuals served as their own controls.

B) murine exposures

Murine PM exposures were accomplished with a modified VACES system[1] at the Inhalation Facility of the University of Louisville (Online Fig. I) and with IACUC approval. Initially, a high volume exhaust pump mounted on the roof of a 7-story research building drew 330 lpm ambient air and $PM_{2.5}$ was then separated by a cyclone generator, humidified and aggregated in a heated stainless steel chamber (~28-30 °C) coupled to a chilled ouflow (-4 °C) before concentration through a virtual impactor and diffusion dryer to disaggregate and dry particles to original shape/size. These CAPs were delivered to 30-l stainless steel exposure chambers for murine exposure by a secondary exhaust pump and by a Gast floor pump delivering charcoal- and HEPA-filtered room air for complete drying of particles. HEPA-filtered air was delivered to the animal chambers through similar steps, bypassing the initial cyclone generator and virtual impactor. Samples of $PM_{2.5}$ particles delivered to exposure chambers were collected using inline Teflon filters (Online Fig. IIA) and their mass and concentration quantified using a ultrasensitive balance and a DataRAM 4 (ThermoElectron) dual-wavelength nephelometer, (Online Fig. IIB) where particle concentration ($\mu g/m^3$) was defined as mass divided by filter air flow. Gravimetric analyses indicated the VACES $PM_{2.5}$ concentration factors were ~10x (July 2009) and ~6x (May 2010) with no major alterations in elemental composition (Online Fig. II C, D).

C) pollution data

During Utah Valley winter inversions, various locally emitted air pollutants are trapped in a stagnant air mass on the valley floor. Daily monitoring of airborne fine particulate matter ($PM_{2.5}$) was conducted by the State of Utah Division of air Quality at two sites in the valley, the North Provo site and the Lindon Elementary site. At each site 24-hr monitoring according to the U.S. Environmental Protection Agency's (U.S. EPA) federal reference method (FRM; U.S. EPA 1997) was conducted. Also at each site, real-time $PM_{2.5}$ mass concentrations were determined using tapered element oscillating microbalance (TEOM) monitors. Additional weather parameters including temperature, dew point, and clearing index were collected from the National Weather Service (Salt Lake City International Airport location).

Louisville, Kentucky $PM_{2.5}$ and PM_{10} levels in July 2009 were measured and reported hourly at several air pollution monitoring stations by both filter-based and continuous monitoring systems in the metropolitan area (Online Fig. IIIA). The hourly data are available at: http://services.louisvilleky.gov/MetroAirNet/HourlyData.aspx. Levels of additional gaseous pollutants (ozone, CO, SO_2, NOx) were monitored daily by the Air Pollution Control District (APCD) in the Louisville area (Online Fig. IIIB). VACES-concentrated $PM_{2.5}$ data was collected every 10 seconds using DataRAM4 over the whole sampling duration (6h/d x 9 days). Hourly average $PM_{2.5}$ values were compared with ambient $PM_{2.5}$ levels as measured by Louisville Air Pollution Control Board (Online Fig. IIIC). These particles were characterized (Online Fig. IIID, E) by a DataRAM4 instrument. This provided a single mass median diameter (MMD; geometric particle size) value for collected individual PM samples where the MMD value is calculated based on a log-normal distribution with a geometric standard deviation of two. Estimated $PM_{2.5}$ mass size distributions were created from this algorithm and particle number distributions were directly converted from particle mass distributions on the assumptions that collected particles have an equal PM density regardless of particle size and the single particle volume of detected spherical particle. An aerodynamic particle size was determined from measured geometric particle size multiplied by square root of particle density.

D) blood collection and processing

Human blood samples were collected at Brigham Young University by venipuncture at the same approximate time of each collection day. A total of 20 ml was collected in three tubes: a Vacutainer® CPT Mononuclear Cell Preparation Tube (Becton Dickinson), a Vacutainer® Plus PST™ plasma separator tube (Becton Dickinson), and a Vacutainer® whole blood ACD tube (Becton Dickinson). The blood withdrawn into the CPT tube was used for EPC analysis. After collection, it was centrifuged at 2750rpm for 30 min and then packaged for overnight shipping at room temperature to the University of Louisville for analysis. In pilot experiments, there was no apparent difference in EPC number in samples kept overnight at room temperature and subject to simulated handling/shipping versus samples that were analyzed immediately after collection. The blood withdrawn into the plasma separator tube was used for analysis of plasma components. This tube was centrifuged at 100g for 15 min and then packaged for shipment at 4°C. Finally the sample collected in the ACD tube was used to analyze the abundance of platelet-monocyte aggregates. Blood in this tube was fixed in paraformaldehyde (1% final) for 30 minutes on ice and red cells lysed by addition of 5 volumes of water. The samples were then centrifuged at 400g for 10 min, the pelleted cells resuspended in Tyrode's buffer, and packaged for shipment at 4°C. All blood samples were labeled such that subsequent analysis was blinded to participant name and pollution level.

At the end of murine exposure periods, the animals were euthanized and blood collected by cardiac puncture using EDTA as an anticoagulant. In one aliquot used for EPC analysis, red blood cells were lysed with a proprietary solution (Becton-Dickinson), the cells pelleted by centrifugation and washed once with PBS containing 2% BSA (PBS/BSA). A second aliquot of

whole blood was used for analysis of plasma components. This sample was centrifuged to pellet cellular material and plasma collected.

E) identification of EPCs

Human EPCs were identified by FACS using modifications of a published approach[2]. Initially, serum and mononuclear cells were withdrawn from the CPT tube and centrifuged at 1500rpm for 10 min. The pelleted cells were washed twice in PBS/FBS and incubated with Fc block (Miltenyi Biotec) for 10 min on ice. The cells were then incubated for 30 min on ice with a panel of fluorescently-conjugated antibodies. These included PE-labeled anti-CD34 (Becton Dickinson), APC-labeled anti-AC133 (Miltenyi Biotec), PE-Cy5.5-labeled anti-CD14 (abCAM), APC-AlexaFluor 750-labeled anti-CD45 (Invitrogen), PE-Cy7-labeled anti-CD16 (Becton Dickinson), and FITC-labeled anti-CD31 (Becton Dickinson). Also included in these incubations were anti-CD41a (Becton Dickinson) and anti-CD235a (Becton Dickinson) antibodies labeled in the laboratory with Pacific Blue (Pacific Blue monoclonal antibody labeling kit; Invitrogen) and a marker for dead cells (LIVE/DEAD fixable dead cell stain; Invitrogen). After these incubations, the cells were pelleted, washed once in PBS/FBS, resuspended and 500,000 events collected on an LSR II flow cytometer (Becton Dickinson).

Collected events were analyzed with the FloJo software package as illustrated in Online Fig. IV. Initial gating selected a lymphocyte population that was negative for CD235a, CD41a, and the dead cell marker (all stained with Pacific Blue). This population was then analyzed for CD14 and CD16 staining, and cells negative for both markers selected. Finally, EPCs were minimally defined within this population as those cells positive for CD34 and CD31. Additional selection criteria subdivided this population of EPCs into those with monocytic/non-monocytic characteristics ($CD45^{+/-}$) and into those with an immature or mature phenotype ($CD133^{+/-}$). Positive/negative boundaries for all gating was accomplished using fluorescence minus one controls[3]. EPC numbers were quantified and normalized to the volume of sample used for analysis.

Pelleted murine cells were resuspended in PBS/FCS, incubated with Fc block for 10 min and then stained with a FITC-labeled anti-Sca-1 antibody (Becton Dickinson) and an APC-labeled anti-Flk-1 antibody for 30 min on ice. An identical sample was stained with isotype control antibodies. At this time the cells were washed with PBS/FBS, resuspended, and 50,000 events analyzed by FACS. EPCs were defined as the number of events double positive for Flk-1 and Sca-1 and normalized to the volume used to collect these events.

F) analysis of serum components

Lipid levels, protein abundance and the presence of inflammatory markers in plasma from the human subjects were analyzed upon receipt of the plasma separator tubes from Brigham Young University. Triglycerides, cholesterol, HDL, LDL, fibrinogen, albumin, total protein, and C reactive protein were determined by a Cobas Mira 5600 Autoanalyzer. Measurements of PF-4, VEGF, SDF-1, IL-6, and IL-1β (R&D systems), cotinine (International Diagnostics Systems), and SAA (Invitrogen) were performed using commercially available ELISA kits. Murine plasma components (hematocrit, cholesterol, HDL, LDL, triglycerides, total protein, albumin, alanine aminotransferase, aspartate aminotransferase, and creatinine) were measured on the Cobas Mira 5600 Autoanalyzer.

G) identification of platelet-monocyte aggregates

The abundance of platelet-monocyte aggregates was identified using a FACS strategy. Upon receipt from Brigham Young University, Tyrode's-resuspended cells were incubated with Fc block for 5 minutes on ice and then with FITC-labeled anti-CD41a (Becton Dickinson) and PerCPCy5.5-labeled anti-CD45 (Becton Dickinson) antibodies for 30 minutes on ice. A second aliquot of cells were incubated with the appropriately-labeled isotype control antibodies. After these incubations, the cells were washed, resuspended and analyzed by FACS (Online Fig. V). Those events positive for both FITC and PerCPCy5.5 staining were indicative of the presence of platelet-monocyte aggregates and were expressed as percentage of total events.

H) isolation, culture and analysis of bone marrow-derived cells (BMDCs)

The femurs and tibias of exposed mice were isolated and their marrows aspirated in 1mL HBSS (Lonza). Mononuclear cells were separated by Ficoll gradient centrifugation and $8x10^5$ viable cells plated on fibronectin-coated 8-well chamber slides (Sigma-Aldrich) in 500 µL endothelial basal media (Lonza) supplemented with 20% FBS (Invitrogen) and appropriate supplements (SingleQuot®: Lonza). Media was changed after three days and at day 7, cells were washed with PBS, fixed in 4% paraformaldehyde and blocked with 1% BSA in PBS. The cells were then stained with an APC-conjugated Flk-1 antibody (Becton Dickinson) and a FITC-conjugated Sca-1 antibody (Becton Dickinson) for 1h at RT. Additional wells were stained with DiI-labeled acetylated LDL (acLDL) (Invitrogen) and FITC-conjugated UE lectin (Accurate Chemical and Scientific Corp.). At this time, the cells were washed three times with PBS and the slides mounted with the DAPI-containing *Slow Fade*® Gold anti-fade reagent (Invitrogen). Positively-staining cells were counted in 5 random fields per chamber (Online Fig. VI).

I) statistical analysis

The primary analysis of the data involved a regression analysis where various measures were regressed on $PM_{2.5}$ controlling for subject-specific fixed effects. The estimated standard errors and p-values were based on heteroskedasticity-consistent covariance matrix estimators as proposed by MacKinnon and White[4]. Based on recommendations by Long and Ervin[5], and because the sample size was small (<250), we used the third proposed heteroskedasticity-consistent estimator (HC_3). Specifically, the statistical analysis was conducted using SAS 9.2 software (SAS Institute Inc., Cary, NC); regression coefficients were estimated using fixed effects models using PROC REG; and the HC_3 standard error estimates and corresponding p-values were estimated using the HCC (or WHITE) HCCMETHOD=3 options in the MODEL statement.

Supplemental References

1. Maciejczyk P, Zhong M, Li Q, Xiong J, Nadziejko C, Chen LC. Effects of subchronic exposures to concentrated ambient particles (caps) in mice. II. The design of a caps exposure system for biometric telemetry monitoring. *Inhalation Toxicology.2005;17:189-97*.
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4. MacKinnon JG, White H. Some heteroskedasticity-consistent covariance-matrix estimators with improved finite-sample properties. *Journal of Econometrics*. 1985;29:305-325
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Online Table I. Baseline Subject Characteristics

Characteristic	Value (n=16)
age	18-26
gender (male/female)	8/8
cotinine	2.58 ng/ml
cholesterol	163 mg/dl
high density lipoprotein (HDL)	49.2 mg/dl
low density lipoprotein (LDL)	81.1 mg/dl
triglycerides	88.6 mg/dl
serum amyloid A (SAA)	188 μg/ml
fibrinogen	500 mg/dl
stromal cell derived factor -1 (SDF-1)	2.63 ng/ml
interleukin 6 (IL-6)	0.92 pg/ml
interleukin 1β (IL-1β)	3.44 pg/ml
vascular endothelial growth factor (VEGF)	118 pg/ml (n=8)
platelet factor 4 (PF-4)	989 pg/ml (n=8)
C reactive protein (CRP)	0.16 mg/dl
total plasma protein	6.68 g/dl
albumin	4.47 g/dl

Online Figure Legends

Online Figure I: Experimental setup for exposing mice to concentrated air particulates. Ambient air was collected from downtown Louisville and passed through a HEPA filter or a Cyclone with a PM cut-off of 2.5 mM. The air was passed through a humidifier and a diffusion dryer before ingress into the animal chamber. Each of the major components of the exposure set up is labeled.

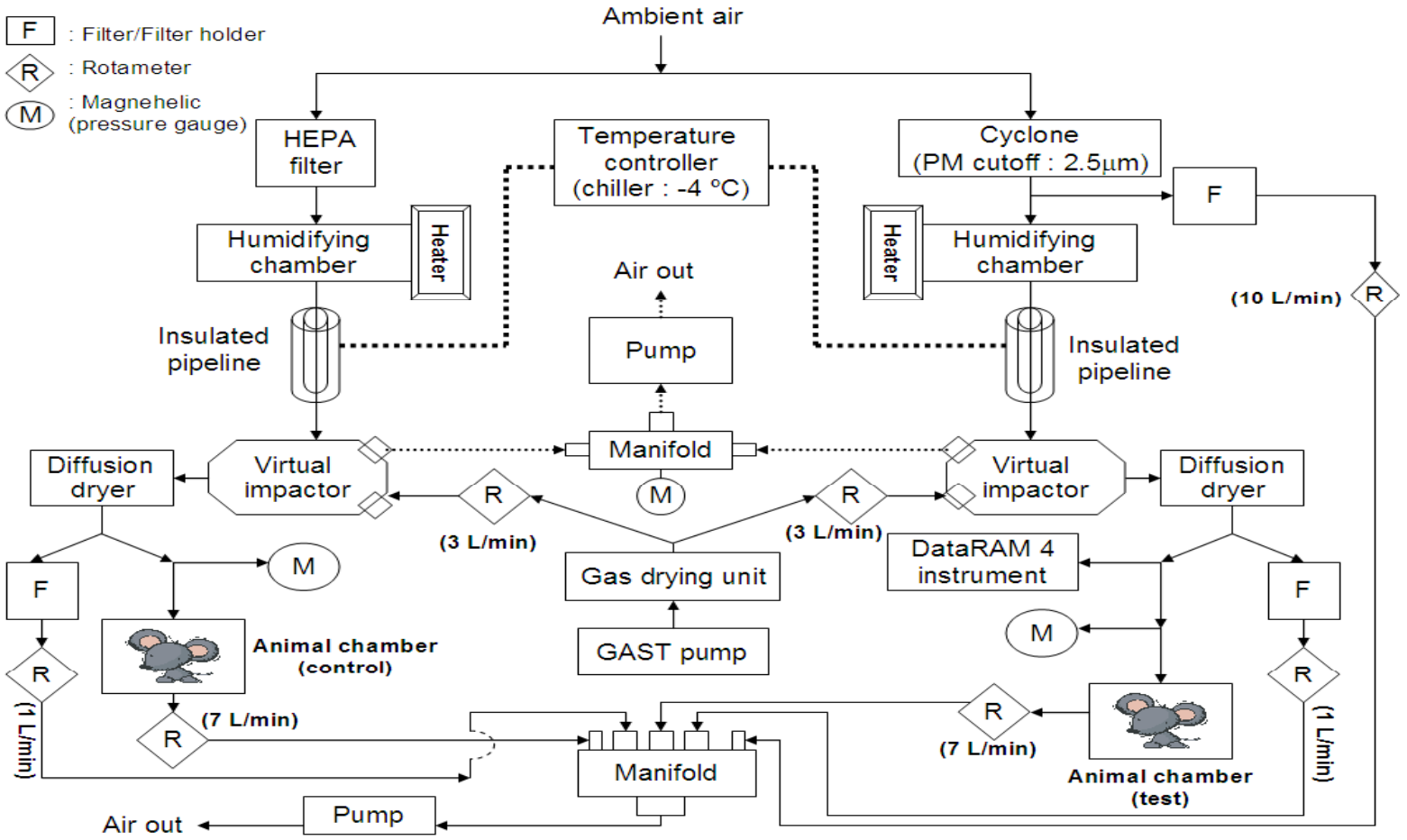
Online Figure II: Analysis of VACES-concentrated particles. A) Representative photograph of Teflon filters from HEPA-filtered air, pre-concentrator air (ambient $PM_{2.5}$ fraction), and post-concentrator air (chamber $PM_{2.5}$ fraction; note: $1/10^{th}$ air flow of pre-concentrator), respectively. Representative filter mass and estimated particle concentration for VACES operation are illustrated in panel B. Also illustrated are pie charts of X-ray flame ionization detection-measured elemental composition of Louisville downtown air in $PM_{2.5}$ fractions of ambient (C) (ie. pre-concentrator) and post-concentrator (D) sources, respectively.

Online Figure III: PM concentration, size and co-pollutants in July 2009. (A) $PM_{2.5}$ and PM_{10} levels as measured at several monitoring sites in the Louisville metropolitan area (insert) between July 16-23, 2009. (B) Levels of various gas-phase air pollutants in the same area during the same time period. Concentrated $PM_{2.5}$ levels, as generated by the University of Louisville VACES during these 9 days, are shown in panel C (note: the July 21, 4 P.M. data were not available.) The mass distributions (D) and number distributions (E) for VACES-generated $PM_{2.5}$ are also represented.

Online Figure IV: Flow cytometric analysis of EPCs. Analytical scheme used for the identification and quantification of EPC populations. Lymphocytes selected from an FSC vs. SSC dot blot (panel A) were analyzed for the presence of red blood cells, platelets and dead cells by Pacific Blue staining (panel B). A negative population from this analysis was further analyzed for the presence of monocytes/granulocytes by staining with antibodies against CD14 and CD16 (panel C). A negative population from this analysis was finally selected and analyzed for defined EPC markers (ex. CD45, CD31) as illustrated in panel D.

Online Figure V: PM exposure induces platelet activation. Representative scatter plots showing the abundance of platelet-monocyte aggregates as assessed by FACS. Events positive for both CD41 and CD45 (red) were determined in peripheral blood from the same individual on days of relatively high (85 $\mu g/m^3$) and low (15 $\mu g/m^3$) PM.

Online Figure VI: Cultured EPC levels. Bone marrow-derived cells isolated from mice exposed to filtered air or PM were cultured for one week on fibronectin and then stained with anti-Flk-1 and Sca-1 antibodies (A) or assessed for binding of DiI-acLDL and FITC-labeled UE lectin (B). Using either assay, there was no significant difference in cell number between air or PM exposures.



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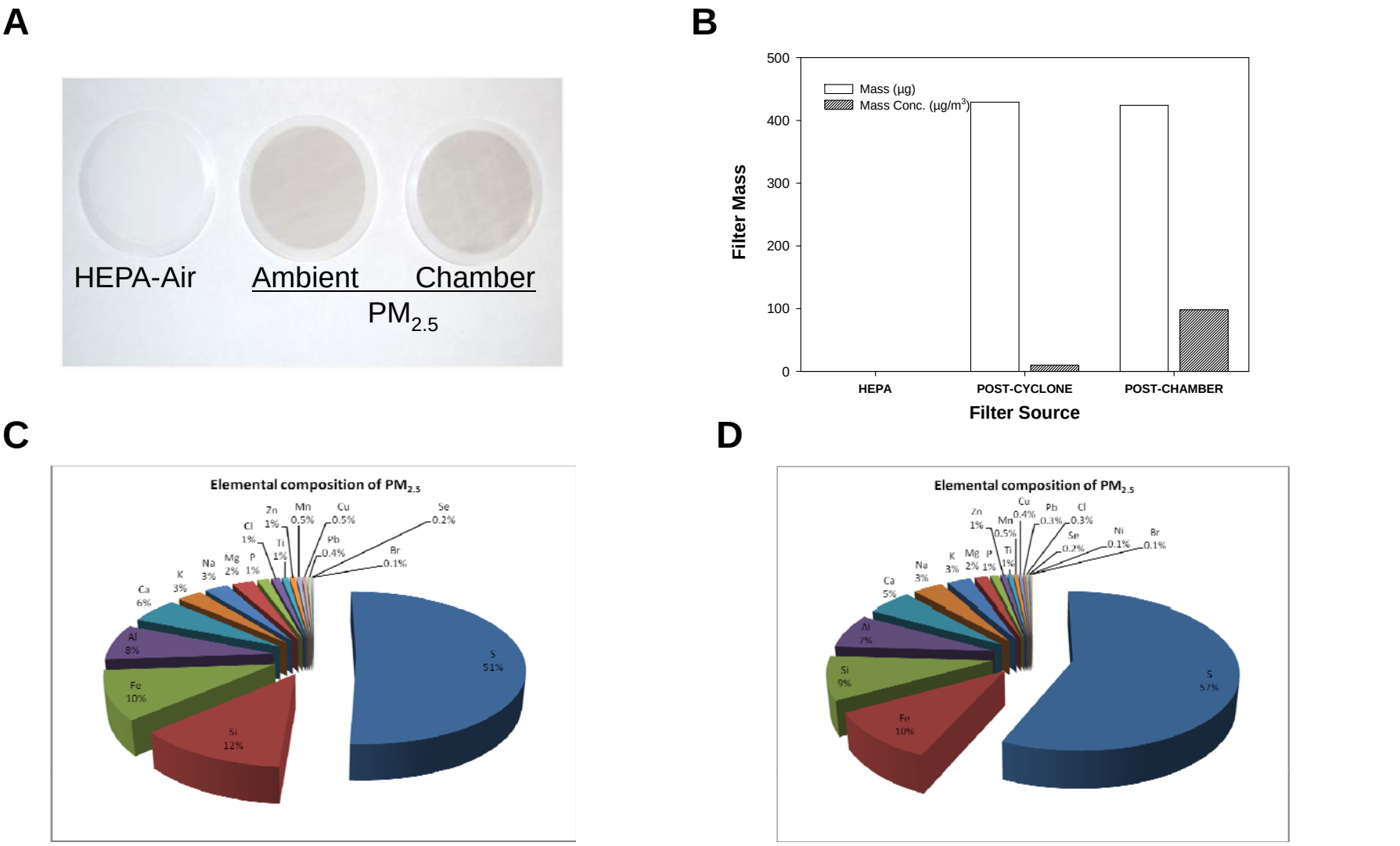

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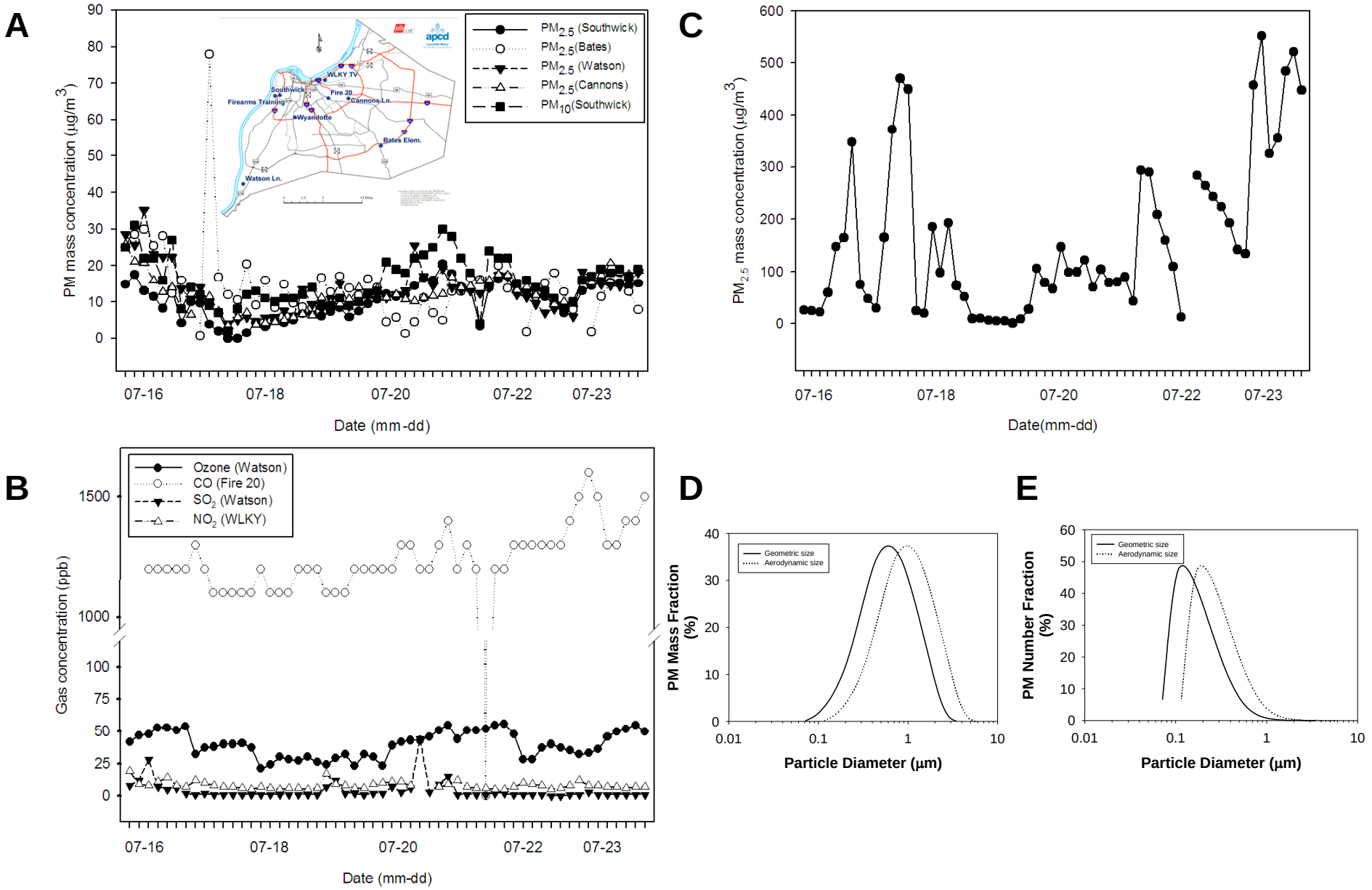

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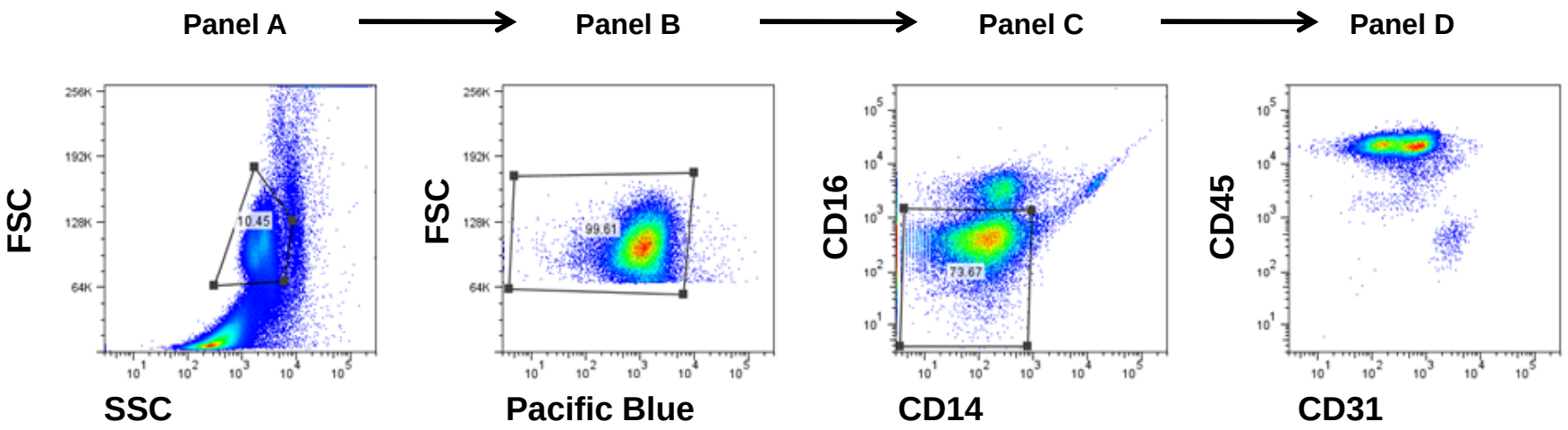

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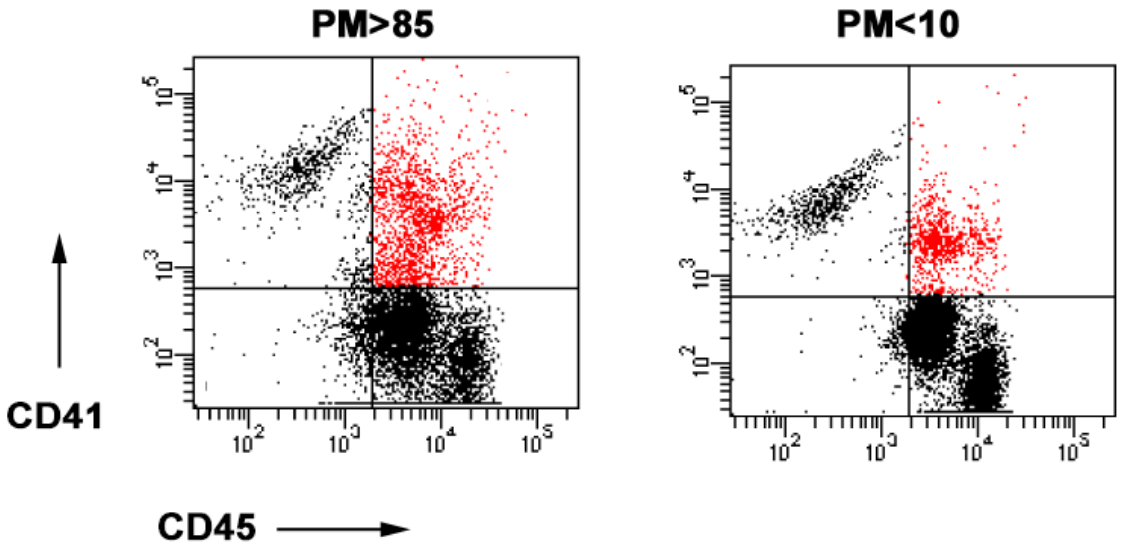

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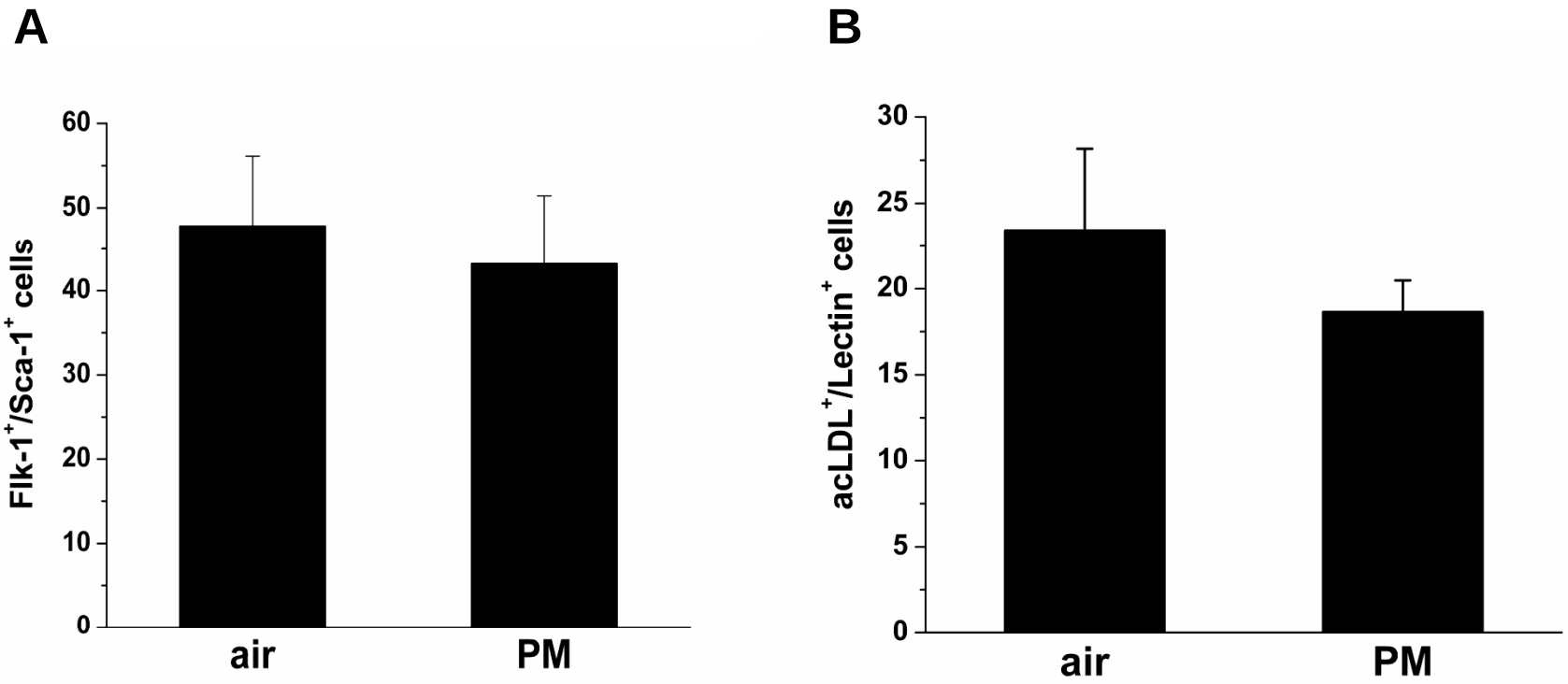

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