Persistent Immune Effects of Wildfire PM Exposure During Childhood Development

Final Report

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Abstract

The objective of this study was to determine the impact of early life episodic ozone and particulate matter (PM) exposure on parameters of immunity that affect responses to infectious disease and lung function. We investigated a cohort of 50 California National Primate Research Center (CNPRC) outdoor colony rhesus monkeys that were born within three months prior to the Trinity and Humboldt County wildfires of June/July 2008. We hypothesized that combined ozone and wildfire $PM_{2.5}$ exposure during early life would result in detrimental effects on immunity and lung physiology. To test this hypothesis, we conducted a nonterminal and minimally invasive study in three-year old adolescent monkeys exposed to combined ozone and wildfire $PM_{2.5}$ as infants by (1) evaluation of the peripheral blood response to microbial ligands and (2) measurement of lung mechanics. Compared with a cohort of 50 age-matched control monkeys, peripheral blood cells from exposed animals showed reduced cytokine synthesis when cultured with microbial ligands. Increased airways hyperresponsiveness and reduced lung compliance correlated with reduced peripheral blood cell cytokine synthesis in exposed female animals (less response to microbial challenge). We conclude that early life exposure to combined ozone and wildfire $PM_{2.5}$ can result in immune and lung function decrements that persist with maturity. In addition, CNPRC rhesus monkeys could serve as biologic sentinels for chronic human health effects of ambient air pollution.

Executive Summary

Background

Although epidemiologic studies provide important correlative data in human subjects, the biologic mechanism for how early life air pollution exposures contribute towards respiratory health decrements in adults is not well understood. Direct scientific evaluation of children is restricted due to limitations in experimental assessment and methodology. As with adults, it is clear that inflammation is a consistent effect of air pollutant exposure in children. Yet, the establishment of a persistent effect on lung function distinguishes early life air pollutant exposures in children, with evidence to suggest that health outcomes are retained with maturity and precede the development of chronic obstructive pulmonary disease in adults. In addition to identification of the antecedent events leading to adult pulmonary disease, measures of air pollution exposure are limited in the very young. Immunological markers of environmental challenge have been evaluated in children, but there are few biomarkers for air pollution exposure that are consistent amongst multiple cohorts. There are currently no data available on the long-term impact of air pollutant exposures on immune system function in infants and school-age children. Further, we do not have biomarkers (immune or otherwise) that are predictive of lung function decrements.

Methods

To address knowledge gaps in our understanding of the biologic effects of air pollutants during early life, we conducted a nonterminal and minimally invasive study with a cohort of 100 outdoor colony rhesus macaque monkeys at the California National Primate Research Center (CNPRC). We assessed long-term biologic effects of ambient air pollutant exposure on immunity using peripheral blood samples collected from 50 three-year old adolescent rhesus monkeys that were born and housed in an outdoor environment within three months prior to the summer wildfires of July 2008. In parallel, we conducted pulmonary function testing on the same cohort of animals to determine if airway mechanics were compromised with exposure. Control animals consisted of 50 age-matched monkeys that were also born and housed in the CNPRC outdoor environment, but were not exposed to summer wildfires.

Results

Our data show that peripheral blood cells from rhesus monkeys born in 2008 produced significantly less cytokine in response to stimulation with microbial ligands in culture, as compared with control animals born in 2009. We did not observe a significant effect of exposure on lung function overall, but did find significant correlation of increased airways hyperresponsiveness and reduced lung compliance in association with reduced cytokine synthesis in peripheral blood cells from animals born in 2008. An unexpected finding was that the degree of cytokine attenuation observed in peripheral blood assays was gender dependent, with female monkeys exhibiting the most significant correlation between reduced cytokine synthesis and reduced lung function. Our data show that early life exposure to episodic ozone and wildfire $PM_{2.5}$ can result in reduced immunity and lung function decrements that persist with maturity. These results also suggest that

rhesus monkeys may serve as biological sentinels for human health effects of ambient air pollution.

Conclusions

The observation of significant compromise of immune function in association with lung function decrements in rhesus monkeys exposed to wildfire $PM_{2.5}$ as infants suggests that young human subjects who were also exposed to wildfire $PM_{2.5}$ in 2008 could exhibit a similar health profile. Our findings in three-year old adolescent animals provide compelling evidence that wildfire $PM_{2.5}$ during infancy elicits effects in the immune compartment that persist with maturity; future studies that focus on identification of susceptible immune cell phenotypes and epigenetic changes in specific genes will provide further detailed information on the biological mechanisms of wildfire $PM_{2.5}$ effects. Ultimately, these findings have the potential to lead to establishment of definitive biomarkers that can measure degree of wildfire $PM_{2.5}$ exposure and be predictive of immune dysfunction and lung function decline.

Introduction

Of the six criteria pollutants for which National Ambient Air Quality Standards are set by the United States Environmental Protection Agency, ozone and particulate matter (PM) pose the most significant threat to human health based on an extensive body of peer-reviewed literature reviewed by US EPA in the Integrated Science Assessments for PM and Ozone (USEPA, 2006, 2009). There is a pathologic association between ambient air pollutants and childhood respiratory health, including lung function deficits, respiratory allergies, increased hospital admissions and prevalence of asthma (1-4). Multiple physiologic parameters are believed to enhance susceptibility towards adverse health outcomes related to air pollutants in the very young, including increased metabolic rate and larger lung surface area per unit of body weight than adults (5). Airways injury and reparative processes are also likely to differ in children given that the human lung continues to grow until the age of 18-21 (6). It has been estimated that human infants are born with 50% of the alveoli number found in adults; lung development continues through adolescence, primarily due to the addition of alveoli (6-9). Despite epidemiologic evidence to support an association between ambient air pollutant exposures during childhood and chronic lung disease in adults (reviewed in (10)), the biologic mechanisms for this phenomenon remain uncertain.

Scientific investigation of mechanisms is difficult to perform in children due to ethical considerations about intentional exposure to specific pollutants for this age group. In adult human subjects, inhaled ozone or concentrated ambient particles result in a rapid influx of leukocytes into the airways with local release of cytokines and other inflammatory mediators (11, 12). The airway inflammatory cell profile of ozone and ambient particles is predominantly neutrophilic in adults, however there is evidence that children develop an eosinophilic response to ambient levels of ozone (13-15). Much of what is currently known about the effects of ambient air pollution on the human immune system in early life has focused on maternal exposures and measures obtained from cord blood. Despite potential differences in exposure routes to the fetus, there are data to support modest but significant shifts in cord blood lymphocyte populations and IgE synthesis (16-18). Several peripheral blood immunological markers of environmental challenge have been evaluated in children, but they have not been consistent between studies (reviewed in (19)). There has been recent work demonstrating that ambient air pollutant exposures in a Fresno cohort of asthmatic children were associated with impaired T regulatory cell function via hypermethylation of the Foxp3 gene (20). The observation of epigenetic changes in genes associated with function of a T cell population provides strong molecular evidence to support a causal link between air pollutants and impairment of the immune system.

The rationale for this CARB study is based upon previous work in our laboratory, in which experimental exposure of infant monkeys to episodic ozone during postnatal development resulted in significant attenuation of both pulmonary and peripheral blood cell responses to an exogenous mimic of microbe infection (21). In our published study, infant rhesus monkeys starting at one month of age received 11 cycles of 0.5 ppm ozone for 8 hours per day for 5 days, followed by 9 days of filtered air; at 6 months of age when the exposure regimen was completed, the animals remained housed in

filtered air until evaluated at one year of age. At one year of age, animals received a single inhaled dose of lipopolysaccharide, which is a molecule that is a major component of the outer membrane of gram-negative bacteria. Upon inhalation of lipopolysaccharide, one-year old monkeys with prior postnatal exposure to episodic ozone showed attenuation of airways inflammation as compared with controls, indicating that early life ozone exposure resulted in a long-term persistent change in the ability of the immune system to respond to microbiota. Subsequent evaluation of peripheral blood samples from one-year old monkeys with prior postnatal exposure produced similar results; the cytokine response to culture with lipopolysaccharide was attenuated as compared with controls. Because monkeys were housed in a highly controlled filtered air environment for this experimental study, we concluded that prior postnatal exposure to ozone resulted in an impaired innate immune response that persisted for at least six months following the last exposure.

How ozone (or other air pollutants) interfaces with the innate immune system remains enigmatic. Rodent models show impaired pulmonary microbial clearance following acute exposure to ozone, a finding that is enhanced in younger animals (22-24). However, the inhibitory effects of ozone on microbial immunity in rodents are limited to acute exposures and not maintained for an extended duration. Identification of Toll-like receptor four (TLR4) as an essential susceptibility gene for the inflammatory and physiologic effects of ozone exposure in certain mouse strains has provided a potential mechanistic link between innate immunity and air pollutant effects (25, 26). TLR4 is a member of the toll-like receptor family of proteins that are expressed by cells of the innate immune system and bind to common structural motifs of pathogens (reviewed in (27)). Targeted deletion mutant mice for TLR4, Toll-like receptor two (TLR2) and the Toll-like receptor signaling adapter protein myeloid differentiation factor 88 (MyD88) exhibit an attenuated inflammatory response and no airways hyperreactivity following ozone exposure (28). There are limited data in the human population regarding environmental effects on Toll-like receptors, however a recent study by Gold, et al. provides strong evidence that birth seasonality has significant impact on cord blood responses to innate immune ligands; children born in summer months showed reduced cytokine synthesis following stimulation of cord blood with various Toll-like receptor ligands (29). Signaling via flagellin, the ligand for Toll-like receptor five (TLR5), has been recently associated with asthma in humans, but it is currently unknown whether additional Toll-like receptors mediate respiratory health effects of air pollutant exposures (30).

For this current CARB study, we determined whether our previous findings under well-defined experimental conditions of air pollution exposure could be reproduced under ambient conditions. To complete our objective, we evaluated a cohort of rhesus monkeys that are housed within outdoor colonies at the CNPRC and capitalized upon a naturally occurring environmental exposure in the form of a concentrated series of wildfires that took place in the summer of 2008. While wildfires are not a rare occurrence in Northern California, weather conditions and location of wildfires in June/July of 2008 resulted in hazy conditions with multiple days of high concentrations $PM_{2.5}$ exposures within 2 miles of the CNPRC. Because rhesus macaque monkeys are seasonal breeders, the majority of infants within the CNPRC colony are born in the spring, with a peak of birth numbers corresponding to the months April-May of each

year. As such, during the high $PM_{2.5}$ exposures of 2008, there were a large number (greater than 200) of infant animals in the CNPRC outdoor colony. Based upon this acute ambient air pollution event, we proposed to evaluate the innate immune system of exposed infant monkeys in conjunction with parameters of lung function to assess whether this period of acute exposure from ozone and wildfire $PM_{2.5}$ resulted in attenuation of responses. Importantly, we evaluated animals at three years of age, which corresponds to adolescence in humans; observation of significant exposure dependent effects would provide evidence of a long-term chronic effect on the immune and pulmonary systems. Age-matched controls consisted of three-year old monkeys that were born in 2009 and housed exclusively within the CNPRC outdoor colony throughout their lifespan.

The focus of this CARB study was to investigate the early life impact of ozone and wildfire $PM_{2.5}$ exposure on immune parameters that modulate responses to infectious disease and determine if exposure corresponded to lung function decline. We collected peripheral blood samples and conducted pulmonary function testing, and utilized methods to assess immunity and lung mechanics. This non-invasive approach allows longitudinal evaluation of this monkey cohort in the future, and also demonstrates applicability towards translational studies in human subjects. Peripheral blood samples obtained from rhesus monkeys that were born in an outdoor environment within three months prior to the summer wildfires of July 2008 were cultured with the microbial ligands lipopolysacchride and flagellin, followed by evaluation for ability to synthesize cytokines. In parallel, we conducted pulmonary function testing, including airways hyperreponsiveness measures, on the same cohort of animals to determine if lung mechanics were compromised with exposure. Lung mechanics measure how well lungs work with regards to how much air can be inhaled and how much air can be blown out. Airways compliance provides additional information on relative stiffness of the lung; low compliance suggests a fibrotic lung whereas high compliance suggests emphysema. Finally, increased bronchial responsiveness to non-specific stimuli is a hallmark of asthma; asthmatic subjects exhibit a lowered threshold for concentration of agonist required to elicit bronchoconstriction. Immune parameters in this study were also correlated with pulmonary function measures, with the ultimate intent that peripheral blood analysis (which may be more readily completed in a large number of subjects) may be predictive of lung function decrements in the human population. In conjunction with metrics of immune and lung function, we also determined whether ozone and wildfire $PM_{2.5}$ exposures were associated with changes in behavioral phenotype of monkeys, by taking advantage of a pre-existing NIH research resource program at the CNPRC led by John Capitanio. Whether air pollution has a direct role in the development of behavioral disorders is unclear, however there are some recent epidemiologic evidence to suggest a linkage between maternal/childhood exposures and increased rates of autism (31-33).

Materials and Methods

Air Quality Data

During the summer of 2008, the Sacramento valley experienced multiple days of elevated concentrations of particulate matter, due to persistent ambient wildfire smoke from Northern California fires. As shown in Figure 1, a comparison of daily 8-hour ozone concentration detected by a California Air Resources Board sampling station in Yolo County located within 2 miles of the CNPRC shows similar patterns between June/July 2008 versus June/July 2009, with a single day in June 2008 and a single day in July 2008 that exceeded the current NAAQS standard of 0.075 ppm/8-hour period. In comparison, there were two episodes in the months of June and July 2008, consisting of 4-6 days each, where $PM_{2.5}$ levels exceeded the current NAAQS standard of 35 ug/m^3 per 24-hour period (Figure 2). $PM_{2.5}$ levels correlated with a dry low pressure system on June 20-22, 2008 that produced dry lightning igniting approximately 2000 forest fires across Humboldt County in Northern California, which is located approximately 270 miles from the University of California, Davis campus. In Yolo County, where the CNPRC is located, air quality improved June 26-July 5 2008 due to onshore winds and Delta breeze, but declined July 7-10 2008 when winds calmed. In addition to the data presented within the time from of Figures 1 and 2, the 8 hour daily average for ozone concentration was 0.099 ppm in 2008 and 0.082 ppm in 2009. Although the 8 hour daily average ozone declined between 2008 and 2009, it should be recognized that these values still exceed the state standard of 0.070 ppm. While these data represent the most accurate measures of air quality within the immediate vicinity of the CNPRC, it should be acknowledged that other sources of pollutants may have been present during the period of time in which infant monkeys were housed in the outdoor colony.

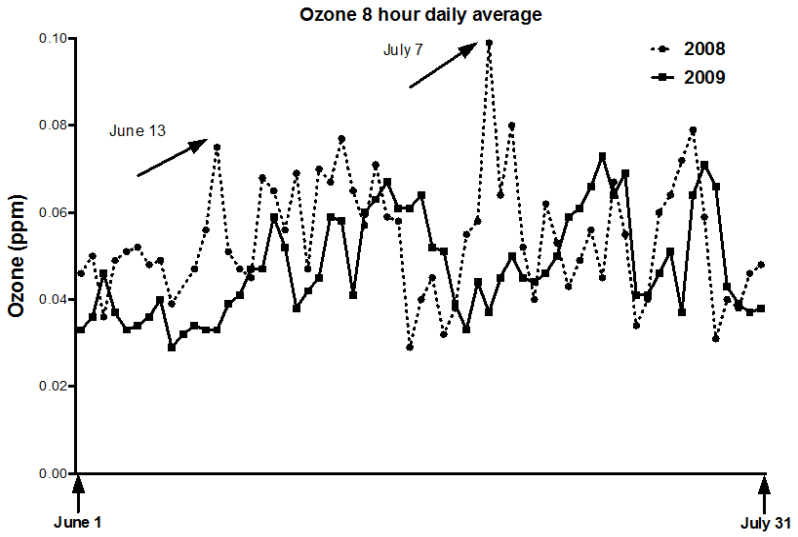

Figure 1. Daily 8 hour average concentration of ozone from June 1-July 31 on UC Davis campus. Daily average readings for ozone were obtained from a California Air Resources Board sampling site located on the UC Davis campus, within two miles of the California National Primate Center. Daily readings for 2008 (dotted line) and 2009 (solid line) are compared over time, arrows point to two different time points that exceed the current NAAQS standard of 0.075 ppm/8 hours. Note that there are 1-3 days in June with missed readings.

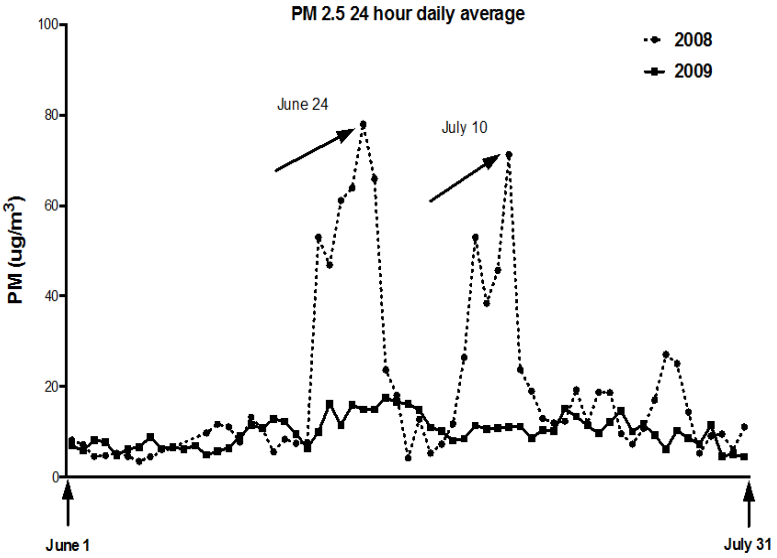

Figure 2. Daily 24-hour average concentration of PM-$_{2.5}$ from June 1-July 31 on UC Davis campus. Daily average readings for $PM_{2.5}$ were obtained from a California Air Resources Board sampling site located on the UC Davis campus, within two miles of the CNPRC. Daily readings for 2008 (dotted line) and 2009 (solid line) are compared over time, arrows point to the peak of two different time periods that exceed the current NAAQS standard 35 ug/m^3 per 24 hour period.

Animals

Male (n=25) and female (n=25) rhesus macaques were born in outdoor field cages at the CNPRC between April 1st 2008 and May 31st 2008. Male (n=25) and female (n=25) rhesus macaques born in outdoor field cages between April 1st 2009 and May 31st 2009 served as the control group. The yearly population density of infants and the ratio of male and female animals at the CNPRC have not fluctuated by more than 10% since 2005. Animals that were selected and enrolled in this study were exclusively housed outdoors prior to the assessment period. Selection of animals was based upon gender and birth date relative to the peak of $PM_{2.5}$ in June/July (approximately 3 months of age). Animals enrolled in this study were selected from more than 15 different outdoor field cages, which individually house multiple genetically diverse family units. Although animals in each family unit are genetically related, none of the enrolled animals were siblings due to the rarity of twin births as well as the polygamous nature of breeding pairs in each field cage. All CNPRC animals in outdoor field cages are fed a commercial diet (Lab Diet #5038, Purina Mills International, St. Louis, MO) supplemented with fruits and vegetables. Dams nurse infant monkeys until approximately 3 months of age. Developmental stages in the rhesus monkey are as follows: newborn, 24 hours postnatal; neonate, 0–1 months; infant, 1–12 months; juvenile, 12–24 months; adolescent, 2–4 years; and young adult, 4–8 years.

Blood collection and pulmonary function testing took place when animals were three years of age. The rationale for selecting the adolescent age group was based upon our interest in evaluating the effect of exposure in animals that were sufficiently mature with regards to the immune system. Peripheral blood samples were collected from animals while sedated for pulmonary function testing (see following section for details of pulmonary function testing). Peripheral blood was obtained from animals by CNPRC animal health technicians using standard venipuncture methods. All animals were returned to outdoor field cages following assessment of lung function and recovery from anesthesia. Evaluations took place in subsequent years in order to collect measures in age-matched animals. Care and housing of animals before, during and after evaluations complied with the provisions of the Institute of Laboratory Animal Resources and conforms to practices established by the American Association for Accreditation of Laboratory Animal Care (AAALAC). The University of California Institutional Animal Care and Use Committee approved all animal procedures.

In Vitro Stimulation of Peripheral Blood Mononuclear Cells With Microbial Ligands

To assess the responsiveness of innate immune function in animals evaluated in this study, we tested cultures of peripheral blood cells by *in vitro* stimulation with microbial ligands. Peripheral blood mononuclear cells were used in the study as this population consisting of lymphocytes and monocytes is known to express Toll-like receptors that recognize a broad range of microbial ligands. Peripheral blood mononuclear cells were prepared from blood samples by Histopaque 1077 gradient centrifugation and cryopreserved prior to culture (34). For consistency, experiments with thawed peripheral blood mononuclear cells were conducted after all blood samples were collected from a single year. Thawed peripheral blood mononuclear cells were cultured in AIM-V medium (Invitrogen, Carlsbad, CA) in 96 well tissue culture plates at a concentration of 2×10^5 cells/100 ml. Lipopolysaccharide (LPS)(E. Coli 026:B6, Sigma-

Aldrich, St. Louis, MO) and flagellin (flagellin from *B. subtilis,* Invivogen, San Diego, CA) were diluted in AIM-V media and added to cultures at the start of incubation. Cultures were maintained for 6 hours at 37°C in 5% CO_2. At the end of 6 hours, media supernatants were collected by centrifugation of cultures to remove cells. ELISA assays were used to assess media supernatants for IL-6 and IL-8 protein concentration. Although LPS and flagellin may stimulate the synthesis of multiple cytokines, IL-6 and IL-8 were selected for evaluation for this study as they are considered to be prototypic proinflammatory cytokines. RNA from peripheral blood mononuclear cells were obtained by extraction with TRIzol reagent (Invitrogen, Carlsbad, CA) and evaluated by using a PCR array.

IL-6 and IL-8 ELISA

IL-6 and IL-8 protein concentration in peripheral blood mononuclear cell culture supernatant were measured by ELISA Ready-SET-Go! Kits (eBioscience, San Diego, CA). The limit of detection for ELISA assays was 2 pg/mL (IL-6), and 4 pg/mL (IL-8).

Pulmonary Function Testing

Pulmonary mechanics were measured in sedated and anesthetized monkeys intubated and placed in a whole body plethysmograph with the cuffed endotracheal tube attached to a pneumatic four-way valve/pneumotachograph assembly. Monkeys were sedated using Telazol (8 mg/kg, IM) and anesthetized using Diprivan (0.1-0.2 mg/kg/min, IV) with the dose adjusted as deemed necessary by the attending veterinarian. Measurements include quasi-static respiratory system compliance, total lung capacity, vital capacity, inspiratory capacity, functional residual capacity, residual volume, forced expiratory flow at 25% vital capacity, and forced expiratory flow at 50% vital capacity. Monkeys spontaneously breathe through the valve/pneumotachograph to measure frequency and tidal volume. Monkey lungs are then inflated to 30 cm H_2O and allowed to deflate to functional residual capacity to measure total lung capacity. Lungs are subsequently deflated to -10 cm H_2O to determine residual volume. Lungs are reinflated to 30 cm H_2O, and then rapidly deflated to -10 cm H_2O to determine residual volume. Monkeys are subsequently allowed to spontaneously breathe against a closed valve. Measurement of both mouth pressure and pressure inside the plethysmograph makes it possible to calculate quasi-static lung compliance.

For measurement of airways hyperresponsiveness, animals were challenged with doubling concentrations of histamine, with the initial concentration being 0.0625 mg/ml. The final concentration of histamine aerosol delivered was 32.0 mg/ml or the dose that causes arterial O_2 saturation to fall below 75%. All challenges were administered as aerosols at a set tidal volume and breathing frequency (15.0 ml/kg and 20 breaths per minute) using a compressed air neubulizer in series with a positive pressure ventilator. Histamine challenges were done using repeated 30 second challenge periods separated by 240 second data collection periods. The final concentration of histamine to be delivered is one that will produce a 200% increase in airway resistance (Raw) with the data being expressed as the concentration that doubles Raw (EC200Raw). The CDA200Raw and EX200Raw were determined by linear interpolation on the log-log plot of the dose response curve with the response being expressed as the percent of the baseline Raw. Pulmonary mechanics were

measured using a transfer impedance method. Briefly, the monkey breathes spontaneously through the pneumotachograph while the thorax of the monkey is vibrated using a pseudo-random noise waveform encompassing frequencies of 2 to 128 Hz by two speakers mounted in the walls of the head-out plethysmograph. The small changes in flow produce at the mouth are measured along with the changes in pressure inside the plethysmograph using a Microswitch transducer. This technique allows the monkey to breathe spontaneously while making pulmonary mechanics measurements at 4 second intervals. Transfer impedance is calculated as the ratio of the Fourier transform of pressure inside the box versus the Fourier transform of airway flow at each frequency. Finally, tidal volume and breathing frequency were recorded on a breath-by-breath basis by integrating the output of the pneumotachograph using a digital data acquisition system. Arterial oxygen saturation values were recorded at the beginning and ending of each data collection system.

Behavioral Testing

A subset of animals born in 2008 underwent evaluation through the Bio-behavioral Assessment Program, which is a colony-wide program that monitors infant monkeys for innate temperament phenotypes. Because the Bio-behavioral Assessment Program evaluates a limited population of CNPRC animals, not all of the animals evaluated in in this study (2008 and 2009 animals). The program is directed by Dr. John Capitanio and is supported by an NIH R24 resource granting mechanism. The program has been described in detail in (35). In brief, a cohort of 3-4-month old infants within the CNPRC colony participate each year in a Bio-behavioral Assessment program designed to characterize behavioral and physiological responsiveness in young animals. More than 2000 infants have been tested since 2001 and an electronic database has been prepared with data from the Bio-behavioral Assessment program. Infants between 90 and 120 days of age were separated from their dams and relocated from their home cages to individual indoor cages in the morning on the day of testing. Five to 8 infants, comprising a single cohort, were tested at the same time. The individual indoor cages contained a cloth diaper, a stuffed terry cloth duck and a novel manipulatable object containing activity sensors. Infants were provided with water, a fruit-flavored drink, commercial monkey diet and fresh fruit throughout the Biobehavioral Assessment. During the 24 hour assessment period, behavior was evaluated under four conditions: undisturbed in the living cage at the beginning and end of the 24 hour period, response to novel objects (separate test cage), response to monkey videos of aggressive and nonsocial behaviors (separate test cage), response to human intruder (separate test cage). A trained staff member observed cages, while videotaping was used for the response to human intruder (the technician conducting the test dressed in protective clothing) and monkey video segments. In addition, adrenocortical response was monitored by serum cortisol. At the end of the 24-hour observation period the observer rated affect quality from a list of 26 adjectives.

PCR Array

To investigate whether genes associated with the TLR4 and TLR5 pathways might be affected by $PM_{2.5}$ exposure, we conducted PCR array analysis on peripheral blood mononuclear cells from a subset of animals in this study cohort. Reverse transcription

was performed on total RNA collected from peripheral blood mononuclear cell cultures using random hexamers and MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA). To obtain a broad survey of genes associated with TLR4 and TLR5 pathways, we used the RT2-Profiler PCR Array Rhesus Macaque Toll-Like Receptor Signaling Pathway (SA Bioscience, Valencia, CA) as recommended by the manufacturer and analyzed with the SA Bioscience PCR Array Data Analysis Web Portal. The RT2-Profilter PCR Array consists of 84 different genes associated with TLR signaling pathways.

Statistical Analysis

All data are reported as mean +/- SD or mean +/- SEM as appropriate for each measured parameter. Treatment and exposure differences were evaluated using ANOVA (one way or two way) with GraphPad Prism software (GraphPad 5.0, La Jolla, CA). A p <value of 0.05 or less was considered statistically significant.

Results

Summary of Animals Evaluated

As shown in Tables 1-2, animals surveyed within this study were identified based upon age relative to the peak $PM_{2.5}$ exposure period in 2008. Age-matched animals born in 2009 were selected in an analogous fashion (Tables 3-4). Given the constraints of evaluating large numbers of animals for pulmonary function testing, assessment of female monkeys took place between July-August, whereas male monkeys were assessed between August-October for both 2008 and 2009 animal groups. Because rhesus macaques are seasonal breeders and breeding takes place during the fall season, females were collectively assessed for pulmonary function first to avoid pregnancy as a potential confounder in the analysis. All blood samples from each animal were collected at the time of pulmonary function testing. There were no significant differences in age of animals between 2008 and 2009 relative to June 24 (selected as peak of $PM_{2.5}$ exposure in 2008). There were no significant differences in weight between 2008 and 2009 females at the time of assessment (2008: 4.868 kg $\pm$ 0.5847 SD; 2009: 4.589 kg $\pm$ 0.4669 SD). Males in 2008 weighed significantly more than males in 2009 ($p<0.05$) at the time of pulmonary function testing (2008: 6.155 kg $\pm$ 0.9823 SD; 2009:5.635 kg $\pm$ 0.6294 SD), however this may be attributed to modest differences in timing of assessment; 2008 males were evaluated in September/October of 2011 versus August of 2012 for 2009 males. Because infant monkeys are not consistently weighed at the same chronological age, exact age comparable data for animals during the first year of life is not available.

Date of Birth	Gender	Weight (kg)	Age at June 24 (days)
04/08/08	F	4.88	77
04/09/08	F	5.03	76
04/09/08	F	4.47	76
04/10/08	F	4.35	75
04/11/08	F	5.23	74
04/12/08	F	4.54	73
04/13/08	F	4.50	72
04/14/08	F	3.87	71
04/14/08	F	4.16	71
04/14/08	F	6.02	71
04/15/08	F	5.46	70
04/18/08	F	5.26	67
04/22/08	F	5.82	63
04/24/08	F	5.24	61
04/26/08	F	5.82	59
04/27/08	F	4.08	56
04/28/08	F	5.64	55
05/03/08	F	4.68	50
05/05/08	F	5.09	48
05/06/08	F	4.2	47
05/07/08	F	5.00	46
05/07/08	F	4.64	46
05/08/08	F	4.55	45
05/10/08	F	4.66	47
05/15/08	F	4.50	42

Table 1. Age and Weight Distribution of CNPRC Female Monkeys Born in 2008.
The average weight for evaluated female monkeys born in 2008 was 4.868 kg $\pm$ 0.5847 SD.

Date of Birth	Gender	Weight (kg)	Age at June 24 (days)
04/08/09	F	4.47	77
04/08/09	F	5.02	77
04/08/09	F	4.28	77
04/08/09	F	4.25	77
04/09/09	F	5.20	76
04/10/09	F	4.05	75
04/10/09	F	3.93	75
04/12/09	F	5.45	73
04/12/09	F	4.83	73
04/15/09	F	4.50	70
04/16/09	F	4.57	69
04/17/09	F	4.70	68
04/18/09	F	5.70	67
04/19/09	F	4.80	66
04/20/09	F	5.05	65
04/23/09	F	4.48	62
05/02/09	F	4.52	53
05/04/09	F	4.40	51
05/05/09	F	4.02	50
05/07/09	F	3.92	48
05/09/09	F	4.70	46
05/11/09	F	3.89	44
05/17/09	F	4.88	38
05/18/09	F	4.55	37
05/18/09	F	4.57	37

Table 2. Age and Weight Distribution of CNPRC Female Monkeys Born in 2009.
The average weight for evaluated female monkeys born in 2009 was 4.589 kg $\pm$ 0.4669 SD.

Date of Birth	Gender	Weight (kg)	Age at June 24 (days)
04/02/08	M	7.12	83
04/12/08	M	6.75	73
04/12/08	M	5.00	73
04/17/08	M	6.75	68
04/17/08	M	6.27	68
04/18/08	M	5.67	67
04/18/08	M	7.00	67
04/18/08	M	5.51	67
04/20/08	M	5.31	65
04/21/08	M	4.18	64
04/23/08	M	7.75	62
04/24/08	M	5.64	61
04/24/08	M	4.90	61
04/24/08	M	5.97	61
04/25/08	M	6.53	61
04/29/08	M	7.00	57
05/02/08	M	5.67	54
05/07/08	M	4.61	49
05/07/08	M	6.98	49
05/11/08	M	6.37	45
05/15/08	M	6.70	41
05/17/08	M	8.1	39
05/20/08	M	5.8	36
05/20/08	M	6.97	36
05/20/08	M	5.32	36

Table 3. Age and Weight Distribution of CNPRC Male Monkeys Born in 2008.
The average weight for evaluated male monkeys born in 2008 was 6.155 kg $\pm$ 0.9823 SD.

Date of Birth	Gender	Weight (kg)	Age at June 24 (days)
04/07/09	M	6.76	78
04/07/09	M	4.86	78
04/08/09	M	6.62	77
04/08/09	M	5.26	77
04/08/09	M	6.13	77
04/09/09	M	4.93	76
04/09/09	M	6.64	76
04/13/09	M	4.73	72
04/13/09	M	6.45	72
04/15/09	M	6.08	70
04/17/09	M	5.83	68
04/19/09	M	5.81	66
04/21/09	M	5.04	64
05/02/09	M	5.32	53
05/03/09	M	4.72	52
05/05/09	M	5.82	50
05/07/09	M	5.65	49
05/12/09	M	5.32	44
05/12/09	M	5.81	44
05/13/09	M	5.43	43
05/13/09	M	5.35	43
05/14/09	M	5.97	42
05/15/09	M	4.60	41
05/16/09	M	6.15	40
05/19/09	M	5.60	37

Table 4. Age and Weight Distribution of CNPRC Male Monkeys Born in 2009.
The average weight for evaluated male monkeys born in 2009 was 5.635 kg $\pm$ 0.6294 SD.

Effect of Combined Ambient Ozone and Wildfire $PM_{2.5}$ Exposure on TLR4 and TLR5 Ligand Stimulation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were cultured with either the TLR4 ligand lipopolysaccharide or the TLR5 ligand flagellin as described in Materials and Methods; culture supernatants were evaluated for cytokine protein secretion by ELISA assay. As shown in Figure 3, peripheral blood mononuclear cells cultured with lipopolysaccharide resulted in a significant dose dependent induction of IL-6 (Fig. 3A) and IL-8 (Fig. 3B) protein secretion at 6 hours post TLR ligand addition *in vitro* ($p<0.01$). Comparison of lipopolysaccharide-induced cytokine values obtained from animals (combined males and females) born in 2008 versus 2009 showed a significant exposure dependent effect; peripheral blood mononuclear cells from animals born in 2008 produced less IL-6 and IL-8 protein following culture with lipopolysaccharide. Comparatively, peripheral blood mononuclear cell cultures with flagellin elicited a dose dependent effect on both IL-6 (Fig. 3C) and IL-8 (Fig. 3D). However, only IL-8 protein expression was significantly affected by exposure; peripheral blood mononuclear cells from animals born in 2008 produced less IL-8 protein following 6-hour culture with flagellin.

To determine whether gender impacted upon the peripheral blood mononuclear cell cytokine response to TLR4 and TLR5 ligands, we compared data obtained from male monkeys versus female monkeys for all exposure groups in this study. As shown in Figure 4, peripheral blood mononuclear cell cultures generated from male monkeys born in 2008 produced significantly less IL-6 protein following culture with lipopolysaccharide as compared with male monkeys born in 2009 (Fig. 4A), whereas the effects of flagellin stimulation on IL-8 synthesis were significantly reduced in peripheral blood mononuclear cultures generated from female monkeys born in 2008 as compared with female monkeys born in 2009 (Fig. 4D).

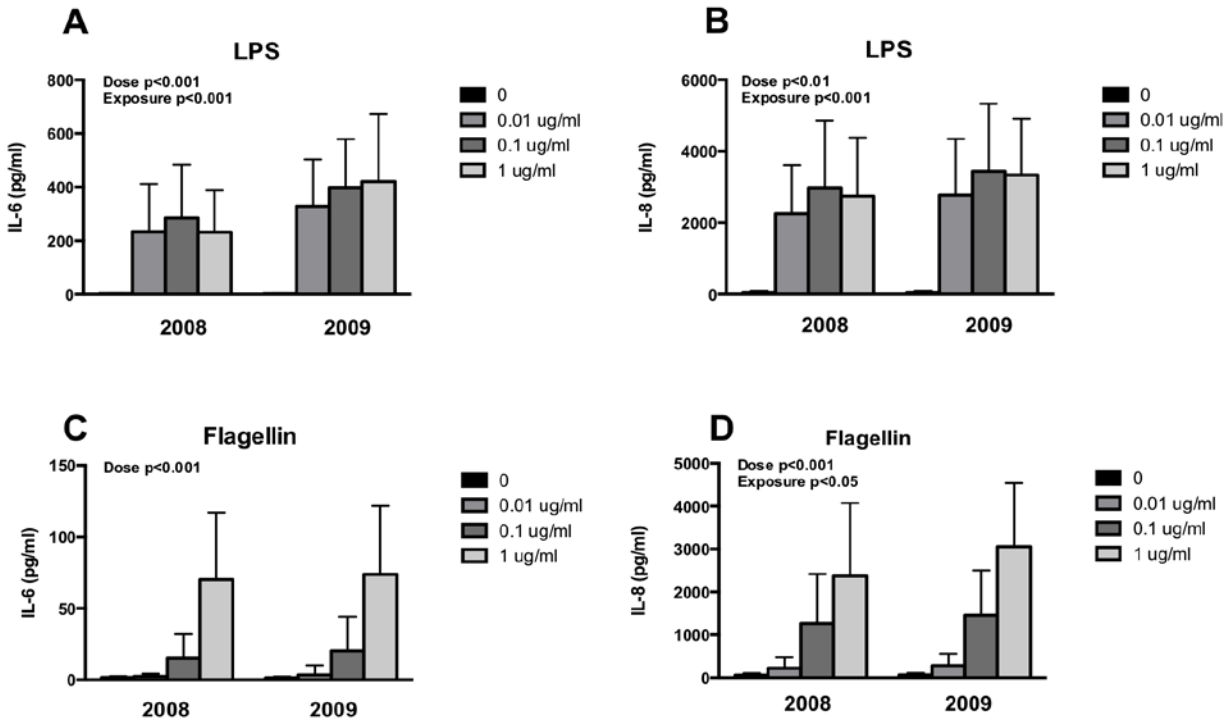

Figure 3. Effect of combined ozone and wildfire $PM_{2.5}$ exposure on Toll-like receptor ligand stimulation of peripheral blood mononuclear cells from three year old monkeys. Peripheral blood mononuclear cells from three year old animals previously exposed to wildfire smoke PM2.5 (2008) or age-matched controls (2009) were treated with either lipopolysaccharide (LPS) or flagellin *in vitro* and evaluated at 6 hours for cytokine protein secretion. Concentrations of IL-6 (A, C) and IL-8 (B, D) protein were measured in culture supernatant. Columns represent mean $\pm$ SE values obtained from both male and female blood samples for each animal group. Dose and exposure dependent effects were measured by two-way ANOVA.

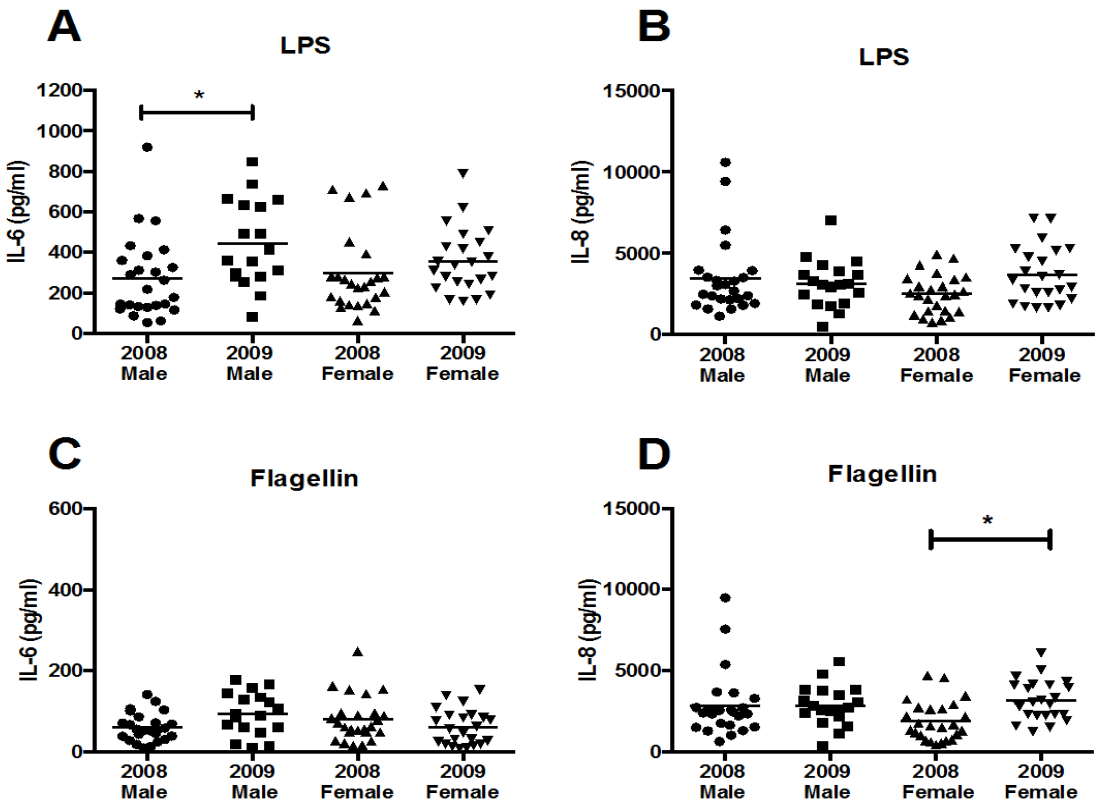

Figure 4. Gender dependent effect of combined ozone and wildfire PM 2.5 exposure on Toll-like receptor ligand stimulation of peripheral blood mononuclear cells from three year old monkeys. Peripheral blood mononuclear cells from three year old animals previously exposed to wildfire smoke PM 2.5 (2008) or age-matched controls (2009) were treated with either lipopolysaccharide 0.1 ug/ml) or flagellin (1 ug/ml) *in vitro* and evaluated at 6 hours for cytokine secretion. Concentrations of IL-6 (A, C) and IL-8 (B, D) protein were measured in culture supernatant. Each data point represents a single animal. *$p<0.05$ by one way ANOVA and Tukey's multiple comparisons.

Effect of Combined Ozone and Ambient Wildfire $PM_{2.5}$ Exposure on Pulmonary Function

All animals in this study underwent pulmonary function testing to measure multiple parameters of lung mechanics, including vital capacity, forced respiratory capacity and total lung capacity. There was no significant effect of exposure or gender on values obtained for vital capacity, forced respiratory capacity, and total lung capacity. There was also no evidence of increased airways hyperresponsiveness to a non-specific airways challenge in association with combined ozone and wildfire $PM_{2.5}$ exposure, however male monkeys born in 2008 showed a significant increase in lung compliance compared with male monkeys born in 2009, although this may be attributed to slight differences in animal age or weight as 2009 animals underwent pulmonary function testing in August 2012, whereas 2008 animals underwent pulmonary function testing in September/October 2011 (Figure 5). Despite the lack of an overall effect of exposure on parameters of lung function, we assessed whether cytokine synthesis by peripheral blood mononuclear cells correlated with lung function parameters. We found that the peripheral blood mononuclear cell culture cytokine response to flagellin significantly correlated with degree of airways hyperresponsiveness and lung compliance in female monkeys. As shown in Figure 6, lower IL-8 synthesis in response to flagellin corresponded to increased airways hyperresponsiveness (increased sensitivity to histamine dose) in female monkeys born in 2008 ($p<0.0455$). Reduced lung compliance in female monkeys from 2008 also correlated with reduced synthesis of IL-6 in response to flagellin stimulation of peripheral blood mononuclear cells ($p<0.0117$). In contrast, there was no significant association of lipopolysaccharide stimulation with any parameter of lung function.

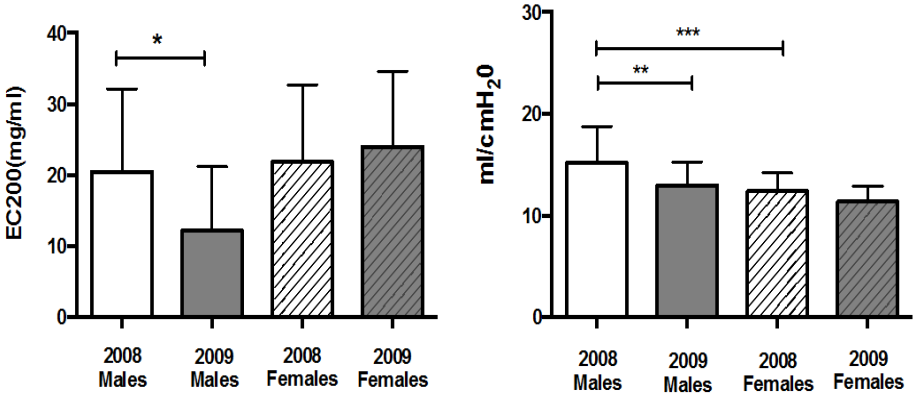

Figure 5. Gender dependent effect of combined ozone and wildfire $PM_{2.5}$ exposure on airways hyperresponsiveness and lung compliance in three year old monkeys. Airways hyperresponsiveness and lung compliance were measured in three year old animals previously exposed to wildfire $PM_{2.5}$ 2008) or age-matched controls (2009). *p<0.05, **p<0.01, ***p<0.001 by one way ANOVA and Tukey's multiple comparisons test.

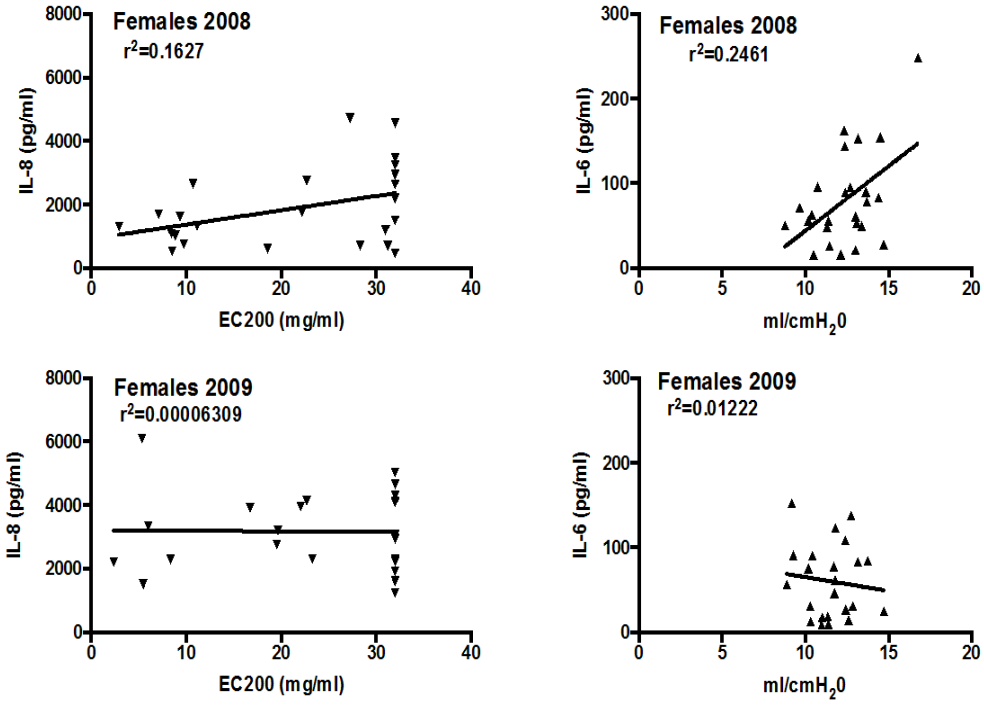

Figure 6. Correlation of flagellin induced cytokine synthesis with airways hyperresponsiveness and lung compliance in three year old female monkeys. Airways hyperresponsiveness and lung compliance were measured in three year old animals previously exposed to combined ozone and wildfire $PM_{2.5}$ (2008) or age-matched controls (2009). Values were correlated with maximal cytokine synthesis elicited in peripheral blood mononuclear cell cultures with flagellin. For 2008 female monkeys, IL-8 significantly correlated with EC200 values whereas IL-6 significantly correlated with lung compliance.

Correlation of Temperament with Peripheral Blood Mononuclear Cell Cytokine Synthesis

In addition to surveying animals for immune and lung function in this study, a subset of animals (n=14) born in 2008 were enrolled in the CNPRC Biobehavioral Assessment Program for assessment of temperament at infancy as well as at maturity. Animals that exhibit a high anxiety phenotype are characterized as having an inhibited temperament. Animals that exhibit behavioral inhibition are characterized by decreased social interaction and increased risk of anxiety and depression. As shown in Figure 7, peripheral blood mononuclear cell cultures generated from animals that exhibited significantly reduced IL-6 synthesis following culture with lipopolysaccharide corresponded to an inhibited behavior phenotype ($p<0.05$ by two way ANOVA). In comparison, IL-8 synthesis by peripheral blood mononuclear cells following culture with lipopolysacchride showed no significant association with behavior. IL-6 and IL-8 synthesis by peripheral blood mononuclear cell cultures with flagellin also did not correlate with behavioral phenotypes. Because animals born in 2009 were not enrolled in the CPRC Biobehavioral Assessment Program, we are not able to conduct a comparative analysis.

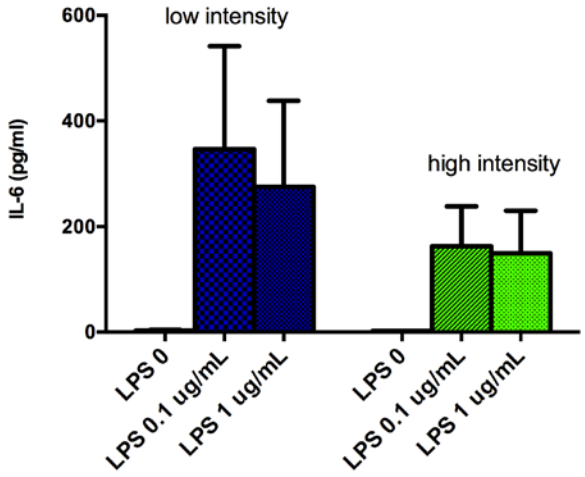

Figure 7. Peripheral blood mononuclear cell cultures from animals that exhibit high intensity behavior produce less IL-6 following LPS stimulation. Animals born in 2008 were evaluated as described in Materials and Methods for behavior.

Effect of Combined Ozone and Wildfire $PM_{2.5}$ on Toll-like Receptor Pathway Gene Expression

To begin to identify the specific alterations in TLR4 and TLR5 signaling pathways that are compromised by combined ozone and wildfire $PM_{2.5}$ exposure, RT-PCR arrays were utilized to assess expression of genes associated with Toll-like receptor signaling in peripheral blood mononuclear cell cultures. Upregulation/downregulation of RT-PCR array genes in peripheral blood mononuclear cells in females born in 2008 relative to females born in 2009 would suggest that exposures resulted in epigenetic alterations of genes associated with Toll-like receptor signaling pathways. Because female animals exhibited the most pronounced effect on pulmonary lung function testing, assessment by RT-PCR array was limited to peripheral blood mononuclear cell cultures generated

from a subset of females born in 2008 (n=6) and 2009 (n=6). Using a random number generator, females enrolled in the RT-PCR array experiments were randomly selected from Tables 1 and 2. As shown in Table 5, comparison of peripheral blood mononuclear cell cultures at baseline showed multiple genes that were upregulated in females born in 2008 compared with females born in 2009. Table 6 shows a comparison of peripheral blood mononuclear cells cultured with lipopolysaccharide for 3 hours; the transcriptional factor Fos was significantly downregulated in females born in 2008 relative to females born in 2009. Table 7 shows a comparison of peripheral blood mononuclear cells cultured with flagellin for 3 hours. RelB is a member of the NF-kappa B transcription factor family and is consistently upregulated in 2008 female monkey blood samples, regardless of culture condition. Additionally, mRNA for MyD88, an adaptor protein essential to multiple Toll-like receptor families, is also upregulated at baseline as well as with lipopolysaccharide culture. Mal, a T cell differentiation protein, is upregulated with flagellin culture; this may reflect the known expression of TLR5 on T cells.

Gene Symbol	Fold Regulation	P value
RELB	1.3993	0.000262
REL	1.3264	0.041019
NFKB1	1.283	0.050861
MYD88	1.2735	0.056359
RIPK2	1.2092	0.067343
TBK1	1.0743	0.070121

Table 5. Baseline differential expression of Toll-like receptor signaling pathway genes in peripheral blood mononuclear cells from 2008 female monkeys relative to 2009 female monkeys.

Gene Symbol	Fold Regulation	P value
MYD88	1.4988	0.006957
RELB	1.2373	0.00071
FOS	-1.6234	0.038431

Table 6. Effect of lipopolysaccharide on Toll-like receptor signaling pathway genes in peripheral blood mononuclear cells from 2008 female monkeys relative to 2009 female monkeys.

Gene Symbol	Fold Regulation	P value
MAL	2.7498	0.057449
CHUK	1.3229	0.043995
RELB	1.2741	0.00173

Table 7. Effect of flagellin on Toll-like receptor signaling pathway genes in peripheral blood mononuclear cells from 2008 female monkeys relative to 2009 female monkeys.

Discussion

In this CARB study, multiple parameters were used to assess both immune and lung function in a cohort of rhesus monkeys that were acutely exposed to both ozone and $PM_{2.5}$ as a result of Northern California wildfires in the summer of 2008. We first investigated peripheral blood mononuclear cell responses to microbial ligands *in vitro*, with the rationale that this approach would mimic an infectious agent. We also assessed animals for respiratory health by measuring lung mechanics and conducting airways challenge using a non-specific stimulus. Overall, we observed that peripheral blood samples collected from monkeys that were infants during the 2008 wildfire smoke exposure period showed a significant reduction in cytokine synthesis following *in vitro* culture with either lipopolysaccharide and flagellin, as compared with age-matched controls. Although lung function parameters did not appear to be significantly affected by exposure, we observed increased airways hyperresponsiveness and reduced lung compliance in association with reduced cytokine production in peripheral blood cultures, specifically in conjunction with flagellin stimulation. It is important to note that the exposure dependent effects observed in this study were measured in animals that were adolescent in age, indicating that the combined ozone and wildfire $PM_{2.5}$ exposures during infancy resulted in health effects that persisted with maturity.

Wildfire smoke contains both particulate matter and toxins. The health effects of particulate matter exposure have been well documented, yet wildfire smoke PM may be far more toxic than PM associated with combustible sources such as traffic in urban areas (36). In rodents, intratracheal instillation of PM collected during a peak period of wildfire smoke resulted in increased pulmonary neutrophilic inflammation and alveolar macrophage cytotoxicity relative to an equal mass dose of PM collected under normal ambient conditions (36, 37). While rodent studies have provided important biologic confirmation of wildfire PM toxicity, much remains to be determined regarding the specific chemical constituents of wildfire air pollution that appear to enhance pulmonary toxicity. In addition, because wildfire smoke may travel long distances, we do not yet know whether aged and presumably diluted wildfire PM elicits comparable biologic effects. To the best of our knowledge, this is the first reported evaluation of systemic innate immune responses following wildfire PM exposures. Based upon our studies in the CNPRC outdoor monkey colony, we have demonstrated significant immune and lung effects following exposure to wildfire PM that was carried over 200 miles from the combustion source. While we have measures of wildfire $PM_{2.5}$, a potential limitation of our findings is that we do not know what other air toxicants were present during the wildfire smoke exposure; it is plausible that other toxicants may be mediating the observed immune, respiratory and behavioral effects in animals born in 2008.

An unexpected finding from this CARB study was the observation of enhanced susceptibility to immune and lung physiology effects by gender. There have been numerous reports of adult and childhood respiratory conditions associated with air pollutants in a gender specific fashion, with inconsistent findings (reviewed in (38)). A recent multi-city survey of over 30,000 children from northeast China has reported that the association of air pollution effects with asthma was dependent upon predisposition to allergy; air pollution had a positive effect on asthma in males without allergy, whereas females with allergy were more likely to develop respiratory symptoms, suggesting an

immunological basis for gender dependent effects (39). Our data suggests that female monkeys were more susceptible to the effects of wildfire air pollutant exposure, particularly with regards to immunological changes associated with lung function. While we do not yet understand the basis for the gender outcome of this study, it could be speculated that reduced physical weight may enhance susceptibility to inhaled environmental challenges by increasing toxicant deposition relative to tissue mass; male rhesus monkeys weigh approximately 20% more than female monkeys of comparable age. A comprehensive study of alveolar growth in the rhesus monkey has shown no significant relationship between body weight and alveolar volume, although the trajectory of growth was accelerated in males, indicating that alveolar number does not correlate with increased susceptibility in female monkeys (40). While beyond the scope of this study, it might be speculated that the interaction of sex hormones on immune system development may be sensitive to the effects of air pollutants during infancy. Indeed, gonadal sex hormones regulate multiple aspects of B cell development and may regulate establishment of antibody responses to vaccines (reviewed in (41)).

How air pollutants impose direct and persistent effects on the immune system remains unclear. Multiple immune cell types in peripheral blood express the TLR4 and TLR5 receptors, but it is primarily the circulating monocyte that is responsible for the robust cytokine storm triggered by systemic endotoxin. We cannot at this time confirm which blood cell type is most sensitive to exposure in this study, but it should be emphasized that frequent bone marrow turnover and release of white blood cells would suggest that the cell(s) of interest in this study are derived from a stem cell population. In an experimental exposure, we have also previously shown that postnatal ozone results in an attenuated lipopolysaccharide response in peripheral blood mononuclear cells that persists with maturity, which is consistent with our findings from ambient exposures (21). Recent studies with diesel exhaust particles have shown that the cytokine NF-K β pathway in peripheral blood mononuclear cells following M. tuberculosis treatment is suppressed with exposure, further supporting the dampening effects of pollutants on immunity (42). To begin to investigate specific molecular targets in peripheral blood mononuclear cells, we used RT-PCR arrays to explore a number of gene targets in Toll-like receptor pathways that might be persistently modulated by early life exposure. We consistently observed increased expression of Rel and RelB in peripheral blood cells from female monkeys born in 2008, suggesting that transcriptional factor regulation might be a target for air pollutant exposures. Interestingly, RelB has been associated with establishment of endotoxin tolerance, which would be consistent with our findings of diminished cytokine response to microbial ligands in peripheral blood mononuclear cell cultures (43). Because we observed constitutive changes in gene expression within peripheral blood mononuclear cells from female monkeys born in 2008 following *in vitro* culture, as opposed to measurements *in vivo* that can be influenced by environmental exposures immediately preceding the sampling period, we speculate these effects were mediated by ozone and wildfire PM exposure in an epigenetic fashion.

The health implication of an attenuated innate immune response towards pathogens is profound. Innate immunity is elicited when an inflammatory response is generated towards any environmental challenge, whether it is a toxicant or a microbe. In the presence of a microbe, the innate arm of the immune system is also critical for

communication to the adaptive immune system, which generates a robust T and B lymphocyte response. Attenuation of innate immune responses is translated into delayed or attenuated lymphocyte mediated immune protection. While we have not yet observed a significant increase in microbial infections within animals born in 2008, it should be emphasized that CNPRC monkeys have limited exposure to animal and human pathogens by virtue of housing and use of personal protective equipment by animal care staff. Future studies might explore this avenue of investigation by measuring antibody titers towards hepatitis B vaccination, which is a standard practice for the CNPRC colony. In addition, the antibody response to cytomegalovirus (CMV), which is frequently observed in this colony, may also be evaluated.

An additional novel finding in this CARB study was the observation of behavioral phenotypes associated with attenuated cytokine production by peripheral blood mononuclear cells from animals born in 2008. While the number of animals evaluated for behavior within this study was limited, the data are intriguing on the basis of recent studies suggesting that urban air pollution exposure is significantly linked to increased rates of autism (31-33). Autism spectrum disorders by definition exhibit a range of behavioral characteristics. In light of the variable behavioral phenotype of autism, the peripheral blood response to microbial ligands in children diagnosed with autism spectrum disorders has been inconsistent. In one cohort of children diagnosed with autism spectrum disorder, microbial ligand stimulation of peripheral blood monocytes resulted in upregulation of cytokines as compared with controls (44). In contrast, a separate study of children diagnosed with autism spectrum disorder who also exhibit a deficiency of polysaccharide antibody reported attenuation of cytokine synthesis by peripheral blood mononuclear cells following microbial ligand stimulation (45).

Summary and Conclusions

In summary, the objective of this study was to determine the impact of early life episodic ozone and particulate matter (PM) exposure on parameters of immunity that affect responses to infectious disease and lung function. To complete this objective, we investigated a cohort of California National Primate Research Center outdoor colony rhesus monkeys that were born within three months prior to the Trinity and Humboldt County wildfires of June/July 2008. We hypothesized that combined ozone and wildfire $PM_{2.5}$ exposure during early life would result in detrimental effects on innate immunity and lung physiology that persisted with maturity. To test this hypothesis, we (1) evaluated the peripheral blood response to microbial ligands and (2) measured lung mechanics in three-year old adolescent animals born in 2008. Our data show that peripheral blood cells from animals born in 2008 produced significantly less cytokine in response to stimulation with microbial ligands, as compared with animals born in 2009. We did not observe a significant effect of exposure on lung function overall, but did find significant correlation of increased airways hyperresponsiveness and reduced lung compliance in association with reduced cytokine synthesis in animals born in 2008. An additional unexpected finding was that the degree of innate immune dysregulation was gender dependent and correlated with lung function in female monkeys. Based upon these data, we conclude from this study that early life exposure to episodic ozone and wildfire $PM\text{-}_{2.5}$ can result in dysfunction of innate immune responses towards infectious agents and lung function decrements at adolescence in a cohort of rhesus monkeys. Our data suggests that children who underwent similar exposures to episodic ozone and wildfire $PM\text{-}_{2.5}$ in 2008 as infants/toddlers may exhibit a similar health profile. Further, the ability to quantitatively assess exposure impact on immune parameters using a non-invasive peripheral blood assay also makes our approach developed for rhesus monkeys feasible for a large population-based human study.

Recommendations

The observation of significant immune effects in association with lung function decrements in rhesus macaque monkeys exposed to combined ozone and wildfire $PM_{2.5}$ as infants suggests that children who experienced similar exposures in 2008 may exhibit a similar health profile. Because data from this study were collected from isolated leukocyte cultures, our findings reflect a permanent alteration in the immune compartment of exposed animals and provide evidence that combined ozone and wildfire $PM_{2.5}$ exposure can elicit irreversible epigenetic changes that persist with developmental maturity. Despite the linkage of our findings with ozone and wildfire $PM_{2.5}$, a potential limitation of this study is that wildfire emissions contain many additional chemical constituents that could travel with particulate matter but were not measured. These data may lead to development of peripheral blood biomarkers that can readily measure degree of exposure and be predictive of immune system dysfunction. Future studies that focus on identification of susceptible immune cell populations and epigenetic mechanisms in specific genes will determine how combined ozone and wildfire $PM_{2.5}$ exposure results in functional deficiencies in both immunity and lung physiology. Because the animals in this study were evaluated as adolescents, tracking of health status across the life span would be important to assess whether exposure (and associated immune/lung function deficiencies) result in enhanced susceptibility towards chronic conditions such as chronic obstructive pulmonary disease, diabetes, atherosclerosis and cancer. Further, the heritability of ozone and $PM_{2.5}$ exposure effects could be addressed by evaluation of progeny from animals enrolled in this study. Ultimately, the outcome of this study would support greater public health efforts to educate parents and caregivers to modify behavior in the presence of wildfire smoke to limit exposure of young children.

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