FINAL REPORT

Location Specific Systemic Health Effects Of Ambient Particulate Matter

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Disclaimer

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Abstract:
Previous work by us and others demonstrates induction of a systemic pro-inflammatory and pro-coagulant state in response to inhalation of environmental particulate matter. Our work demonstrated PM exposure activated platelets to a enhanced state of reactivity and suggested this could be a key factor in adverse cardiovascular events. This project evaluated pulmonary inflammation and systemic inflammatory and platelet responses to fine and ultrafine ($PM_{2.5}$) ambient particulate matter collected from an urban (Sacramento) and rural (Davis) location. The objective of these experiments was to determine whether prior chelation of transition metals with deferoxamine mesylate (DFM) or binding of bacterial source endotoxin by polymyxin B reduced pulmonary and systemic responses to $PM_{2.5}$. We hypothesized those inflammatory responses to a more transition metal rich urban source $PM_{2.5}$ would be inhibited more by metal chelation while rural source $PM_{2.5}$ would be more affected by endotoxin binding. Adult mice were given intratracheal instillations of collected $PM_{2.5}$ and pulmonary pathology, systemic cytokine concentrations, and platelet activation were evaluated 24 hours later. To better assess contributions of aromatic hydrocarbons, oxidants or pro-inflammatory responses to biologically active material like endotoxin, we used laser capture microscopy to probe specific anatomic locations in lung for gene responses associated with each of these components. Our results support the following conclusions:

1) Urban source $PM_{2.5}$ was significantly more pro-inflammatory than an equivalent dose by mass of rural source $PM_{2.5}$.
2) DFM pre-treatment of urban source $PM_{2.5}$ did not decrease PM induced pulmonary inflammation.
3) Treatment with DFM alone led to significant systemic platelet activation.
4) Pulmonary inflammatory responses to both urban and rural source $PM_{2.5}$ were inhibited by pre-incubation of $PM_{2.5}$ with polymyxin B.
5) In contrast to several prior animal experiments that demonstrated significant activation of circulating platelets after exposures to concentrated ambient particulates for two-weeks in the field,, this study with a single exposure dose found no evidence of platelet activation in response to instilled $PM_{2.5}$ at 24 hours after exposure.
6) Urban source $PM_{2.5}$ elicited transcription of genes associated with polycyclic aromatic hydrocarbon (PAH) metabolism (CYP 1A1, Aldehyde dehydrogenase), reactive oxygen species (ROS) response elements (ATF3, HOX-1) and inflammation (CCL-20, IL-1β, GM-CSF) in small airways, pulmonary arterioles and alveolar parenchyma.

Executive Summary:

Background

Environmental particulate matter, particularly the subset with a size range less than 2.5 microns in diameter ($PM_{2.5}$), is a significant public health concern due to its strong correlation with exacerbation of both pulmonary and cardiovascular disease. While regulation of PM levels is done on the basis of mass concentration within specific size ranges, there is much interest in the relative contribution of PM components and sources to PM-induced health effects. Classes of chemicals thought to contribute to PM toxicity include transition metals that generate reactive oxygen species (ROS), polycyclic hydrocarbons that alter cellular metabolism of xenobiotics and inflammatory molecules, and pro-inflammatory biologic material such as endotoxin.

While ROS generation resulting from transition metal catalysis as well as polycyclic aromatic hydrocarbon (PAH) metabolism has wide acceptance as a mechanism leading to pulmonary injury resulting from urban sources of PM such as vehicular exhaust and power generation, the role of endotoxin in eliciting inflammation has primarily been addressed in PM from agricultural sources. Our previous concentrated ambient particulate field studies demonstrated significant systemic platelet activation in two-week mouse exposures to both rural and urban source PM. Cell culture studies with PM collected from these exposures showed that responses to urban source PM were more inhibited by ROS scavengers, while binding of endotoxin was a more effective inhibitor of rural source PM-induced cellular inflammatory responses.

Consistent with prior cell-based studies, we hypothesized that location specific differences in endotoxin- and ROS- mediated responses would be evident in animals treated with urban or rural source PM. The present study tested that hypothesis using oropharyngeal aspiration of urban or rural source PM pretreated with a transition metal chelator, deferoxamine mesylate (DFM), or polymyxin B, an antibiotic that specifically binds endotoxin. A secondary objective of this study was to compare pulmonary and systemic inflammatory responses to urban and rural source PM using equivalent gravimetric doses, something not possible in CAPs experiments.

Methods

We compared the pulmonary and systemic responses to collected ambient PM from rural (Davis CA) and urban (Sacramento) sampling locations. Mice were given gravimetrically equal amounts (200 µg) by oropharyngeal aspiration. We used laser capture microscopic dissection (LCM) to characterize and compare gene responses of airways, pulmonary arteries and alveolar parenchyma using probes for genes associated with responses to PAH, ROS and inflammation. We determined the relative contribution of endotoxin to pulmonary and systemic responses by pre-incubating PM with polymyxin B, an antibiotic that specifically binds endotoxin. Similarly, we compared responses to PM pre-treated with the transition metal chelating agent deferroxamine (DFM). We evaluated endpoints including the nature and extent of pulmonary inflammation by histopathology, regionally specific pulmonary gene responses by LCM and real time Polymerase Chain Reaction (rt PCR), systemic cytokine concentrations by bead-based ELISA assays, and platelet activation using flow cytometry and probes for platelet surface molecules altered in pro-thrombotic states. Platelet studies used expression of GP41b, LAMP-1 and P-Selectin, surface markers of activation in both unstimulated platelets and in response to the platelet agonists ADP and thrombin. In addition, we

evaluated platelet-monocyte aggregates, a pro-thrombotic and pro-atherogenic consequence of platelet activation.

Results

While pulmonary inflammatory responses were present 24 hours after instillation of PM from both sources, histologic inflammation and gene responses were greater in mice treated with urban source PM. Pre-treatment with DFM did not affect biological responses to PM instillation. DFM studies were further complicated by evidence that DFM increased systemic platelet responses in synergy with $PM_{2.5}$. Consequently, we limited further experiments to endotoxin-binding studies with polymyxin B. Contrary to expectations, polymyxin B not only inhibited pulmonary inflammatory responses to rural PM, but also PM from the urban source.

To determine whether pulmonary inflammation initiated a systemic inflammatory response, we evaluated serum concentrations of a panel of pro-inflammatory cytokines and immune response proteins. In summary, of the few cytokine changes found to be statistically significant, all but GM-CSF in the urban source experiment were decreased relative to control animals, and there was no effect of rural source $PM_{2.5}$ on any serum cytokine.

Platelet activation studies similarly showed little response to PM instillation at 24 hours. Experiments using DFM chelation showed that DFM increased platelet activation parameters while polymyxin B had the opposite effect, decreasing the percent of activated platelets in both control and PM treated mice. There were no changes in the numbers of platelet-monocyte aggregates in any treatment group.

Only urban source $PM_{2.5}$ induced transcription of genes probed in these experiments. Untreated urban source $PM_{2.5}$ increased expression of the PAH response element CYP1A1 in lung airways and parenchyma. Untreated urban $PM_{2.5}$ increased aldehyde dehydrogenase 3a, another PAH response element in airways. ATF3, a transcription factor activated by the ROS sensitive stress activated MAP kinase and the ROS response element HOX-1 were both increased in the polymyxin B experiment and their expression was suppressed by polymyxin B pre-treatment. A similar HOX-1 response was evident in pulmonary arterioles. Untreated urban source $PM_{2.5}$ induced increased airway expression of CCL-20, a cytokine associated with immune responses. In airway epithelium in the experiment investigating polymyxin B inhibition, IL-1β, a master regulator pro-inflammatory cytokine, was increased by urban source $PM_{2.5}$ and inhibited by polymyxin B treatment. GM-CSF, a cytokine important in bone marrow activation, was similarly up-regulated in airways by urban PM and inhibited by polymyxin B pre-treatment.

Conclusions

This study examined the relative role of transition metals and bacterial-derived endotoxin in inducing pulmonary and systemic inflammation in response to $PM_{2.5}$ derived from urban and rural sources. Our findings confirm that urban source $PM_{2.5}$ induces greater responses on a mass equivalent basis than $PM_{2.5}$ collected from rural areas. Pulmonary inflammation and gene responses were diminished by pretreatment intended to bind endotoxin in $PM_{2.5}$ but not by pretreatment to chelate transition metals. In contrast to prior studies demonstrating platelet activation in longer-term field exposures to concentrated ambient particles, no $PM_{2.5}$ induced platelet activation was detected in

these single dose studies. We conclude endotoxin may be a key factor in both urban and rural $PM_{2.5}$ toxicity.

Recommendations:

1) This study provides additional evidence for the importance of $PM_{2.5}$ composition for influencing biological responses. Experiments with equal gravimetric doses of PM from differing regions, i.e urban as compared to rural led to markedly different inflammatory responses.
2) Endotoxin must be considered an important constituent in both rural and urban source PM.
3) The nature and time course of systemic responses to $PM_{2.5}$ inhalation deserves further investigation, particularly relative to the length of the post-exposure period necessary to induce these responses.
4) The observation that a specific constituent of PM, namely endotoxin might contribute to short-term inflammatory pulmonary responses in a rodent model of exposure raises the possibility that specific constituents of PM might drive health effects in humans and contribute to the societal health burden of ambient PM. Such an situation if proven would have implications regarding policy and regulatory decision making

Detailed Report:

Introduction:

Environmental particulate matter, particularly that less than 2.5 microns in diameter ($PM_{2.5}$), is a significant public health concern due to its strong correlation with exacerbation of both pulmonary and cardiovascular disease[1, 2]. While regulation of PM levels is principally done on the basis of mass concentration within specific size ranges, there is much interest in the relative contribution of PM components and sources to PM-induced health effects. Classes of chemicals thought to contribute to PM toxicity include transition metals that generate reactive oxygen species (ROS), polycyclic hydrocarbons that alter cellular metabolism of xenobiotics and inflammatory molecules, and pro-inflammatory biologic material such as endotoxin.

Generation of ROS through both endogenous inflammation and exogenous sources is often considered a key mechanism in PM induced injury[3]. ROS generation from PM is a consequence of redox active organic hydrocarbons and can also be catalyzed by transition metals[4]. Both of these PM components are derived from products of fuel combustion in vehicles and power generation, and thus are associated with urban sources of PM. Metal chelators decrease PM- induced *in vitro* responses including arachadonic acid release [5] and ROS generation[6]. Inclusion of ultrafine iron particles in exposures of rats to soot markedly increased evidence of inflammation and cytotoxicity in broncho-alveolar lavage[7].

Metals of particular importance to PM toxicity include iron, nickel, copper, zinc and vanadium[8]. Iron is most frequently cited as catalyzing production of ROS through a chemical process known as the Fenton reaction. Iron, copper and zinc are present in relatively similar concentrations in $PM_{2.5}$ collected in California[9]. Seasonal variation in iron concentration has been demonstrated and was correlated with increased markers of systemic inflammation in a two-week concentrated ambient particle (CAPs) study[9]. Iron concentrations are generally higher in urban source particulates, with an expected two fold difference from rural source atmospheric particles[10].

Increases in pulmonary antioxidant systems are the key findings suggesting that ROS contributes to responses to $PM_{2.5}$ inhalation[3]. While it is commonly thought that transition metal-catalyzed ROS generation is the key mechanism leading to ROS mediated injury, other sources of ROS generation related to $PM_{2.5}$ toxicity are recognized. These alternative means of ROS generation complicate the justifications for regulatory decisions based on reducing transition metal generation. Polycyclic aromatic hydrocarbons (PAH), particularly the quinone derivatives, are highly capable of redox activity and have been proposed as significant contributors to $PM_{2.5}$-mediated ROS generation in the lung[11].

While ROS-related responses are often attributed to metal-based generation through catalysis of Fenton-like redox activity, endogenous sources of ROS as part of inflammatory responses may also contribute to ROS mediated injury. For example, experimental inhibition of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a key enzyme in endogenous ROS generation, significantly decreases inflammatory cytokine generation in response to various sources of $PM_{2.5}$ *in vitro* and *in vivo*.[12]

Despite much interest in transition metal-induced ROS in the mechanisms of $PM_{2.5}$ mediated health effects, epidemiologic evidence investigating the relationship between source specific composition and adverse effects of $PM_{2.5}$ shows inconsistent correlations between concentrations of transition metals and physiologic alterations such as extent of inflammation in the lung, altered heart rate variability and increases in inflammatory proteins in blood[13, 14]. Markers of inflammation in blood from elderly humans with cardiovascular disease were associated with ultrafine PM concentrations of PAHs and hopanes, but not other organics or

transition metals[4]. Individuals with polymorphisms enhancing the iron sequestration activity of the hemochromatosis (HFE) gene product were protected from $PM_{2.5}$-induced decrease in the high-frequency component of heart rate variability[13]. In contrast, a study evaluating pulmonary inflammation resulting from instillation of mice with $PM_{2.5}$ collected from several sites in Europe demonstrated correlations with oxidized organic compounds and transition metals, but an inhibitory effect was associated with PAH concentration[14].

Given the contrast between *in vitro* studies implicating transition metals and ROS generation in cellular responses and the variability of findings from epidemiologic studies, the role of transition metals and ROS in $PM_{2.5}$ induced health effects remains uncertain. This project used a transition metal chelator, deferoxamine mesylate (DFM) to examine the effect of selective inhibition of metals on pulmonary and systemic responses to urban source $PM_{2.5}$.

DFM is an iron scavenging protein (siderophore) derived from bacteria that effectively binds ferric iron and prevents oxidative tissue damage. DFM also binds aluminum and calcium, and exhibits iron-independent anti-oxidative effects on hydroxyl radicals and tyrosine nitrosylation[15]. DFM chelation abrogated 90% of *ex vivo* hydroxyl radical formation in collected ambient particulate matter[8]. Fly ash particle-induced formation of ROS, liberation of aracadonic acid and prostacyclins (PGE2/TXB2) and activation of stress-associated MAP kinase pathways was decreased in macrophages pretreated with DFM[16]. Similarly, DFM (but not polymyxin B) pretreatment of particles collected in a subway inhibited $PM_{2.5}$-induced TNF production in murine macrophages[17]. While DFM has been used in clinical trials as an anti-inflammatory agent, its effect on $PM_{2.5}$ toxicity *in vivo* has had limited study. In one experiment, decreases in whole blood clotting time in response to water soluble metals extracted from $PM_{2.5}$ were prevented by DFM treatment[18]. Exposure to the water-soluble fraction of $PM_{2.5}$, at doses as low as 50 ng/ml original particle, significantly diminished the whole-blood coagulation time. Inclusion of DFM prolonged coagulation time following the exposures to the water-soluble fraction[18]. The present project investigated the effect of DFM chelation on mice treated with $PM_{2.5}$ collected from an urban region in Sacramento California. We evaluated pulmonary and systemic inflammation, as well as the effect on platelet activation 24 hours after intratracheal instillation.

Multiple lines of evidence suggest a significant role for endotoxin in pro-inflammatory responses to inhaled particulates. Many of these studies emphasize agricultural occupational exposures[19]. Endotoxin concentrations are high in both dairy and swine confinement operations[20] and workers in these areas have decreased forced expiratory volume in 1 second and decreased forced vital capacity during and after their work shifts[21].

Because the standard bioassay for endotoxin lacks specificity, the role of endotoxin in $PM_{2.5}$-induced health effects has been little studied. Reduction in endotoxin levels was a more consistent factor than transition metal concentrations in driving differences in *in vitro* toxicity of $PM_{2.5}$ collected after stringent air pollution controls were applied in preparation for the 2008 Olympic games[22]. Similarly, endotoxin was strongly associated with cytokine secretion in nasal fluid of humans exposed to $PM_{2.5}$ from a variety of locations, while oxidant potential was not correlated with inflammation[23]. Principal component analysis of $PM_{2.5}$ collected from multiple sites in Mexico City correlated *in vitro* secretion of the inflammatory cytokines TNFα and IL-6 with elements derived from soil, including endotoxin, but not with PAH or most anthropogenic transition metals[24]. Endotoxin concentrations are likely higher in rural source $PM_{2.5}$, with one study demonstrating three to ten-fold higher endotoxin levels in $PM_{2.5}$ collected from rural agricultural regions compared with urban sources[25].

Polymyxin B is commonly used to bind endotoxin for *in vitro* experiments[26] and is proposed as a treatment for septic shock[27]. Polymyxin B treatment selectively attenuated the effects of coarse urban PM to reduce acetylcholine-mediated relaxation of isolated pulmonary arteries[28] and reduced cytokine responses in coarse PM but not $PM_{2.5}$ treated monocytes[26]. In contrast, previous studies in our laboratory showed polymyxin B to selectively inhibit monocyte cytokine

secretion and adhesion to endothelial cells in response to treatment with urban $PM_{2.5}$ from Fresno, California[29]. In preliminary studies we also found that *in vitro* responses to rural source $PM_{2.5}$ were much more attenuated by polymyxin B treatment than responses to urban source $PM_{2.5}$ (unpublished data). While these cell culture studies implicate endotoxin as an important pro-inflammatory component of $PM_{2.5}$, confirmation of this in animal exposures has not previously been done. Our preliminary studies combined with the reported higher concentrations of endotoxin in rural source $PM_{2.5}$ led to our hypothesis that polymyxin B treatment would be much more effective in attenuating pulmonary and systemic inflammatory responses to rural source $PM_{2.5}$ compared with urban sources.

While many animal studies of $PM_{2.5}$ are done with freshly collected concentrated ambient particles (CAPs), comparison between studies is complicated by variability in the overall dose of PM to which the animals are exposed because CAPs technology allows control of the PM enrichment factor but not the actual concentration. Our prior CAPs study demonstrated up-regulation of inflammatory and xenobiotic metabolism genes in specific regions of lung that varied by season, and urban or rural location[9]. We also found that intratracheal instillation of collected $PM_{2.5}$ elicits pulmonary inflammation and generates a similar up-regulation of inflammatory and xenobiotic metabolism genes in specific regions of pulmonary parenchyma. Using treatment with equivalent masses of $PM_{2.5}$, we showed that these responses varied in intensity and specificity between $PM_{2.5}$ collected from urban or rural sites, but with opposite site-specific intensity relative to responses in CAPs studies (manuscript in preparation). The present study asked whether location specific differences in responses to $PM_{2.5}$ could be related to differences in the transition metal or endotoxin content of $PM_{2.5}$. We hypothesized that inflammation resulting from instillation of urban source $PM_{2.5}$ is primarily related to metal components in the $PM_{2.5}$ mixture that would be inhibited by DFM treatment, while inflammation induced by rural source $PM_{2.5}$ would be primarily dependent on endotoxin content that would be inhibited by polymyxin B.

Methods:

Project Design:

Our original project design included four experiments, each consisting of an animal exposure to urban or rural source $PM_{2.5}$, either unaltered or pre-treated with either polymyxin B to remove endotoxin, or desferoxamine, as an antioxidant and to chelate metals. Health-related endpoints included several pulmonary and systemic responses. Our proposal was modified from the original contract through consultation with the ARB contract manager in two ways. First, we changed the source of $PM_{2.5}$ originally proposed because there proved to be an insufficient amount of archival PM2.5 from urban and rural Fresno to complete the study. Therefore, we used more recently collected samples from an urban and a rural site close to Sacramento. Second, based on the negative results using deferoxamine pretreatment of urban source $PM_{2.5}$, we eliminated Task 2 (Deferoxamine pretreatment of rural source $PM_{2.5}$). Tasks 1, 3, and 4 were completed as outlined below. Funds originally intended for Task 2 were redirected to expanding the number of endpoints and enlarging the sample sizes in the remaining three experiments.

Task 1: Intratracheal instillation of rural source $PM_{2.5}$ +/- Polymixin B.

a. Animal study, Flow Cytometry
b. Cytokine analysis, Histopathology
c. Laser Capture Microdissection RT-PCR

Task 2: Intratracheal instillation of rural source $PM_{2.5}$ +/- Deferoxamine.
(This task was eliminated)

a. Animal study, Flow Cytometry
b. Cytokine analysis, Histopathology

c. Laser Capture Microdissection RT-PCR

Task 3: Intratracheal instillation of urban source $PM_{2.5}$ +/- Polymixin B.

a. Animal study, Flow Cytometry
b. Cytokine analysis, Histopathology
c. Laser Capture Microdissection RT-PCR

Task 4: Intratracheal instillation of urban source $PM_{2.5}$ +/- Deferoxamine.

a. Animal study, Flow Cytometry
b. Cytokine analysis, Histopathology
c. Laser Capture Microdissection RT-PCR

Collection of ambient particulate matter: Experiments in this study used archival samples of ambient particulate matter collected by the UC Davis Air Quality Research Center at an urban site in Sacramento and rural sampling site near Davis, CA. The urban site is located on top of the CARB building at the corner of T Street and 13th Street in downtown Sacramento in a high traffic area with a predominance of gasoline fueled automotive traffic The rural site is located at the University of California Center for Health and the Environment and is adjacent to the University's animal science research facilities. The facility operates as an agricultural research station with mixed crop plantings. Ambient $PM_{2.5}$ was collected simultaneously at both sites using the combination of a $PM_{2.5}$ high-volume sampler and 4-stage high-volume cascade impactor in the winter of 2011 (12/04-12/12). The sampling was done during a dry period with predominately high pressure modulated by very light precipitation during the middle of the sampling period. During the sampling period there were 6 moderate ($PM_{2.5}$ between 13-35 $\mu g/m^3$) and 3 unhealthy for sensitive groups ($PM_{2.5}$ between 36-55 $\mu g/m^3$) days (source: http://www.sparetheair.com). Bulk $PM_{2.5}$ samples were collected with prebaked (48 hrs at 550°C) glass fiber filters. Samples were collected for 24 hours a day. Filters were stored sealed in an argon atmosphere at -80°C until analyzed or used for biologic experiments.

Particle composition and preparation for use: Particles were collected over 8 contiguous days 24 hours a day during the winter season at an urban location adjacent to multiple freeways in Sacramento, CA and simultaneously at a rural location adjacent to both animal and plant agricultural fields south of Davis, CA. Glass fiber filters were used for both extraction for animal experiments and analysis. The particle elemental carbon (EC) and organic carbon (OC) concentrations were determined by thermo-optical analysis following the NIOSH temperature protocol[30] and reported as mass of carbon per unit volume of sampled air; e.g. ug/m^3. Total carbon is calculated as the sum of EC and OC. Organic matter is calculated from organic carbon (OC) using a simple multiplication factor (1.6) to account for all of the other non-carbon elements in organic matter (i.e. H, O and N0. Water-soluble ions were quantified with Ion Chromatography (IC) analysis and trace elements were quantified with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) analysis[30]. Polycyclic aromatic hydrocarbons (PAHs) were extracted from samples via sonication in organic solvents followed by concentration by nitrogen evaporation and quantification using Thermal Desorption-Gas Chromatography Mass Spectrometry (TD-GCMS). PAH concentrations were quantified using isotopically labeled internal standards.

$PM_{2.5}$ was extracted from filters by probe sonication in saline as previously described [31]. Particles for intratracheal instillation were obtained by probe sonication of collected filters followed by filtration through Quiagen spin filters (Quiagen, Valencia, CA) to remove fibers derived from collection filters. The resulting PM suspension was frozen to -80˚C and then lyophilized to obtain dry PM. Particle mass used to calculate dilutions for treatment doses was determined by lyophilizing PM in tared storage vials and weighing the vial post lyophilization. For oropharyngeal aspiration, PM was resuspended by sonication in 30µl phosphate buffered saline (PBS). Control mice were treated with a similarly prepared extract of clean filters. Preliminary experiments with defined size iron oxide particles assayed by dynamic light

scattering demonstrated probe sonication was necessary but adequate for close reconstitution of size distributions (data not shown).

Animal studies: Male C57BL/6 mice were obtained from Jackson laboratories at 8 weeks of age. Mice were acclimated in American Association of Laboratory Animal Care (AALAC) accredited facilities for one week before experiments were begun. The UC Davis institutional animal care and use committee (IACUC) approved all experimental protocols. Groups of six mice were anesthetized with ketamine/xylazine and given 200 µg of $PM_{2.5}$ from either urban or rural archival samples by pharyngeal aspiration of $PM_{2.5}$ suspensions in 30 µl phosphate buffered saline (PBS). Additional groups of mice received urban PM2.5 pretreated for one hour with DFM (500 µM, EMD Biosciences), urban PM2.5 pretreated with Polymyxin B (100 µg/ml, Sigma-Aldrich) or rural source $PM_{2.5}$ treated with Polymyxin B. The relatively high dose of $PM_{2.5}$ is comparable to that used in several prior studies[32-34]. It is approximately equivalent to the total exposure of an adult human breathing a high ambient concentration of 20 µg/m^3 for 24 hours (calculated based on assumptions of 0.5 l tidal volume and 15 breaths/min.). Control animals were similarly instilled with PBS alone. Mice were monitored during recovery from anesthesia (approximately 60 min.) and kept for 24 hours before euthanasia after which blood and tissue samples were collected.

A tracheal cannula was inserted and secured by ligation with suture material. The lungs were removed from the thorax. The left lung lobe was ligated at the mainstem bronchus and removed. The left lung lobe was inflated with polyethylene glycol and polyvinyl alcohol cryosectioning matrix (OCT) (50% OCT: PBS) and three transverse sections flash frozen in 100% OCT by immersion in liquid nitrogen cooled isobutane. These three sections were cryosectioned for use in laser capture microdissection experiments. The remaining right lung lobes were inflated with 10% neutral buffered formalin at 20 cm H_2O pressure. The formalin instilled right lung lobes were fixed overnight and transverse sections of each lung was embedded in paraffin using standard histotechnology approaches and an automated processor. These blocks were then used to prepare standard hematoxylin and eosin stained slides.

Histologic Evaluation: A veterinary pathologist blinded to treatment group assignment evaluated each section of lung. Subjective lesion scores were generated for overall inflammation and epithelial hypertrophy/hyperplasia in small airways, parenchymal inflammation and alveolar wall thickening, and arteriolar inflammation and mural thickening. Lesions were scored relative to severity on a 0-4 scale with 4 representing the most prominent or intense lesion in the study.

Laser Capture Microdissection (LCM): The left lung lobe from experimental mice were perfused with 50% polyethylene glycol and polyvinyl alcohol (OCT) in saline and flash frozen in labeled plastic cassettes. Sections 15 µm thick were cut using a cryostat, mounted on a glass slide coated with a thin layer of polyethylene naphthalate foil and preserved with RNA*later* ICE (Ambion, Fischer Scientific, Pittsburg PA) at -20°C. LCM was performed at the UC Davis Center for Health and the Environment using a Leica LMD6000 system equipped with an ultraviolet laser and an upright microscope with moving prisms to guide the laser over a stationary sample. Based on the location of histologically evident inflammatory responses, specific tissue regions including terminal bronchioles, venous and arterial blood vessels, and surrounding parenchyma were micro-dissected from each mouse and sorted into separate microfuge tubes.

Quantification of gene expression levels: Total RNA was immediately isolated from pooled samples of each tissue type from individual lung lobes using an RNeasy Micro Kit (Qiagen), including a DNA digestion step, following manufacturer's instructions. Total RNA was quantified and mRNA was reverse transcribed into cDNA using a Superscript III First Strand Synthesis System (Invitrogen). TaqMan assays (Applied Biosystems) specific for genes of interest were used to quantify gene expression changes using qRT-PCR according to the following amplification parameters: initial denaturation for 10 min at 95°C, followed by 40 cycles at 95°C

for 15 s (melting) and 60°C for 1 min (annealing and extension). Transcript levels were measured using the ABI Prism 7700 system (Applied Biosystems) and normalized to β2-microglobulin expression levels. Results were expressed as fold changes using the comparative C_T method for relative quantification. Target genes were selected based on *in vitro* experiments with collected particulate matter[31, 35, 36] .

Bioplex Assay: A multiplex assay was employed in order to perform a broad screening assay for systemic cytokine activation in response to PM inhalation. Blood samples were collected from the inferior vena cava of sodium pentobarbital anesthetized mice at euthanasia, under institutionally approved protocols, and allowed to clot. Using the resulting serum, cytokine assays were done using a commercial immuno-bead based analytical system and mouse specific assay kits (Bioplex, Biorad Life Science, Hercules CA). Growth factors assayed included Eotaxin, Fibroblast Growth Factor (FGF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), Interferon-γ(IFNγ) Interleukin-1α(IL-1α), Interleukin-1β (IL-1β), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-9 (IL-9), Interleukin-10 (IL-10), Interleukin-12 protein 40 (IL-12p40), Interleukin-12 protein 70 (IL-12p70), Interleukin-13 (IL-13), Interleukin-15 (IL-15), Interleukin-17 (IL-17), Interleukin-18 (IL-18), Keratinocyte chemotactic factor (KC), Leukemia Inhibitory Factor (LIF), Monocyte Chemotaxtic Protein-1 (MCP-1), Monocyte Colony Stimulating Factor (M-CSF), Monokine Induced by Gamma Interferon (MIG), Macrophage Inflammatory Protein-1α (MIP-1α), Vascular endothelial growth factor (VEGF), Macrophage Inflammatory Protein-1β (MIP-1β) Macrophage Inflammatory Protein-2 (MIP-2), Platelet Derived Growth Factor (PDGF), Regulated Upon Activation, Normally T-Expressed, and presumably Secreted (RANTES), and Tumor Necrosis factor-α (TNFα□□

Flow Cytometry Studies: Blood samples were collected into acid citrate dextrose (ACD) coated syringes with a ratio of 1:6 ACD: blood from the inferior vena cava of sodium pentobarbital anesthetized mice, under institutionally approved protocols. Following the addition of 10 □g/ml PGE_1 whole blood was incubated at 37°C for 30 min prior to analysis. All cell counts were determined using an automated blood counter (ActDiff, Beckman-Coulter, Miami, FL).

Platelet alpha granule proteins and integrins: Mouse platelet activation was analyzed in ACD-A anticoagulated whole blood (collected from the vena cava at euthanasia) by flow cytometry using the following anti-mouse antibodies, along with appropriate isotypes for each: a biotin conjugated monoclonal antibody to the α_{2b} subunit for the major platelet integrin $\alpha_{2b}\beta_{3a}$ (CD41, BD Pharmingen) followed by Streptavidin Alexa 633 (in vitrogen), a FITC-conjugated monoclonal antibody to P-selectin for alpha granule secretion (CD62P, BD Pharmingen), and a PE-conjugated monoclonal antibody to LAMP-1 for lysosomal granule secretion (CD107a, eBioscience). A resting (unstimulated) sample and samples stimulated with either 10 □M ADP or 0.1U/ml thrombin were examined for each animal. After stimulation of whole blood, platelets were labeled with the preceding antibodies for one hour and fixed in 1% (final) paraformaldehyde prior to analysis by flow cytometry (FC500, Beckman-Coulter, Miami, FL). Platelets were defined by forward (FSC), and side scatter (SSC) characteristics, and ten thousand events were collected within the platelet gate for each animal and each condition. Platelet-derived (CD41positive) membrane microparticles were identified based on FSC with the threshold set at 10^1 representing the lower FSC boundary for resting platelets.

Platelet activation was assessed by the presence of either alpha granule secretion resulting in P-selectin expression on the platelet surface, or by lysosomal granule secretion resulting in LAMP-1 expression on the platelet surface. Generally resting platelets express very little of either protein. Stimulation by agonist (either ADP or thrombin) results first in alpha granule secretion, followed by lysosomal granule secretion. Platelets were evaluated using two parameters: (1)the percent positive cells, the number of cells present within the pre-defined platelet gate which express the protein of choice on their cell surface, and (2) the mean fluorescence intensity, the relative number of molecules on the platelet surface. Below are

general definitions and information regarding platelets that should clarify interpretation of the data.

When considering flow cytometric data there are two things collected for each antibody studied: the percent positive and the mean fluorescence intensity (MFI). The percent positive are the number of events (cells) that are positive for the antibody. The mean fluorescence intensity (MFI) is an indicator of how many molecules of the particular antigen are present on the surface of the cells.

Unstimulated or "resting" platelets have large amounts of the major platelet integrin alpha2b beta3a on their surface and this should be reflected by the high percent positive cells (between 85-99%) and the high MFI that is arbitrary, as it is based on the titration of the antibody. The value of MFI generally runs between 100-300 arbitrary fluorescence units (AFUs). Resting platelets should not have either alpha or lysosomal granule membrane proteins on their surface, as they should not have secreted their granules when in a resting state. Thus they should have little P-selectin both with regard to %+ and MFI, as well as little LAMP-1.

When platelets are stimulated with the physiological agonists thrombin or ADP, there is usually secretion of both alpha granules and lysosomal granules with resultant increase in both %+ and MFI for both P-selectin and LAMP-1. With regard to CD41b there is a more complicated phenotype. CD41b is present in large numbers on the platelet surface and on the membranes of alpha granules. When platelets are stimulated by an agonist one, or more of the following events may occur: 1) CD41b (along with its beta subunit) can be shed on microvesicles, which resulting in a decrease in %+ and decrease in MFI of the platelets; 2) CD41b and its beta subunit can be recycled (internalized) also resulting in a decrease in %+ and MFI; and 3) when the platelets are only slightly activated there can be an increase in CD41b on the platelet surface, simply reflective of alpha granule secretion and the CD41b associated with those alpha granules. It should be noted that it is very difficult to differentiate between these processes without evaluating the number of CD41b positive microvesicles. In either case, however, the decrease in %+ and decrease in MFI are strong and well recognized indicators of platelet activation.

Leukocytes and Monocytes: Leukocytes were identified with a monoclonal antibody to CD11b (eBioscience) and monocytes were specifically labeled with antibody to CD115 (eBioscience). Separate samples were prepared to evaluate platelet-monocyte and platelet-leukocyte interactions. After processing with antibodies, blood was fixed in 1% paraformaldehyde and analyzed by flow cytometry. Platelet-monocyte or -leukocyte aggregates were defined as populations that were positive for CD41, and either CD11b or CD115. All flow cytometric data were analyzed in FloJo (TreeStar, Oregon).

Statistics: For cytokine and PCR assays, the Student's T-test was used to compare control and particle-instilled animals. Subjective histologic scores were compared using the Kruskal Wallace non-parametric ranking statistic. Differences in platelet populations and platelet monocyte aggregates were compared using a Student's T-test. Statistical calculations were done with GraphPad Prism. All tests used a significance level of $p \leq 0.05$.

Results:

Particle Composition:
Urban source particles had equivalent elemental carbon but a greater proportion of organic carbon than that in rural source PM2.5. Urban source particles also had greater proportions of iron, copper, barium, and aluminum. Overall polycyclic hydrocarbons (PAH) concentrations were 2 fold higher in urban compared with rural source $PM_{2.5}$ (appendix 1). Rural source PM had greater amounts of water-soluble ions (27+/- 0.5% vs 16 +/- 0.6% in urban source PM).

Histology:
To compare responses to urban and rural $PM_{2.5}$ (untreated, and pre-treated with DFM or polymyxin B) on pulmonary inflammation, sections of lung were subjectively scored for the extent of small airway hyperplasia and inflammation in the terminal bronchiole, alveolar parenchyma, and surrounding the pulmonary arterioles at the terminal bronchiole. Compared with control animals (Fig. 1A), mice instilled with urban source $PM_{2.5}$ had marked infiltration of neutrophils surrounding aggregates of homogenous fine black particulates admixed with lesser refractile crystalline or amorphous brown particles (Fig. 1B). Particles were both extracellular and within macrophages. Neutrophils also infiltrated the walls and adventitia of bronchiolar arterioles. Mice given urban source $PM_{2.5}$ pretreated with DFM did not differ from $PM_{2.5}$ treated mice in the location or extent of pulmonary inflammation (Fig. 1C), while mice given urban source $PM_{2.5}$ pretreated with polymyxin B had similar amounts of histologically evident $PM_{2.5}$ as the $PM_{2.5}$ only group but markedly reduced inflammatory cell infiltrates (Fig. 1D).

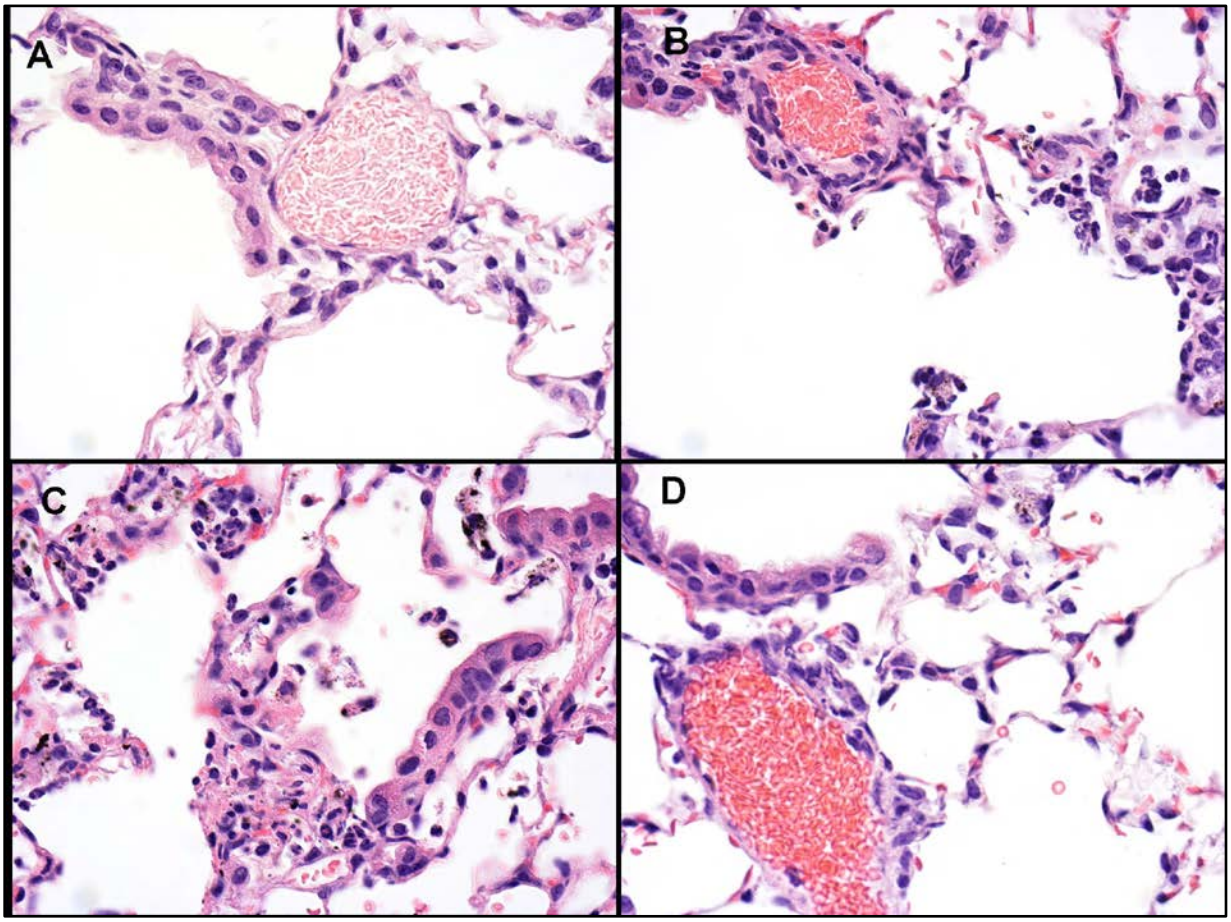

Figure 1: Histopathology of terminal bronchiolar junction with pulmonary arteriole from mice given intratracheal instillations of 200 ug/mouse of: A) PBS only; B) Urban source $PM_{2.5}$; C) Urban source $PM_{2.5}$ pretreated with DFM; or D) Urban source $PM_{2.5}$ pretreated with polymyxin B. $PM_{2.5}$ alone treatment elicited significant neutrophil accumulation around evident $PM_{2.5}$ in proximal alveolar ducts and in the adventitia of pulmonary arterioles. DFM pre-treatment did not alter the location or extent of inflammatory response but polymyxin B pre-treatment significantly decreased the neutrophil influx.

Subjective scores for pulmonary inflammation are presented in Fig. 2. There was a significant increase in airway and parenchymal inflammation resulting from urban $PM_{2.5}$ treatment compared with DFM alone and equivalent increases in all regions from animals given DFM pretreated $PM_{2.5}$ (Fig. 2A). Similar increases in inflammation scores were evident in a separate experiment using polymyxin B inhibition (Fig. 2B). Polymyxin B pre-treatment of $PM_{2.5}$

prevented much of the inflammatory infiltrate in response to both urban (Fig. 2B) and rural (Fig. 2C) source $PM_{2.5}$.

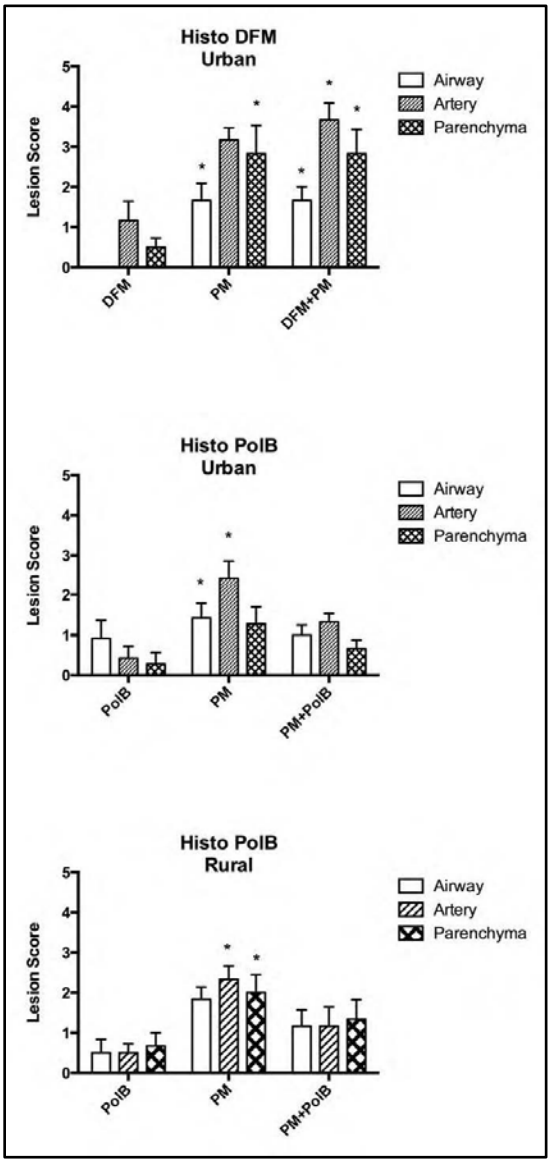

Figure 2: Subjective histologic analysis of airway, alveolar and arteriolar inflammation comparing responses to intratracheally administered: A) urban source $PM_{2.5}$ pre-treated with polymyxin B; or B) DFM; or C) rural source $PM_{2.5}$ pretreated with polymixin B.

Histologically, there was a significant difference in the nature of inflammatory infiltrates between mice treated with urban when compared to rural source $PM_{2.5}$. Mice treated with urban source $PM_{2.5}$ had marked neutrophilic infiltration surrounding $PM_{2.5}$ particles that were both extracellular and intracellular in macrophages. Inflammation in lungs of mice treated with rural source $PM_{2.5}$ was predominantly composed of macrophages with most $PM_{2.5}$ located intracellularly. The proportion of neutrophils in rural source induced exudates was markedly less than that in urban source treated animals (Fig. 3).

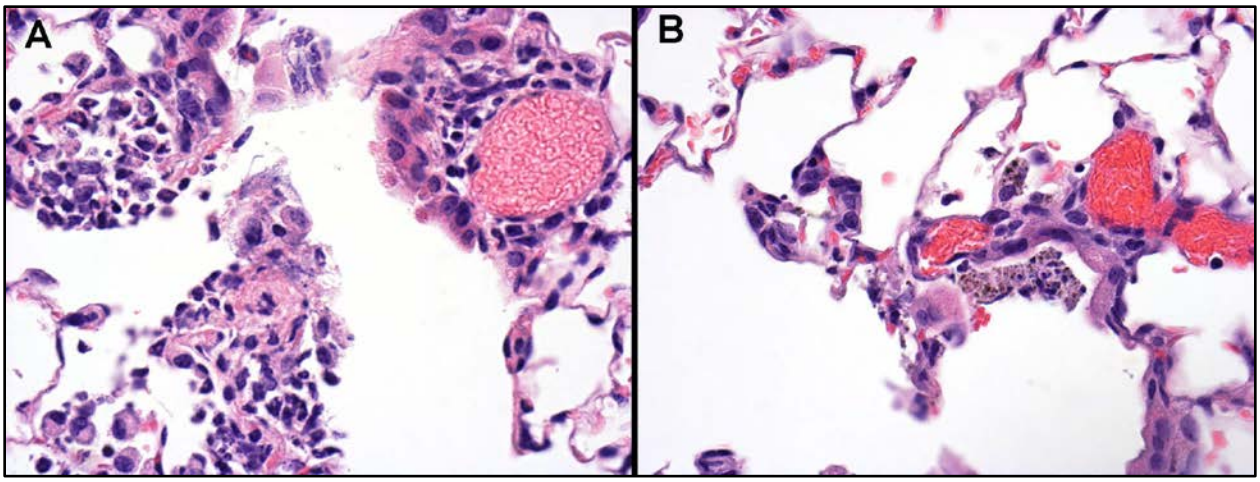

Figure 3: Histopathology of terminal bronchiolar junction with pulmonary arteriole from mice given intratracheal instillations of 200 ug/mouse of: A) urban source; or B) rural source $PM_{2.5}$. While there is no difference in apparent $PM_{2.5}$ densities, urban source $PM_{2.5}$ elicits a significantly greater neutrophil response while rural source $PM_{2.5}$ is more evident intracellularly in macrophages. Similarly, periarteriolar inflammatory cell infiltrates are more prominent in lungs of mice treated with urban source $PM_{2.5}$.

Histologic findings demonstrate that DFM pretreatment had little effect on pulmonary inflammation induced by urban source $PM_{2.5}$, while polymyxin B pretreatment significantly reduced pulmonary inflammation. In addition, there were significant differences in the nature and extent of neutrophilic inflammation in response to equivalent mass treatments of urban when compared to rural source $PM_{2.5}$ with urban source being much more pro-inflammatory, as evidenced by higher subjective inflammation scores in the mice treated with urban PM, and a relatively less prominent neutrophil infiltrate in response to rural PM treatment.

Systemic Cytokines:

To determine whether pulmonary inflammation initiated a systemic inflammatory response, we evaluated serum concentrations of a panel of pro-inflammatory cytokines and immune response proteins. The general classification of these activities are presented in Appendix 3. Results are presented in Table 1. In summary, of the few cytokine changes found to have changed significantly (Table 2) all but GM-CSF in the urban source experiment were decreased relative to control animals, and there was no effect of rural source $PM_{2.5}$ on any serum cytokines. There were few changes more than 25% of control values and no consistent effect of pre-inhibition with either DFM or polymyxin B.

Table 1:Cytokine analysis in serum of mice given intratracheal instillations of urban or rural source $PM_{2.5}$ pretreated with either polymyxin B or DFM.

	Urban			Urban				Rural			
Serum	Value (pg/ml)			Value (pg/ml)				Value (pg/ml)			
Mediator	PBS+DF	PBS+PM	PBS+DF+PM	PBS	PBS+PolB	PM	PM+PolB	PBS	PolB	PM	PM+PolB
Basic FGF	505	529	405	822	686	276	795	551	397	591	353
Eotaxin	522	317	370	ND	ND	ND	ND	ND	ND	ND	ND
G-CSF	516	1251	2585	ND	ND	ND	ND	ND	ND	ND	ND
GM-CSF	231	140	174	76	121	132	95	96	57	55	73
IFN-g	21	11	13	11	14	14	12	9	10	9	10
IL-1a	ND	ND	ND	9	9	11	8	7	6	7	7
IL-1b	253	214	206	179	179	164	163	138	137	159	142
IL-2	24	23	26	22	23	20	20	15	ND	19	19
IL-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-5	39	34	35	20	23	20	22	19	14	17	15
IL-6	9	15	20	ND	ND	ND	ND	ND	ND	ND	ND
IL-9	554	424	461	201	250	184	203	193	147	197	118
IL-10	77	58	55	30	33	32	26	28	21	29	25
IL-12p40	73	52	58	75	77	95	66	67	75	67	64
IL-12p70	128	75	101	56	50	54	61	47	36	52	50
IL-13	205	149	153	120	117	112	116	99	83	104	86
IL-15	1220	1485	1064	962	677	182	858	505	280	533	252
IL-17	246	173	186	88	97	84	100	99	97	95	109
IL-18	988	976	976	1661	1473	739	1557	1247	927	1391	939
KC	121	157	193	23	18	29	28	30	30	61	21
LIF	140	165	117	85	68	22	77	44	26	50	25
M-CSF	2312	2044	1868	1065	968	860	992	1107	967	913	797
MCP-1	316	264	253	162	164	158	148	139	118	145	124
MIG	2860	3085	2528	3059	2488	926		1822	1371	2142	1193
MIP-1a	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1b	50	33	40	45	37	32	37	32	24	35	27
MIP-2	87	98	75	46	37	11	42	28	17	28	15
PDGFbb	12674	13906	12487	17438	16286	7504	12343	7245	6404	9952	3297
RANTES	34	32	34	32	31	18	32	16	13	15	11
TNFa	777	496	464	423	440	486	377	362	291	328	304
VEGF	743	810	605	629	489	130	575	349	201	380	183

Table 2: Statistical analysis of cytokine results from mice treated with urban source $PM_{2.5}$. Changes in black represent decreased concentration. Changes in red are increased concentrations.

Serum		
Mediator	**Significant Changes**	
Basic FGF	**PBS+PM vs PBS+DF+PM**	
Eotaxin		
G-CSF		
GM-CSF	**PBS+DF vs PBS+PM**	**PBS vs PM (Urban, PolB) Upreg**
IFN-g	**PBS+DF vs PBS+PM**	
IL-1a		
IL-1b		
IL-2		
IL-3		
IL-4		
IL-5		
IL-6		
IL-9		
IL-10		
IL-12p40		
IL-12p70		
IL-13		
IL-15	**PBS+PM vs PBS+DF+PM**	
IL-17		
IL-18		
KC		
LIF	**PBS+PM vs PBS+DF+PM**	**PBS+DF vs PBS+DF+PM**
M-CSF	**PBS+DF vs PBS+PM**	
MCP-1		
MIG	**PBS+PM vs PBS+DF+PM**	**PBS+DF vs PBS+DF+PM**
MIP-1a		
MIP-1b		
MIP-2	**PBS+PM vs PBS+DF+PM**	
PDGFbb		
RANTES	**PBS vs PM Urban (polB)**	**PM + PolB vs PM (Urban)**
TNFa	**PBS+DF vs PBS+DF+PM**	**PBS+DF vs PBS+PM**
VEGF	**PBS+PM vs PBS+DF+PM**	

Deferoxamine effects on platelet activation by urban source $PM_{2.5}$

Resting platelets did not show any differences, either in the percent positive or MFI for CD41b or for LAMP-1. However, there was a significant increase in the %+ platelets for P-selectin in the PBS+PM2.5+DFM group relative to control (PBS+DFM) ($p \leq 0.05$) (Fig. 4a). There were no differences in the P-selectin MFI for any condition.

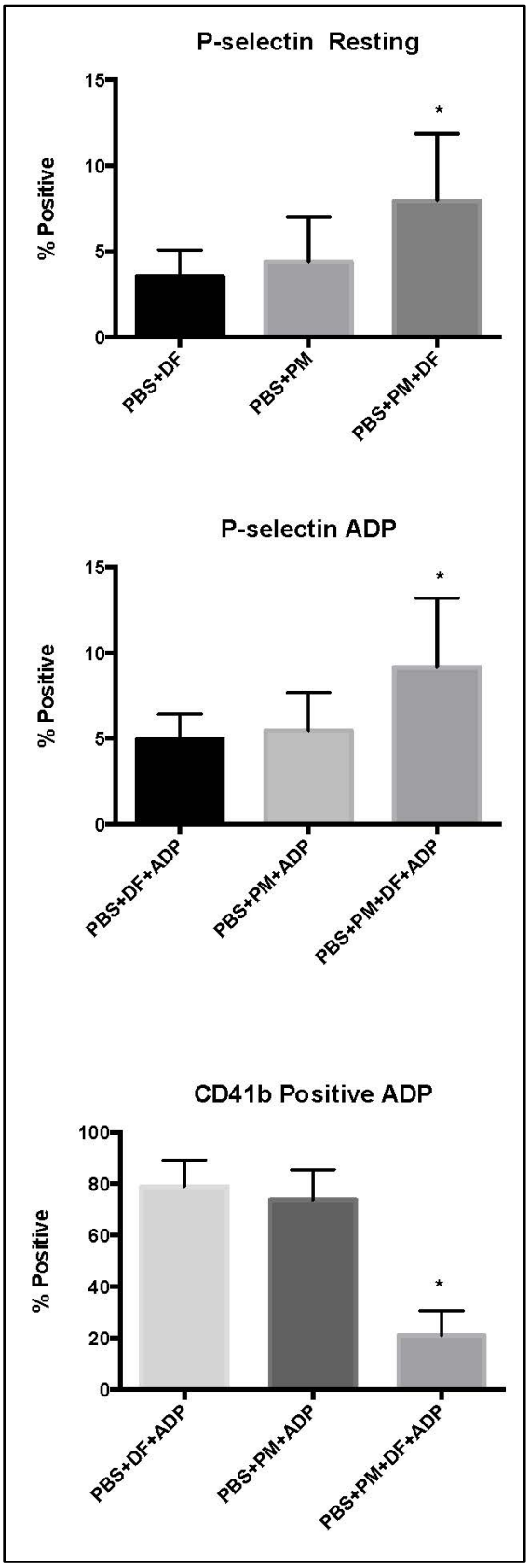

Figure 4: Platelet responses to DFM treatment. The percent of platelets positive for P-selectin was increased significantly in DFM + $PM_{2.5}$ treated groups in both resting (A) and ADP stimulated (B) platelets. The expression of the platelet surface marker CD41b was significantly reduced in the DFM + $PM_{2.5}$ treated group (C). All three changes are consistent with platelet activation (See text).

Platelets were stimulated with one of two physiologic agonists: either ADP or thrombin. ADP is a weaker agonist than thrombin, however there were differences in both P-selectin and CD41b. There were no differences in the %+ or the MFI for expression of LAMP-1 under any condition. There was a significant increase in P-selectin %+ in the PBS+PM2.5+DFM+ADP vs. control (PBS+DFM+ADP ($p \leq 0.05$) experiments (Fig. 4b), but no differences for MFI. With regard to the expression of CD41b, both the control and PM samples showed high expression of the integrin, while the PBS+PM+DFM sample had greatly reduced expression of CD41b (Fig. 4b). There were significant differences in the percent CD41b positive between PBS+DFM+ADP and PBS+$PM_{2.5}$+DFM+ADP and PBS+$PM_{2.5}$+ADP and PBS+$PM_{2.5}$+DFM+ADP (both $p \leq 0.001$) however there were no changes in MFI. The percent of cells expressing CD41b in PBS+$PM_{2.5}$+DFM+ADP was significantly lower than that in both PBS and PBS+ $PM_{2.5}$ groups(Fig 4c). This is additional evidence of platelet activation as activated platelets shed their surface CD41b.

Incubation with thrombin, a potent physiological agonist, resulted in increases in P-selectin and LAMP-1 expression in all groups for both the %+ as well as the MFI. There was decreased %+ and integrin expression (MFI) in all groups as well. None of these differences were statistically significant. Thus, it can be concluded that there was no alteration of the effect of agonist by incubation either with DFM or with PM and/or PM+DFM.

Our prior CAPs studies had shown an interaction between platelets and monocytes, thus we determined platelet-monocyte and platelet- -leukocyte interactions by flow cytometry. There were no differences in platelet-leukocyte or platelet-monocyte aggregates under any condition.

Polymyxin B Effects on Platelet Activation by Urban Source $PM_{2.5}$
Similar to our DF studies, we saw no differences between any of the treatments in the number of platelet-monocyte aggregates or the number of platelet-leukocyte aggregates.

Resting platelets showed no evidence of alpha granule secretion, as there was no difference either the %+ or the MFI of P-selectin for any of the conditions. There was, however, a significant difference between the %+ for LAMP-1 between $PM_{2.5}$ and PB+$PM_{2.5}$ ($p<0.05$), but there were no differences in MFI. All conditions had roughly equal numbers of platelets that were positive for CD41b. The MFI of the control (PBS) was significantly lower than PB ($0<0.001$), and was significantly lower than PB+PM ($p<0.01$). In addition the MFi of PM was significantly decreased when compared with the MFI from PB ($p<0.05$).

ADP stimulation showed no differences in either the percent positive cells or MFI for both P-selectin and LAMP-1. Additionally there were no differences in the percent positive cells for CD41, but similarly to resting platelets, there were differences in CD41b MFI. The control MFI (PBS) was significantly decreased relative to the $PM_{2.5}$ ($p<0.01$), as well as being decreased compared with polymyxin ($p<0.001$) and decreased compared with $PM_{2.5}$+PB ($p<0.0001$). The increased expression of CD41b on the surface is very similar to that seen in the resting platelets.

When platelets were stimulated with thrombin there were changes seen measured in all three parameters. There were significant differences in the percent positive P-selectin cells between PBS and PB ($p<0.05$) and PBS and PM+PB ($p<0.05$). There were no differences in P-selectin MFI. There were no significant changes in percent positive LAMP-1 cells or the MFI under any condition. There were no significant changes in the percentage of CD41b positive cells, however there was a difference between all of the conditions for the CD41b MFI ($p=0.042$).

Polymyxin B Effects on Platelet Activation by Rural Source $PM_{2.5}$
Resting PB treated platelets (both PB and PM+PB) had decreased expression of P-selectin, both with regard to percent positive cells ($p<0.05$) as well as the MFI ($p<0.001$). Interestingly,

PB had no detectable effect on LAMP-1 expression, nor did it effect CD41b expression or the percentage positive cells.

ADP stimulation resulted in increased numbers of P-selectin positive cells, however there was no difference between any of the conditions. There was, however, a slight decrease in MFI for $PM_{2.5}$, PB and $PM_{2.5}$+PB expression ($p<0.01$ for all conditions). There were no difference seen for either parameter of LAMP-1, nor were changes seen in CD41b percent positive or MFI.

Thrombin stimulation resulted in small changes with both granular membrane proteins. There was a significant decrease in percent positive P-selectin in $PM_{2.5}$+PB, ($p<0.05$) but no effect on MFI. There were no significant differences for any LAMP-1 parameters. There was a significant reduction in the percent positive CD41b cells in the $PM_{2.5}$ samples ($p<0.001$) when compared with PBS controls (consistent with platelet activation). There were also significant decreases in CD41b percent positive $PM_{2.5}$ compared with PB ($p<0.05$) as well as a significant decrease in percent positive $PM_{2.5}$ compared with $PM_{2.5}$+PB ($p<0.01$). There was a significant difference in MFI between PBS and $PM_{2.5}$+PB (decreased) ($p<0.01$).

Neither the number of platelet-monocyte aggregates nor the number of platelet-leukocyte aggregates differed among the various conditions studied.

Results of polymyxin B effects on platelet activation suggest the following conclusions: 1) PB knocks down the platelet response, 2) there is no evidence of platelet activation in circulating blood 24 hours after intratracheal instillation of $PM_{2.5}$ from either urban or rural sources, and 3) no platelet-leukocyte or platelet-monocyte interactions of significance were found in these studies.

Laser Capture Microscopy and rt-PCR.
Changes in specific lung regions are often not apparent in evaluations of whole lung gene responses. To achieve greater specificity for anatomic location specific responses, we isolated terminal bronchioles, alveolar parenchyma and pulmonary arterioles and probed each with a set of gene markers of PAH, ROS, and inflammatory response elements (Fig. 5). In contrast to mice treated with rural source PM only urban source $PM_{2.5}$ induced transcription of genes probed in these experiments, relative to controls. Untreated urban source $PM_{2.5}$ increased expression of the PAH response element CYP1A1 in airways and parenchyma in presence of DFM or polymyxin B. Aldehyde dehydrogenase 3a, another PAH response element, was increased in airways of animals given untreated urban $PM_{2.5}$ in the polymyxin B inhibition experiment. ATF3, a transcription factor activated by the ROS sensitive stress activated MAP kinase, and the ROS response element HOX-1 were both increased in the polymyxin B protocol and their expression was suppressed by polymyxin B pre-treatment. Untreated urban source $PM_{2.5}$ induced increased airway expression of CCL-20 in both experiments. In the experiment investigating polymyxin B inhibition, IL-1beta, a central pro-inflammatory cytokine, was increased by urban source $PM_{2.5}$ and inhibited by polymyxin B. GM-CSF, a cytokine important in bone marrow activation, was similarly up-regulated in the polymyxin B experiment and inhibited by polymyxin B pre-treatment.

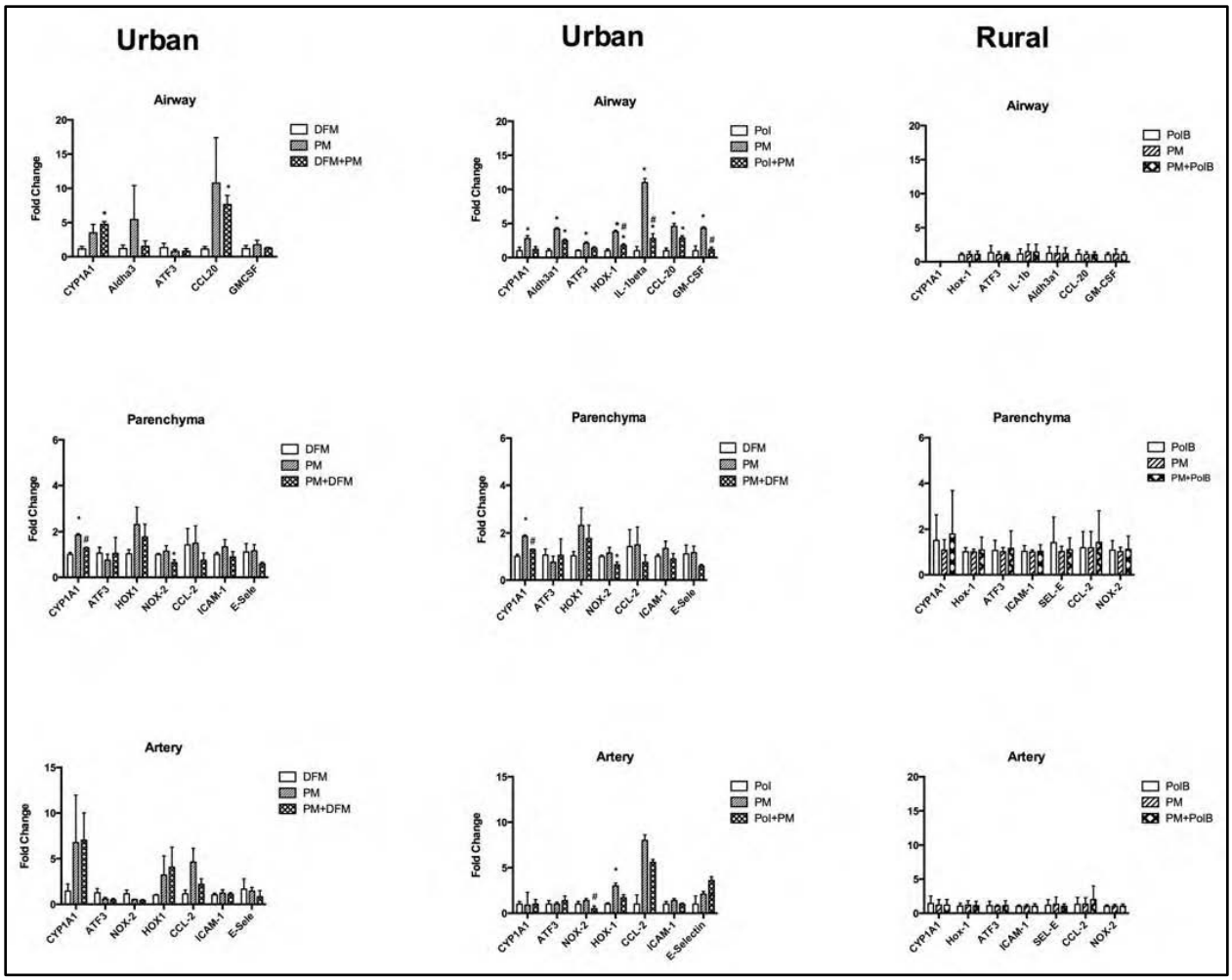

Figure 5: Results of rt-PCR measurement of transcripts from terminal bronchioles, alveolar parenchyma or pulmonary arterioles isolated by laser capture microscopy. Significant responses were only evident in tissues isolated from mice treated with urban source $PM_{2.5}$. Significant increases in PAH response elements (CYP 1A1, Aldh3a1) ROS response elements (ATF3, HOX-1) and inflammatory markers (CCL-20, IL-1 beta and GM-CSF) were present in urban $PM_{2.5}$ treated mice. While DFM pretreatment had little effect on responses, polymyxin B pretreated $PM_{2.5}$ inhibited transcription of CYP 1A1 in airways and parenchyma and ATF3, HOX-1, IL-1beta and GM-CSF in airways.

NOX-2, an NADPH dependent oxidase and source of endogenous reactive oxygen species, was probed in parenchyma and arterioles. While no $PM_{2.5}$ treatment effect was evident, interestingly both DFM and polymyxin B treatment decreased NOX-2 expression relative to controls. This suggests that DFM and polymyxin B both have some protective effect on background inflammation induced by the instillation process.

Despite significant periarteriolar inflammation in $PM_{2.5}$ treated animals, transcription of the endothelial cell adhesion molecules E-selectin and ICAM-1 were not significantly upregulated in $PM_{2.5}$ treated arterioles or parenchyma in any of these studies.

Discussion:

In a previous CAPs study, we demonstrated location- and season- specific differences in platelet and gene responses to exposure to $PM_{2.5}$ from another region of California[9]. In that study, the greatest number and extent of change occurred in mice exposed to rural source $PM_{2.5}$ during the winter. In contrast, the present study using mass equivalent amounts of $PM_{2.5}$

collected in winter from urban or rural sources shows a much greater response to urban source PM.
The principal findings of the present study are that the antioxidant effects and chelation of iron in $PM_{2.5}$ by DFM had no effect on pulmonary inflammation resulting from instillation with urban source $PM_{2.5}$. By contrast, instillation with $PM_{2.5}$ pretreated with polymyxin B significantly reduced pulmonary inflammation and gene responses in animals instilled with particles from both urban and rural sources.

Subjective histology evaluation showed a trend towards more pulmonary inflammation in animals exposed to urban source $PM_{2.5}$ relative to exposure to equivalent doses of rural source $PM_{2.5}$. This was further substantiated in gene response studies where urban source $PM_{2.5}$ elicited robust increases in both xenobiotic metabolism and inflammatory genes in each of the three lung compartments sampled, whereas rural source $PM_{2.5}$ did not induce significant gene responses. These findings correlate well with chemical analysis of the PM used in these same studies. The urban $PM_{2.5}$ used in these studies had 2-fold more iron and copper and 2-fold greater concentrations of PAH compounds relative to the rural $PM_{2.5}$ (appendix 1, unpublished data).

Previous studies by others and ourselves demonstrate increases in serum cytokines and activation of the clotting system in response to field exposures to concentrated ambient particulates[35, 37]. These changes were not recapitulated in the present single dose aspiration study. The reasons for the disparate findings remain uncertain. However, there were several differences in the protocols used by the two studies that could have influenced the results. Both studies sacrificed the animals about 24-hr following exposure. But, the field studies involved exposure to concentrated ambient particles for several consecutive days, in contrast to the single exposure used in the present study. Because epidemiologic evidence in humans[2] suggests that there is a 2-3 day lag between exposure to $PM_{2.5}$ and onset of morbidity and mortality, we may not have collected tissues for analysis at the most appropriate time post-exposure.

Genes selected for evaluation in this study were chosen based on previous *in vitro* gene response screening studies and represent pathways associated with xenobiotic metabolism (CYP 1A1, AldH2ase), ROS generation or response (NOX-2, ATF3, HOX-1), and inflammation (CCL2, CCL20, GM-CSF, ICAM, E-Selectin). Urban source PM2.5 exposure elicited consistent upregulation of CYP1A1 in both airways and parenchyma. A similar upregulation of AldH2ase occurred in airways. These findings correlate with our previous demonstration of CYP 1A1 as a signature of environmental particulate exposure *in vitro*[31, 38].

While there were no trends towards up-regulation of the endogenous ROS generating gene NOX-2, ROS sensitive genes ATF3 and HOX-1 were both up regulated in airways with similar trends in parenchyma and arteries. ATF3 is a transcription factor activated by the ROS sensitive JNK subset of MAP kinases. HOX-1 acts as an antioxidant through catabolism of free heme. It is transcriptionally regulated in response to cellular oxidative stress. These findings suggest that oxidative stress does occur in PM2.5 exposed lungs but is not a consequence of endogenous ROS generated by NOX-2. Interleukin-1beta (IL-1b), a master cytokine stimulating diverse pro-inflammatory responses and CCL-20, a chemokine enhancing mucosal immune function, and the general leukocyte growth factor GM-CSF were all up-regulated in airways by urban $PM_{2.5}$ treatment and not decreased by metal chelation or endotoxin binding. Up-regulation of pro-inflammatory genes correlates with $PM_{2.5}$-induced airway diseases such as asthma[1].

Up-regulation of xenobiotic metabolism, ROS response, and inflammatory genes was not evident in response to rural source $PM_{2.5}$. Histologic evaluation of lungs from rural $PM_{2.5}$- treated animals showed slightly less but still significant inflammation relative to urban $PM_{2.5}$ treated animals. Estimates of particle density based on visual density of carbon particles in histologic

sections suggested an equivalent particle burden in lungs of rural and urban $PM_{2.5}$ treated animals. This suggests that the gene responses in lung compartments are driven by particle composition rather than simple inflammatory responses.

In contrast to expectations, DFM treatment had little effect on any of the parameters evaluated. There was no difference in subjective histology scores between $PM_{2.5}$ and DFM pretreated $PM_{2.5}$ animals, and only a moderate influence on gene transcription. In the limited number of serum cytokines with significant differences between groups, the majority had higher cytokine levels in animals treated with DFM alone than in either $PM_{2.5}$ treated group. Similarly, mice given DFM pretreated $PM_{2.5}$ had evidences of platelet activation not seen in mice treated with $PM_{2.5}$ alone. This suggested an unexplained synergistic effect between DFM and PM. Unfortunately, a PBS alone group was not included in DFM experiments but comparison with PBS alone groups in later polymyxin B experiments, suggests DFM alone was without significant effect. Polymyxin B pretreatment of $PM_{2.5}$ did, however, significantly blunt pulmonary inflammation and gene responses. While equivalent particle density was present in polymyxin B/$PM_{2.5}$ treated animals, the histologic inflammatory response was markedly reduced in response to both urban and rural $PM_{2.5}$ instillations. Gene responses in urban source treated animals suggest the polymyxin B response is not limited to inflammation alone but also influences the up-regulation of xenobiotic metabolism and oxidative stress genes. Overall, results of these experiments implicate endotoxin as a key element in both urban and rural source $PM_{2.5}$ toxicity. Combined with the lack of response to iron chelation by DFM, the decrease in multiple pulmonary gene responses and subsequent pulmonary inflammation suggest that endotoxin may be a more significant component of $PM_{2.5}$ than transition metals.

This project demonstrates the utility of oropharyngeal aspiration of collected ambient $PM_{2.5}$ in comparative biologic response studies in laboratory animals. Evaluation of selected regions of pulmonary parenchyma greatly enhances sensitivity in characterizing tissue specific gene responses. Doses can be normalized based on mass equivalents thus controlling for differences in environmental conditions that are inherent in CAPs studies. In contrast, there remain potential artifacts in particle collection and processing relative to particle size and extraction of soluble PM components. Given the nature of particle concentrator processes, similar critiques relative to size and composition can be applied to both exposure approaches. Intratracheal exposures are practically limited to single doses so the cumulative effects that can potentially occur in CAPs exposures of greater duration may not be seen. The extent of pulmonary inflammation induced by intratracheal instillation is significantly greater than that seen in CAPs studies where often little pulmonary inflammatory cell infiltrate is evident. Thus, the lack of systemic responses in these studies was unexpected. Our studies were limited to a 24-hour time point after-dosing. The lack of systemic platelet and cytokine responses may reflect a delay between induction of pulmonary inflammation and onset of systemic disease similar to that seen in epidemiologic studies of human populations. While the clear responses to polymyxin B inhibition provide significant evidence for an endotoxin effect in pulmonary inflammatory responses to $PM_{2.5}$, decreased platelet reactivity in polymyxin B treated controls suggests that the possibility of additive effects of endotoxin present in animal housing could be considered in future studies. Despite a record of safe use of DFM in humans, we found DFM treated animals had evidence of platelet activation even in controls. This was unexpected and future experiments with this drug should include untreated controls.

Conclusions

Experiments in this study examined the relative role of transition metals and ROS relative to bacterial derived endotoxin in inducing pulmonary and systemic inflammation in response to $PM_{2.5}$ derived from urban vs. rural sources. Our findings confirm that urban source $PM_{2.5}$ induces greater responses on a mass equivalent basis than $PM_{2.5}$ collected from rural areas. Laser capture microscopic isolation of target pulmonary histological structures demonstrated activation of pro-inflammatory, oxidant metabolism and xenobiotic metabolism consistent with

known biologic functions of airways, pulmonary parenchyma and vasculature. Pulmonary inflammation and gene responses were diminished by pretreatment to bind endotoxin in $PM_{2.5}$ but not by pretreatment to bind iron and diminish ROS responses. In contrast to prior studies demonstrating platelet activation in longer field exposures to concentrated ambient particles, no $PM_{2.5}$ induced platelet activation was detected in these single dose studies. We conclude endotoxin is a key factor in both urban and rural $PM_{2.5}$ toxicity.

Recommendations:

1) This study provides additional evidence for the importance of $PM_{2.5}$ composition for influencing biological responses. Experiments with equal gravimetric doses of PM from differing regions, i.e. an urban site as compared to a rural site led to markedly different inflammatory responses.
2) Endotoxin must be considered an important constituent in both rural and urban source PM.
3) The nature and time course of systemic responses to $PM_{2.5}$ inhalation deserves further investigation, particularly relative to the length of post-exposure period necessary to induce these responses.
4) The observation that a specific constituent of PM, namely endotoxin might contribute to short-term inflammatory pulmonary responses in a rodent model of exposure raises the possibility that specific constituents of PM might drive health effects in humans and contribute to the societal health burden of ambient PM. Such an situation if proven would have implications regarding policy and regulatory decision making

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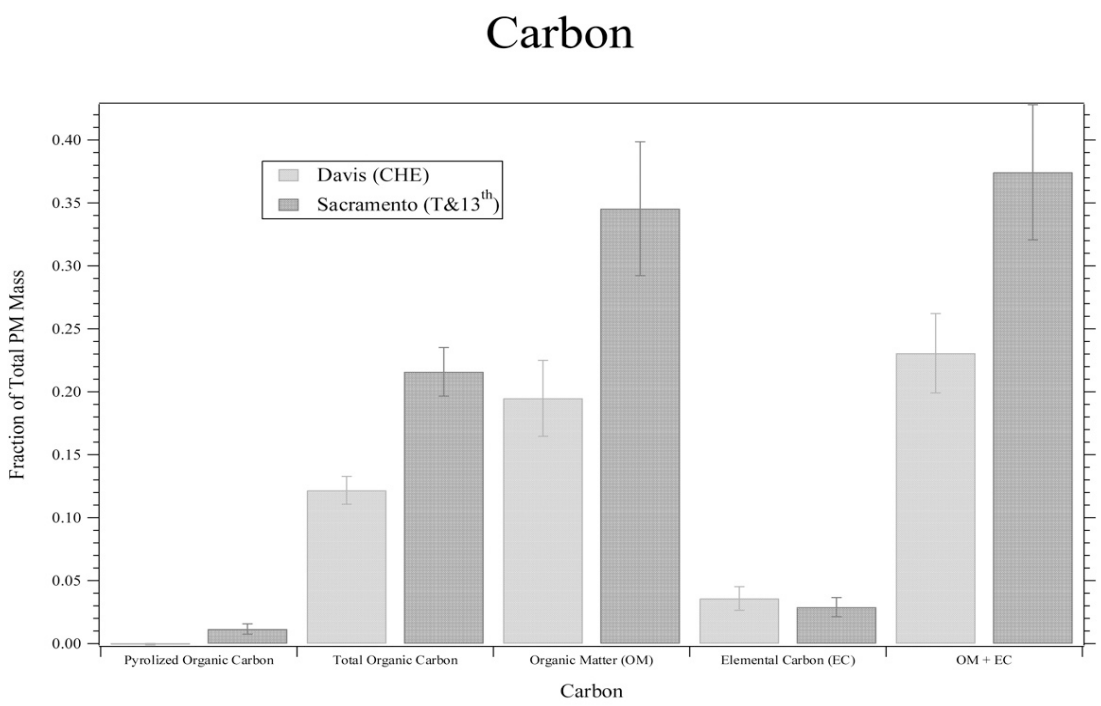
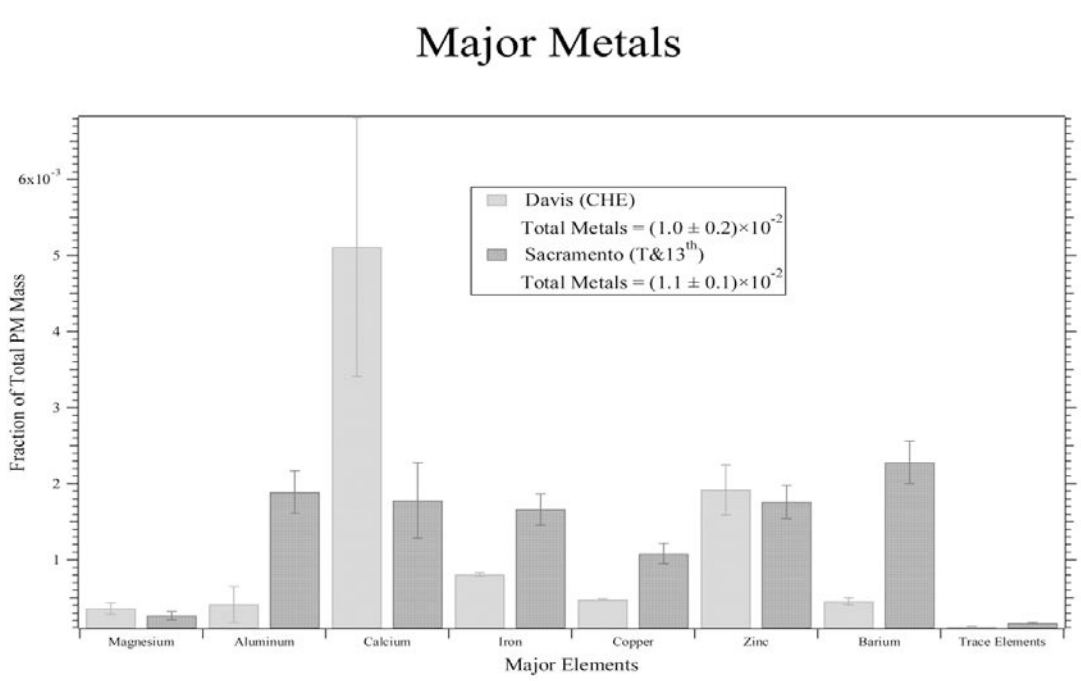
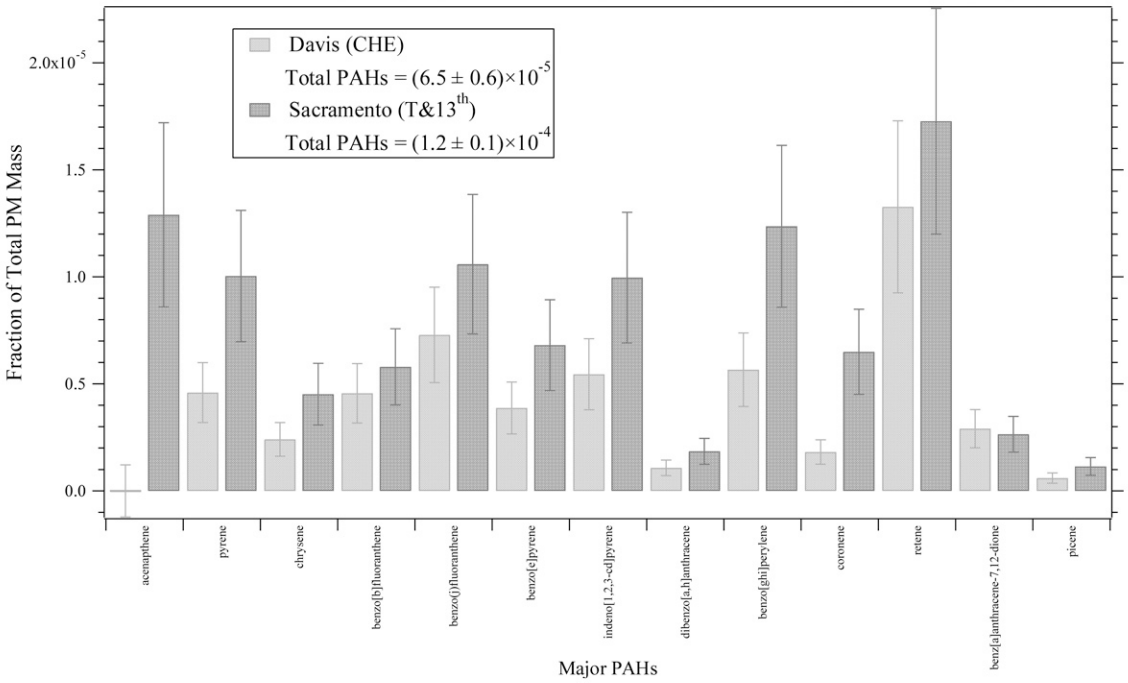
Appendix 1: Relative composition of urban (Sac) and rural (Davis) $PM_{2.5}$.





Major PAHs


Davis (CHE)
Total PAHs = (6.5 ± 0.6)×10^-5
Sacramento (T&13th)
Total PAHs = (1.2 ± 0.1)×10^-4
Fraction of Total PM Mass
2.0x10^-5
1.5
1.0
0.5
0.0
acenaphene
pyrene
chrysene
benzo[b]fluoranthene
benzo(j)fluoranthene
benzo[e]pyrene
indeno[1,2,3-cd]pyrene
dibenzo[a,h]anthracene
benzo[ghi]perylene
coronene
retene
benz[a]anthracene-7,12-dione
picene
Major PAHs

Appendix 2: Table 3: Abbreviations used in this report

PM2.5	Particulate Matter < 2.5 um
CAPs	Concentrated Ambient Particles
DFM	Deferoxamine mesylate
PAH	Polycyclic Aromatic Hydrocarbon
ROS	Reactive Oxygen Species
LCM	Laser Capture Microscopy
Gp41b	Glycoprotein 41b, an integrin marker on platelet membranes
LAMP-1	Lysosomal Associated Membrane Protein-1
rt PCR	Real time Polymerase Chain Reaction
ADP	Adenosine di Phosphate
ATF3	Activating Transcription Factor 3
MAP Kinase	Mitogen Activated Protein Kinase
HOX-1	Heme oxygenase - 1
CCL-20	Ligand for the CC-20 chemokine receptor
IL-1beta	Interleukin 1 beta
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
PGE2	Prostaglandin E 2
TXB2	Thromboxane B2
FITC	Fluorescein isothiocyanate
PE	Phytoerythrin
MFI	Mean Fluorescence Intensity
CD41b	Platelet integrin marker
CD 11b	Leukocyte surface antigen
CD115	Monocyte surface antigen
NOX-2	NADPH Oxidase subunit 2
ICAM-1	IntraCellular Adhesion Molecule-1
CYP 1A1	Cytochrome P 450 isoform 1A1
AldH2ase	Aldehyde dehydrogenase
JNK	Janus Kinase

Appendix 3: Table 4 Systemic activities and associated biologic effects assayed in Bioplex studies of serum

FGF	Fibroblast Growth Factor	Fibrosis Angiogenesis
G-CSF	Granulocyte Colony Stimulating Factor	Bone Marrow Stimulation
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor	Bone Marrow Stimulation
IFN-α	Interferon-gamma	Viral Responses, immune regulation
IL-1α	Interleukin-1α	Inflammatory Cytokine
IL-1β	Interleukin-1β	Inflammatory Cytokine
IL-2	Interleukin-2	Inflammatory Cytokine Allergy
IL-3	Interleukin-3	Inflammatory Cytokine
IL-4	Interleukin-4	Inflammatory Cytokine
IL-5	Interleukin-5	Inflammatory Cytokine
IL-6	Interleukin-6	Inflammatory Cytokine
IL-9	Interleukin-9	Inflammatory Cytokine
IL-10	Interleukin-10	Inflammatory Cytokine Allergy
IL-12p40	Interleukin-12 protein 40	Inflammatory Cytokine Bacterial response
IL-12p70	Interleukin-12 protein 70	Inflammatory Cytokine Bacterial response
IL-13	Interleukin-13	Inflammatory Cytokine Allergy
IL-15	Interleukin-15	Inflammatory Cytokine Allergy
IL-17	Interleukin-17	Inflammatory Cytokine Allergy
IL-18	Interleukin-18	Inflammatory Cytokine Bacterial response
KC	Keratinocyte chemotactic factor	Inflammatory Cytokine
LIF	Leukemia Inhibitory Factor	Inflammatory Cytokine
MCP-1	Monocyte Chemotaxtic Protein-1	Inflammatory Cytokine
M-CSF	Monocyte Colony Stimulating Factor	Bone Marrow Stimulation
MIG	Monokine Induced by Gamma Interferon	Inflammatory Cytokine
MIP-1α	Macrophage Inflammatory Protein-1α	Inflammatory Cytokine
VEGF	Vascular endothelial growth factor	Fibrosis Angiogenesis
MIP-1β	Macrophage Inflammatory Protein-1β	Inflammatory Cytokine
MIP-2	Macrophage Inflammatory Protein-2	Inflammatory Cytokine
PDGF	Platelet Derived Growth Factor	Fibrosis Angiogenesis
RANTES	Regulated Upon Activation, Normally T-Expressed, and presumably Secreted	Inflammatory Cytokine
TNFα	Tumor Necrosis Factor alpha	Inflammatory Cytokine