PRENATAL EXPOSURE TO PARTICULATE AIR POLLUTION AND EFFECTS ON POSTNATAL IMMUNE RESPONSE

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2017

Major Subject: Toxicology

ABSTRACT

There is considerable evidence showing that exposure to particulate matter air pollution during important developmental windows, such as the prenatal period, can cause adverse respiratory outcomes. Mechanisms underlying increased risks from *in utero* exposure are largely unknown. Since epigenetic modifications have been recognized as an important mediator of developmental reprogramming following environmental exposures in early life, the primary objective of this research was to establish a representative model of prenatal air pollution exposure to probe underlying mechanisms leading to adverse respiratory responses in offspring. The preliminary study (aim 1) established the proof-of-principle for differential air pollution-induced epigenetic changes across varying genetic background. Two strains (BALB/c and C57Bl/6 mice) were exposed to diesel exhaust particulate matter (DEPM), a major constituent of outdoor air pollution, throughout pregnancy. Following sacrifice at postnatal day 2, offspring global DNA methylation and hydroxymethylation was quantified in lung tissue. Results indicate differential methylation in BALB/c mice but not C57Bl/6. In aim 2, BALB/c and C57Bl/6 dams were exposed to a representative particulate air pollution mixture throughout gestation using a refined exposure model, and offspring response to allergen challenge was evaluated. After 4 weeks of chronic exposure to house dust mite allergen, offspring from both strains exposed to PM *in utero* demonstrated a reduced inflammatory response compared to filtered air controls. Airway hyperresponsiveness, a typical feature of asthma, was significantly different based on strain; however, air

pollution exposure did not affect this response. In order to investigate the relevance of this model in an exposed human population, we conducted a pilot project (aim 3) evaluating exposure to particulate air pollution during pregnancy in a region in South Texas with a high incidence of childhood asthma. Results demonstrate low levels of air pollution exposure during pregnancy measured by personal sampling of fine particulate matter ($PM_{2.5}$) and polycyclic aromatic hydrocarbons (PAHs). Overall, findings from this work lay a foundation for further clarifying the mechanisms underlying childhood respiratory disease resulting from early life air pollution exposure.

DEDICATION

I would like to dedicate this work to my PaPa, Warren Huckabee. Although he will not get to see me graduate, he always had the utmost faith in me and loved me no matter what. I want to thank him and my Granny, Anna Durham Huckabee, for their support throughout my undergraduate and post-graduate education. This would not have been possible without them.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Natalie Johnson, and my committee members, Dr. Criscitiello, Dr. Harvey, and Dr. Porter, for their guidance and support throughout the course of this research. I would also like to thank Dr. Tim Phillips for his immense input in my doctoral training.

Thanks go to my friends, lab-mates, and colleagues and the Toxicology, VIBS, and PHEO departments' faculty and staff for making my time at Texas A&M University a great experience. I also want to extend my gratitude to our collaborators at Nanjing University, particularly Dr. Wei Shi and Ms. Jing Guo for their amazing hospitality and work ethic. I would like to thank our collaborators at the University of Tennessee Health Science Center, Dr. Cormier and her students.

Finally, I would like to thank my wonderful family, particularly my husband, Steven, and son, Jonathann, for their patience and steadfast love. Thank you to my parents and grandparents. Most importantly, I must give all thanks and credit to my Creator. Thank you, Lord, for carrying me when I needed carrying and for seeking me out when I have been lost.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of chair, Dr. Natalie Johnson, Department of Environmental and Occupational Health, co-chair, Dr. Weston Porter, Department of Veterinary Integrative Biosciences, and committee members, Drs. Michael Criscitiello, Department of Veterinary Pathobiology, and Roger Harvey, Department of Veterinary Integrative Biosciences.

Multiplex analysis (sections 2 and 3) was run in Dr. David Threadgill's laboratory at Texas A&M University with the assistance of Dr. Andrew Hillhouse. The airway hyperresponsiveness analyses depicted in Section 3 were conducted by Dr. Stephania Cormier at the University of Tennessee Health Science Center. Additional assistance on the mouse studies provided by Dr. Renyi Zhang, Texas A&M University, Department of Atmospheric Sciences, and Dr. Aline Rodrigues-Hoffman, Department of Veterinary Pathobiology. Multiple contributors supported Multiple contributors supported the exposure assessment project in South Texas, including Dr. Stephen Sweet (Texas A&M Geochemical and Environmental Research Group), Dr. Genny Carrillo (Texas A&M School of Public Health), Drs. Kirsten Koehler and Misti Zamora (Johns Hopkins Bloomberg School of Public Health), and Dr. Joe Zietsman (Texas A&M Transportation Institute). I would also like to acknowledge all of my fellow students who contributed to this research, including, Jairus Pulczinski, Louise Myatt, Ana Cardenas, Jeremiah Secrest, Rebecca Langley, Muppala Raju, Valery Roman-Cruz, and

Allison Van Cleve. All other work conducted for the dissertation was completed by the student, under the advisement of Dr. Natalie Johnson of the Department of Environmental and Occupational Health.

Funding Sources

This work was made possible in part by the Texas A&M School of Public Health Dean's Research Enhancement and Development Initiative (REDI) and support from the Texas A&M Health Science Center and Texas A&M Transportation Institute. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Texas A&M University School of Public Health.

NOMENCLATURE

DEP(M)	Diesel Exhaust Particulate (Matter)
AHR	Airway Hyperresponsiveness
PM	Particulate Matter
HDM	House Dust Mite
DOHaD	Developmental Origins of Health and Disease
NHANES	National Health and Nutrition Examination Survey
WHO	World Health Organization
AAD	Allergic Airway Disease
OVA	Ovalbumin
PAH	Polycyclic Aromatic Hydrocarbon
TRAP	Traffic-Related Air Pollution
BALF	Bronchoalveolar Lavage Fluid
KO	Knock-Out
PAMP	Pathogen-Associated Molecular Pattern
CDPM	Combustion-Derived Particulate Matter
LPS	Lipopolysaccharide
ROS	Reactive Oxygen Species
BBB	Blood Brain Barrier
HFD	High Fat Diet
DMR	Differentially Methylated Region

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1. INTRODUCTION

The uterine environment is not an impregnable vault. Prenatal exposures in the form of prescribed drugs, such as thalidomide, environmental and occupational exposures, including organic mercury, and dietary factors like insufficient folic acid intake are well known to cause birth defects, growth malformations, and even spontaneous abortions. However, the idea that prenatal exposures can lead to chronic health effects later in life is fairly recent. In 1990, Dr. David Barker hypothesized that intrauterine growth retardation, low birth weight, and premature birth have causal relationships with the onset of hypertension, coronary heart disease, and non-insulin-dependent diabetes in adulthood (Barker 1997). Though simplistic, Barker's hypothesis signaled the beginning of a larger movement that would spur the Developmental Origins of Health and Disease (DOHaD) hypothesis. For instance, while much research has illustrated the severe immediate detrimental effects of too little folate on neural tube development (Blencowe et al. 2010), recent studies have indicated an association between folate intake and an increase in relative risk of chronic diseases later in life, such as childhood asthma (Parr et al. 2017). Due to the public health impact of chronic diseases, environmental factors *in utero* that predispose individuals to adverse health outcomes later in life have gained interest.

Prenatal exposure to air pollution has been associated with an increased risk of asthma development in children (Veras et al. 2016). The exact mechanisms are unexplained, and in many countries, levels of air pollution continue to exceed

recommended standards. For instance, in China, chronic exposure to high levels of air pollution has become a major public health concern driving scientists to call for "urgent action" (Guan et al. 2016). In settings where we cannot eliminate exposure to particulate matter air pollution, it is necessary to determine whether the current standards are sufficient to provide protection to the developing fetus or if there is a possibility for intervention to reduce risk of disease later in life. Therefore, the overarching goal of this research is to lay a foundation to examine the mechanisms underlying childhood respiratory disease resulting from early life air pollution exposure.

1.1 Asthma - Increasing Global Incidence

Asthma is one of the most common chronic childhood diseases, affecting millions of children worldwide. In the U.S., the prevalence of childhood asthma has more than doubled in the past 30 years, increasing from 3.6% in 1980 to 9.3% in 2012 (CDC, Center for Disease Control and Prevention 2015). Globally, the incidence of childhood asthma has increased rapidly in recent years, resulting in an increasing number of hospital admissions for asthma (Braman 2006). According to the 2014 Global Asthma Report from the International Study of Asthma and Allergies in Childhood (ISAAC), 9.4% of children ages 6-7 and 12.6% of children ages 13-14 have been diagnosed with asthma at some point (IUATLD, International Union Against Tuberculosis and Lung Disease and GAN, Global Asthma Network Study Group 2014).

Although historically a disease of developed countries, childhood asthma prevalence has likewise been increasing rapidly in developing countries such as China.

In a study from 2010, children from three cities in China (Beijing, Chonqing, and Guangzhou) reported between 2-7% asthma prevalence (Bai et al. 2010). The Hong Kong Asthma Society recorded levels in 2011 ranging from 7-10% in children ages 6 to 14 (HKAS, Hong Kong Asthma Society 2013). In 2015, researchers from Nanjing Medical University examined the prevalence of asthma-like disease (i.e., asthma, asthmatic bronchitis, chronic bronchitis, and asthmatic pneumonia) and current wheezing in over 12,000 children from the greater Nanjing area. They found that 16.96% of the study population was affected by asthma-like disease and 3.31% were experiencing current wheeze (Yao et al. 2015).

In the United States, asthma disproportionately affects ethnic minorities and populations with low socioeconomic status. Although these factors are partially linked, evidence indicates that genetics could play a role in increased atopy (allergy) and asthma predisposition. In a study examining asthma prevalence and emergency department visits among non-Hispanic black and white children from 1997-2003, McDaniel et al. (2006) found that black children were more likely to have asthma and experience emergency department visits for asthma compared to white children. Differences in measurable child or family characteristics were controlled for in the study and could not account for the measured disparities. Another study assessing children in New York City public elementary schools, found a 70% higher risk of current asthma in individuals residing in low socioeconomic communities. Puerto Rican children had the highest asthma prevalence regardless of school attended or income status (Claudio et al. 2006). Another study investigating risk factors and spatiotemporal patterns of childhood asthma in

Memphis, TN showed that African American patients were more likely to live in a "high risk" area (adjusted odds ratio (OR) of 3.03) compared to their white counterparts (Oyana et al. 2017). Hispanic patients had an increased OR of 1.62 compared to whites, and asthma prevalence was significantly higher in these "high risk" areas compared to "low risk". In sum, their analysis indicated that race, insurance, and admit source (emergency visit v. home or self-referral) were significant factors for childhood asthma in this region.

As asthma is most often atopic, or allergic, sensitization to allergens is a key step in the development and progression of disease. Stevenson et al. (2001) found a significantly increased risk of sensitization to cockroach or house dust mite in both African American and Mexican American children compared with white children following analysis of a representative sample of children age 6 to 16 years of age who participated in the Third National Health and Nutrition Examination Survey (NHANES III). Additionally, the majority of asthma-related deaths occur in low and lower-middle income countries (IUATLD, International Union Against Tuberculosis and Lung Disease and GAN, Global Asthma Network Study Group 2014). Overall, these data indicate the alarming increase in the incidence of asthma worldwide. The contribution of emergency room visits, missed school days, and overall costs related to morbidity represents a major public health concern. Education and access to care are essential steps in helping manage asthma exacerbation; however, in order to counteract or prevent asthma development, more research is needed on the mechanisms of asthma pathogenesis.

1.2 Allergic Asthma - A Complex Inflammatory Disease

Asthma represents multiple clinical phenotypes, which is why it is commonly thought of as a "syndrome." Overall, the common mechanism driving the disease state is chronic inflammation of the airways (Raedler and Schaub 2014). In atopic, or allergic, asthma, individuals exhibit an exaggerated immune response following exposure to an inhaled allergen. This results in the characteristic asthma "attack" including bronchoconstriction, airway hyperresponsiveness, cough, and wheeze. In atopic asthma, patients exhibit an increase in the presence of T helper 2 (Th2) cells in their airways. Th2 cell-associated cytokines [interleukin-4 (IL-4), IL-5, and IL-13] are upregulated (as opposed to Th1 cytokine IFNγ) and collectively induce eosinophilic inflammation, airway hyperresponsiveness (AHR), and circulating immunoglobulin E (IgE) reactivity to specific antigens (Figure 1). Chronic inflammation can lead to airway remodeling.

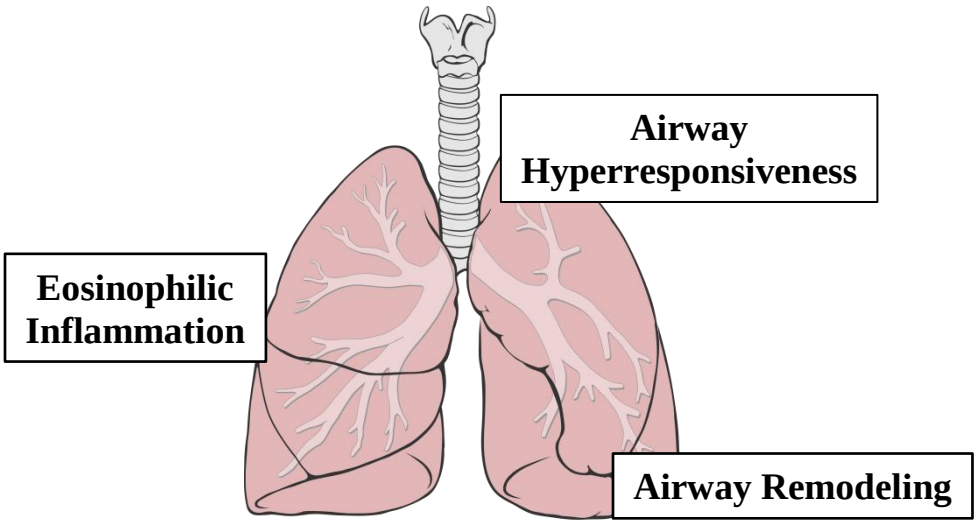


Figure 1. Key Characteristics of Asthma in Humans and Allergic Airway Disease in Mice. Airway hyperresponsiveness is increased as measured by responsiveness to acetylcholinesterase agonists, chronic eosinophilic inflammation due to repeated allergen exposure leads to airway remodeling and buildup of tissue in the bronchoalveolar lining.

These key features of asthma have been replicated in mouse models of allergic airway disease (AAD), typically elicited by allergic sensitization and challenge with ovalbumin (OVA), an egg protein. While effective, this technique does not replicate all of the cellular features evidenced in human allergic asthma. Some models have utilized representative human allergens, such as house dust mite (HDM). The use of this allergen in the mouse model has also been shown to trigger a strong Th17 response resulting in neutrophilia and acute AHR. Th17 cells can also exacerbate the eosinophilic inflammatory response of Th2 cells.

Converse to Th17 cells, regulatory T cells (Tregs) mediate immune homeostasis through anti-inflammatory cytokines, including IL-10 and TGFβ (Figure 2). Treg cell development is dependent on the transcription factor, FOXP3, which, when dysregulated, impacts the expression of this cell population. Furthermore, B cell involvement is key in asthma development, particularly when inhaled allergen exposure is low (Dullaers et al. 2016). Specifically, B cells contribute to the adaptive immune response via allergen-specific Th2 cell expansion in the lungs and mediastinal lymph nodes.

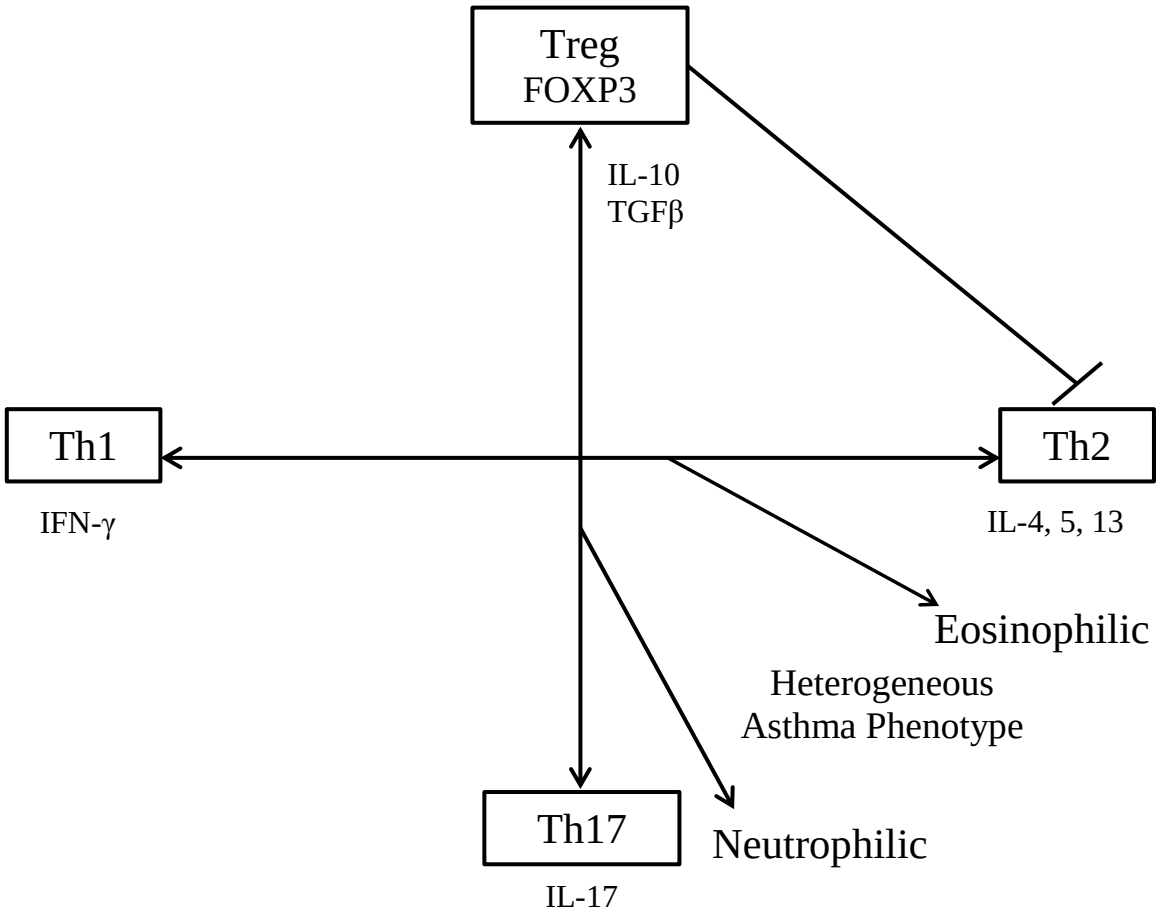


Figure 2. Immune System Dysregulation in Asthmatic Phenotypes. In a normal immune system, there may be a higher percentage of Th1 type cells, regulated by IFN-γ. However, in asthma, there may be more Th2 type cells, induced by IL-4 and IL-5 production. IL-17 may also push toward a higher percentage of Th17 type T cells and fewer Treg cells (induced by presence of IL-10 and TGFβ). FOXP3 is a transcription factor often found to be methylated in asthmatic patients leading to a downregulation of Treg cell population.

It has also been shown that the innate immune system, particularly innate lymphoid cell type 2 (ILC2), plays a large role in eosinophilic airway inflammation of asthma (KleinJan 2016). ILC2 cells are similar in function to T cells, producing IL-5 and IL-13, but they do not express antigen-specific receptors and are, therefore, nonspecific response mediators. Vroman et al. (2016) showed that mice lacking B cells or CD40L signaling (i.e., T cell signaling capabilities) still exhibited a robust eosinophilic infiltration to the lungs following chronic HDM exposure in response from ILC2 cells. This cellular infiltrate was insufficient to produce pulmonary remodeling or airway hyperresponsiveness though. This work indicates that inflammation and hyperresponsiveness may be uncoupled in allergic airway disease. ILC2 activation is contingent on PKCθ (Madouri et al. 2016). Surprisingly, PKCθ has also been shown to activate Treg cell production through the FOXP3 transcription factor (Gupta et al. 2008).

Therefore, the classical Th1-Th2 paradigm and importance of Th17 and Treg cells require careful investigation in mouse models and asthmatics that may vary along the spectrum of disease severity and possess unique genetic factors. Indeed, the development of allergic asthma is influenced by both genetic and environmental factors. Although some genetic predisposing factors have been identified, these alone cannot account for the significant increase in childhood asthma incidence occurring over the past few decades. Therefore, it is likely that environmental factors are playing a larger role than previously believed in asthma etiology (Mukherjee and Zhang 2011).

1.3 Air Pollution

Air pollution consists of a mixture of particles and gases that are classified as either primary (emitted directly into the atmosphere) or secondary (produced via interactions in the atmosphere) (Figure 3) (Zhang et al. 2015). Most primary air pollutants are produced as a result of anthropogenic activity, such as vehicle or industrial emissions. However, even in areas where no humans are present, primary particles are still found in low levels. The particulate phase or particulate matter (PM) is classified by the aerodynamic diameter of the particle. Those with a diameter between 10 μm and 2.5 μm are designated coarse particulate matter or PM_{10}. Particles under 2.5 μm in diameter are designated $PM_{2.5}$ or fine particulate matter. $PM_{2.5}$ presents a greater health threat than PM_{10} because its small size allows penetration deep into the lung. An area of emerging interest (and currently not regulated) are particles less than 100 nm in diameter, termed ultrafine PM. Ultrafine PM have a large surface area, a high capacity for redox reactions, and the ability to form radical species.

Additionally, heavy metals and toxic organic compounds, namely polycyclic aromatic hydrocarbons (PAHs), are often adsorbed to these fine particles. PAHs represent a group of more than one hundred different chemicals that are formed as a result of incomplete combustion. All these factors can contribute to inflammatory effects, cellular DNA damage, or anti-inflammatory inhibition. Therefore, these products, present at high levels in air pollution, are likely to elicit detrimental effects upon exposure, particularly in vulnerable populations such as pregnant women and children.

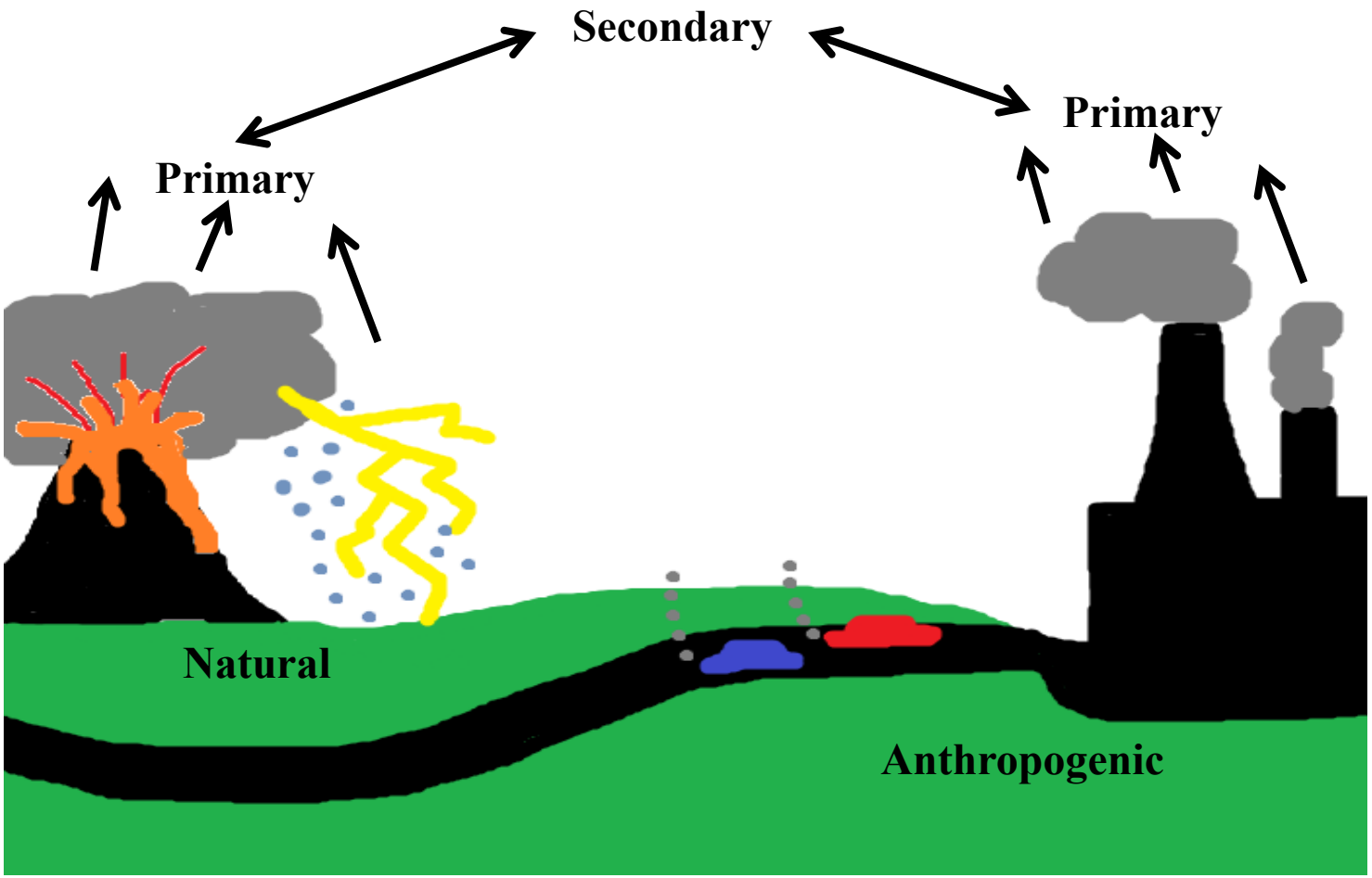


Figure 3. Primary and Secondary Atmospheric Particle Formation. Primary particles are those produced by natural (volcanoes, lightning, etc.) or anthropogenic sources (industrial emissions, traffic-related air pollution, etc.). Secondary particles are formed by gas to particle conversion. These secondary particles can transport and lower into the breathable air space, thereby becoming components of inhaled air pollutants.

Detrimental effects of air pollution exposure can be influenced and modified by particle type and chemical composition. Industrial activity presence and amount of traffic in a metropolitan area will greatly predict particle composition. For example, although Beijing, China and Los Angeles, CA, USA are vastly different, the two cities have similar $PM_{2.5}$ air pollutant chemical compositions. As compiled in Zhang et al. (2015), roughly 44% of $PM_{2.5}$ in LA and Beijing is made up of organics, like black carbon and polycyclic aromatic hydrocarbons (PAHs). Levels of nitrate, sulfate, ammonium and chloride are also comparable between the two sites; however, the density of particles is far greater. 102 $\mu g/m^3$, in Beijing compared to Los Angeles' more closely regulated level of 12 $\mu g/m^3$. The comparability in chemical composition may be attributed to multiple factors including the geographic situation of both cities in a sort of bowl around the harbor area trapping particulate, increasing primary particle interaction and secondary particle formation close to ground level. Further, both cities are highly populous and traffic-related air pollutants are widely emitted.

However, cities with different industrial makeup such as Houston, TX, USA may have similar levels of overall $PM_{2.5}$ levels as Los Angeles but a very different chemical composition. The oil industry in Houston contributes to its higher percentages of sulfate, ammonium, and chloride $PM_{2.5}$ particles and lower traffic reduces the amount of organics attributed to traffic-related air pollution (TRAP). Additionally, levels and composition can also be affected by weather and temporal changes. For instance, if a strong wind is blowing, there will likely be lower particulate on that day. During the

winter months, people will be more likely to have an active heat source in their home and the type of fuel utilized will influence the type and amount of particle emitted.

1.3.1 Health Effects of Air Pollution

Mounting epidemiologic evidence points to the many detrimental health effects of exposure to air pollution, particularly over prolonged periods and in susceptible populations. Overall, the highest risk increases are found for cardiovascular and respiratory diseases. Estimates from the World Health Organization from 2012 place the number of premature deaths caused by exposure to outdoor air pollution at 3 million worldwide (World Health Organization, Media Centre September, 2016). 72% of those were attributed to ischaemic heart disease and strokes, 14% to chronic obstructive pulmonary disease or acute lower respiratory infections, and 14% to lung cancer. Based on a comprehensive literature review, the American Heart Association stated in 2004 that exposure to air pollution is associated with an increased risk of cardiovascular disease (Brook et al. 2004). The International Agency for Research on Cancer classified outdoor air pollution as carcinogenic to humans in 2013, citing the PM_{10} component of air pollution as most closely associated with an increase cancer risk (International Agency for Research on Cancer, WHO 2013). Song et al. (2014) analyzed data from China, the United States, and European Union to show that chronic obstructive pulmonary disease incidence is significantly increased with increases in PM_{10} resulting in increased exacerbation of disease and mortality. Ambient air pollution exposure may also have an effect on metabolic disease and, in particular, susceptibility to type 2

diabetes (Eze et al. 2015; Thiering and Heinrich 2015). A recent review postulated that exposure to air pollution may be associated with cognitive decline in aging populations; however, more work is necessary to confirm this interaction (Peters et al. 2015).

A systematic review recently reconfirmed the detrimental effects of outdoor air pollution exposure on respiratory health in children (Rodriguez-Villamizar et al. 2015). This review emphasizes that while the majority of included studies reported exposure levels below U.S. and Canadian standards, exposure still correlated with decreases in lung function, increased respiratory-related emergency doctor visits and hospitalizations with higher effect levels near industry or refinery areas. Significant association was found between exposure to nitrogen dioxide (NO_2) in the third trimester and an increase in systolic blood pressure in 11-year-old children (Breton et al. 2016) indicating that the cardiovascular effects of air pollution may originate even before birth.

Accumulating evidence suggests that air pollution exposure may cause adverse neurocognitive effects. Increased exposure to PAHs *in utero* were found to be inversely associated with full-scale and verbal IQ scores in children 5 years of age residing in New York City (Perera et al. 2009b). Prenatal exposure to particulate matter may be related to autism spectrum disorder; however, strong evidence for that link is currently absent (Lam et al. 2016). Asthma and allergies are commonly present in children with autism spectrum disorder, indicating a possibility of mechanistic interaction (Lyall et al. 2015). Although there is no question that exposure to air pollution is a public health concern, there is still a necessity to identify a safe level of exposure to protect all members of the

population, particularly the most susceptible such as young children, pregnant women, and the elderly.

1.3.2 Prenatal and Early Life Exposure to Air Pollution and Asthma Development

The link between air pollution exposure during critical developmental windows, e.g., prenatal or early infancy periods, and the development of allergic asthma has become increasingly accepted as exposure assessment techniques have improved in epidemiologic studies and experimental models have been developed (de Planell-Saguer et al. 2014; Finkelman 2014). In a large cohort study conducted in British Columbia (n=37,401), investigators initially highlighted a significantly increased risk of asthma diagnosis for children ages 3-4 years exposed to ambient air pollutants throughout gestation and first year of life (Clark et al. 2010). Traffic-related pollutants, including nitrogen oxides (NO and NO_2), carbon dioxide (CO_2), and black carbon (BC) were associated with the highest risk estimates. Coarse particulate matter (PM_{10}), sulfur dioxide (SO_2), and residence near industrial point sources were also associated with elevated asthma risk. However, fine particulate matter ($PM_{2.5}$), woodsmoke, and road proximity did not show elevated risk. Jedrychowski et al. (2010) found a positive association between prenatal PAH and $PM_{2.5}$ exposures and number of wheezing days during the first two years of life. In another study utilizing repeated measures of geographic information system (GIS) indicators to represent long-term air pollution exposures of young children living in high-density New York City neighborhoods, positive associations were found between proximity to stationary sources of air pollution

and reported asthma (Patel et al. 2011). A more recent study examined the effects of prenatal exposure to $PM_{2.5}$ estimated using spatio-temporal modeling (Hsu et al. 2015). Investigators found that higher prenatal exposure during mid-gestation was associated with asthma development by age 6 in boys. Children 5-18 years of age from the Greater Cincinnati Pediatric Clinic Repository (GCPCR) were found to have a significant association between severe asthma and high DEP exposure. It is worth noting that all children in this analysis suffered from asthma but that frequency of symptoms was the chosen determinant in the association model. A subset of the GCPCR, from the Pediatric Environmental Exposures Study (PEES), was evaluated for serum levels of IL-17A. High exposure to DEP was associated with increased IL-17A levels. Other cytokines were evaluated including IL-4, IL-5, and IL-13, but none were found to be significantly associated with DEP exposure. In an ongoing prospective birth cohort study, Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS), children with positive aeroallergen sensitization had a higher risk of asthma development following early life exposure to DEP. In non-allergic children, this trend was not observed. The variation in these findings could be attributable to many factors including timing of exposure, composition of the pollutants of interest, and age of allergic testing. As these factors can be difficult to control for, animal models have become a useful tool to investigate specific exposure types and susceptible windows as they relate to childhood asthma development. With the knowledge that early life exposure to air pollutants can have profound effects on the immune system, mouse models have been essential components to examine the mechanisms of toxicity of air pollutants.

1.4 Mouse Exposure Models

1.4.1 Mouse Models of Allergic Airway Disease

Many adult mouse asthma models have utilized ovalbumin (OVA) to induce the allergic airway phenotype. OVA has been used to induce both acute and chronic effects (Daubeuf and Frossard 2012). Typically administered systemically upon first dose in the presence of an adjuvant, this exposure model is not extremely relevant to the human disease state. It has been found that inhalation tolerance is common with prolonged exposure; therefore, OVA has been most successful when used as an acute atopic factor. Even so, prenatal pollutant exposure models have utilized the OVA allergen. More recent literature points to the advantages of human-relevant allergen use in allergic airway disease induction.

The human relevant allergen, house dust mite, has been reviewed by Yu et al. (2014) and utilized to induce allergic endpoints. In 1995, the house dust mite Dermatophagoides pteronissinus (Der p) was employed to assess nasal resistance and airway hyperresponsiveness in allergic rhinitis and allergic asthmatic rhinitis in human subjects (Tsai et al. 1995). Use of the two allergens, HDM and OVA, has been compared in a chronic exposure model in BALB/c mice (Johnson et al. 2004). Specifically, female BALB/c mice were exposed to either OVA or HDM without adjuvant 5 days per week for up to seven consecutive weeks. Significantly higher levels of total cell and eosinophilic infiltrates were observed in bronchoalveolar lavage fluid from animals exposed to HDM compared to OVA or controls. Serum total IgE and HDM-specific IgG1 levels in plasma were increased throughout 7 weeks of HDM exposure and

remained high for 7 weeks after the end of the exposure period. The cytokines IL-5 and IL-13 were increased in isolated splenocytes at each collection time point throughout 7 weeks of exposure to HDM. Even after cessation of HDM exposure, isolated splenocytes continued to have increased HDM-induced IL-5 and IL-13 cytokine expression. These effects were not seen in the OVA-exposed animals. The two allergen exposures were compared in another study using BALB/c mice (Hongjia et al. 2010). Findings indicate that exposure to HDM induces a more robust response than OVA. Specifically, HDM-induced effects are shown to be mediated through the toll-like receptor 4 (TLR4) pathway triggering alveolar macrophage responses, similar to those seen in allergic asthma patients. These studies underline the pitfalls of the OVA exposure model and point to the relevancy of HDM exposure to induce chronic allergic airway disease in BALB/c mice.

The HDM exposure model has further been validated in male BALB/c mice in a study showing the integral part IL-13 plays in the HDM chronic response (Tomlinson et al. 2010). In this study, mice treated either prophylactically or therapeutically with anti-IL-13 mouse antibody (mAb) demonstrated a reduced response to intranasal administration of HDM evidenced by reduced total cell infiltrate and eosinophils in the bronchoalveolar lavage fluid (BALF) and reduced goblet cell hypersecretion. Therapeutic treatment did not reduce airway hyperresponsiveness to methacholine whereas prophylactic treatment did so significantly. Another widely used asthma therapeutic, Budesonide (a glucocorticosteroid), was investigated in a C57Bl/6 mouse model of exposure to HDM (Raemdonck et al. 2016). Budesonide pre-treatment was

capable of reducing inflammatory response in bronchoalveolar lavage fluid and airway hyperresponsiveness to methacholine in this model. The group also validated that the HDM allergen exposure model is capable of producing a robust and persistent response in general among the C57Bl/6 strain tested. On the C57Bl/6 background, multiple knockout (KO) mice were also tested in the model. CD4+ T Cell KO mice had significantly reduced BALF inflammatory response and AHR; however, their neutrophilic response remained intact. CD8+ T Cell KO mice also had significantly attenuated response to HDM exposure. Interestingly, B Cell or IgE KO mice had equivalent inflammatory responses compared to wild-type (WT), emphasizing the importance of innate immune response in atopic disease.

House dust mite-induced allergic airway disease has also been studied extensively in relation to other environmental exposures during adulthood. Growing literature is touting the benefits of exposure to farm-related activities on atopy. Hagner et al. (2013) examined the effects of co-exposure to the farm-derived bacterium, *Staphylococcus sciuri* W620, and HDM. Concomitant exposure to the bacterium significantly reduced inflammatory effects of allergen exposure. While bacterium co-exposure elicited a positive effect on asthma, there are many co-exposures that have less favorable outcomes. β-glucan, a pathogen associated molecular pattern (PAMP) found in house dust mite feces, acts as an adjuvant in HDM-induced allergic airway responses (Hadebe et al. 2016). The addition of purified β-glucan during allergen sensitization led to a significantly increased eosinophilic inflammation in the lung.

Although many exposures such as β-glucan are virtually unavoidable, modifiable lifestyle factors may have enhanced effects on asthma outcomes. For instance, vitamin D deficiency can differentially regulate gene expression following HDM exposure (Foong et al. 2016). Surprisingly, PER2, a circadian clock gene, was found to be slightly upregulated in vitamin D deficient mice exposed to HDM. Other variably regulated genes are implicated in inflammatory cell recruitment and airway remodeling including MID1, ACOT1, ADM, ANGPTL4, and HILDPA. High fat diet, also known to interact with clock gene expression, was shown by Everaere et al. (2016) to have an effect on allergic airway inflammation in mice. High fat diet exhibited increased eosinophilic inflammation, airway hyperresponsiveness, and mucus production in lungs of mice exposed to house dust mite compared to low fat diet controls. ILC types 2 & 3 were increased in high fat diet-fed animals as well, indicating that this lifestyle factor may function through induction of the innate immune system. Signaling between the innate and adaptive immune system occurs through antigen presenting cells such as dendritic cells (DCs). In a mouse model of exposure to cigarette smoke during HDM sensitization, challenge, or both, DCs were a potential response mediator (Lanckacker et al. 2013). Concomitant cigarette smoke and HDM exposure over 3 weeks increased eosinophilic recruitment into lungs, mucin-producing goblet cells, Th2 cytokines, and AHR in male BALB/c mice.

The hypothesis that response to certain inhaled pollutants may be mediated by dendritic cells is also supported in a model of early life exposure to combustion-derived particulate matter (CDPM) in C57Bl/6 mice (Saravia et al. 2014). Following exposure to

CDPM alone in early life, increases in IL-10 producing DCs, IL-10, and Treg cells were observed. With the addition of allergen exposure (CDPM/HDM), a reduction in allergic response was observed compared to Air/HDM controls, presumably as a result of the increased Treg presence and reduced Th2 skewing. To further examine the T cell response, investigators utilized OT-II mice and exposed to CDPM and OVA. OT-II mice are transgenic mice on a C57Bl/6 background with expression of OVA-specific T cell receptors which induce a greater response to antigen, allowing for easier interrogation of T lymphocyte response mechanisms. In this model, a similar immunosuppression was observed upon co-exposure in early life, however, a re-challenge later in life elicited an exacerbated immune response to OVA. Similarly, in two studies from the University of Cincinnati, co-exposure of diesel exhaust particulate and HDM resulted in enhanced and persistent allergic response compared to controls exposed to HDM only (Brandt et al. 2013; Brandt et al. 2015). Specifically, response was characterized by a mixture of Th2 and Th17 cell populations. Exacerbated response was seen even after re-exposure 7 weeks following initial sensitization with HDM.

1.4.2 Mouse Models of Air Pollution Exposure

The most common effect found in animal models of exposure to air pollution includes an increase in oxidative stress and systemic inflammation. These outcomes, however, vary depending upon the composition of the exposure administered. For instance, in mice exposed to urban air particulate matter from Buenos Aires, adverse biological effects were observed including recruitment of phagocytes, reduction in air

spaces, and increase in mucous PAS positive cells contributing to increased inflammation in the lungs (Martin et al. 2007). Wan et al. (2010) demonstrated an increase in reactive oxygen species *in vivo* following $PM_{2.5}$ exposure in a mouse model. The response was further characterized by increased expression of superoxide dismutase-1 and heme oxygenase-1 in primary macrophages. Further exacerbation of the systemic inflammatory response can be induced by high fat diet (HFD). Male offspring fed HFD following prenatal exposure to diesel exhaust particles experienced increased weight gain, insulin resistance, and anxiety-like behavior compared to vehicle-treated HFD controls (Bolton et al. 2014). Subsequent exposure to the allergen LPS produced exaggerated IL-1β peripheral response in males. Another study investigating the inflammatory effects of administration of lipopolysaccharide (LPS) found that concomitant exposure to diesel exhaust particulate (DEP) yielded increases in inflammation and endothelial damage, as well as disturbances in coagulation factors (Inoue et al. 2006). Further response characterization in this model revealed increased chemoattractant proteins in the serum of mice exposed to both DEP and LPS 3- to 5-fold compared to LPS alone (Arimoto et al. 2007).

Systemic inflammation can also lead to alterations in adipose. Findings confirm exposure to ambient $PM_{2.5}$ increased reactive oxygen species (ROS) while reducing mitochondrial size in brown adipose tissue and total number of mitochondria in both white and brown adipose tissue in male ApoE knockout mice, a model for dysbetalipoproteinemia (Xu et al. 2011). ApoE knockout mice were also used to probe for activity of mixed vehicle exhaust exposure on the blood brain barrier (BBB)

(Oppenheim et al. 2013). This exposure elicited increased ROS and mixed metallopeptidase activity compared to filtered air controls leading to increased BBB permeability and neuroinflammatory markers. Exposure during early pregnancy to diesel exhaust particulate can reduce viability of an embryo, as evidenced by a disruption in inner cell mass integrity even at low concentrations in a mouse model (Januario et al. 2010). Another study investigated the effects of exposure to diesel exhaust particulate throughout gestation combined with stress (reduced nesting material) (Bolton et al. 2013). Results indicate that male offspring of dams that underwent both stressors experienced impaired cognitive function, increased IL-1B levels in the brain, and increased TLR4 expression. Female offspring were less susceptible to the administered exposure.

DEPs are also known to have an effect on the immune system. Siegel et al. (2004) demonstrated that the components of DEP (particulate v. organic) affect the immune system differently. Two types of engineered particles, diesel-enriched PM and carbon black particles, were tested in a mouse model naïve to PM exposure (Bezemer et al. 2011). It was shown that both exposures activated DCs *in vivo* and upregulated the innate immune response. Interestingly, early life exposure to PM with environmentally persistent free radicals (EPFRs) results in increased oxidative stress in the lung and reduced adaptive immune response to influenza virus (Lee et al. 2014).

Strain	Maternal Exposure (route)	Allergen	Reference
C57BL/6	DEP (oropharyngeal) & DE (inhaled)	Ozone	Auten et al. 2012
C57BL/6	DEP (intranasal)	Ovalbumin	Manners et al. 2013
BALB/c	DE (inhaled)	A. fumigatus	Corson et al. 2010
BALB/c	DEP (intranasal)	Ovalbumin	Fedulov et al. 2008
BALB/c	DEP (intranasal) & LPS (inhaled)	Ovalbumin	Reiprich et al. 2013
BALB/c	DE (inhaled)	Ovalbumin	Sharkhuu et al. 2010

Table 1. Murine Models of Prenatal Exposure to Air Pollution and Allergic Airway Disease. Strain, maternal exposure type and route, and allergen utilized in previous mouse studies. Diesel Exhaust Particulate (DEP); Diesel Exhaust (DE); Lipopolysaccharide (LPS).

1.4.3 Mouse Models of Early Life Air Pollutant-Induced Allergic Airway Disease

Recent *in vivo* experimental studies have begun to explore the association between prenatal exposure to air pollution and allergic airway disease (summarized in Table 1). Across all studies, pregnant mice were exposed to diesel exhaust (DE) or diesel exhaust particulate matter (DEPM) at various times throughout gestation and challenged with different allergens. Four of these studies demonstrated a positive correlation between prenatal exposure and asthmatic response (Auten et al. 2012; Fedulov et al. 2008; Manners et al. 2014; Reiprich et al. 2013); however, one showed no effect (Sharkhuu et al. 2010), while another showed protection by addition of a fungal exposure (Corson et al. 2010). These disparities are likely due to differences in models, as well as mouse strain. Specifically, in the first study of its kind, Fedulov et al. (2008) dosed pregnant BALB/c mice with either DEP or TiO_2 (inert particles) on gestation day (GD) 14 and examined allergic response to ovalbumin (OVA) in the offspring by measuring AHR and airway inflammation. They demonstrated that both "inert" and DEP increased asthmatic and inflammatory responses in offspring. Interestingly, in BALB/c dams chronically exposed to *Aflatoxin fumigatus* via the intranasal route prior to and during gestation, concomitant exposure to generated diesel exhaust appeared to decrease the inflammatory response in offspring compared to controls of DE or *A. fumigatus* alone (Corson et al. 2010). Varying doses of ozone (another component of environmental air pollution) exposure during pregnancy in BALB/c mice reduced sensitivity reactions to bovine serum albumin in offspring (Sharkhuu et al. 2010). Sensitization early in life of the offspring produced a more notable response than

sensitization later in life. Alternatively, diesel exhaust exposure during pregnancy was shown to worsen airway hyperresponsiveness induced by ozone exposure in offspring (Auten et al. 2012). Heightened response persisted for 4 weeks in offspring, and there were indications of impaired alveolar development in animals exposed to both DE and ozone.

One study that included prenatal exposure to lipopolysaccharide (LPS) demonstrated protection from future allergic response to OVA (Reiprich et al. 2013). However, the protection was ameliorated by co-exposure to diesel exhaust particulate during pregnancy. A final compelling finding from this study found that the antioxidant N-acetyl cysteine could reverse the increased asthma risk in offspring of co-exposed dams. The most recent study involving prenatal exposure to diesel exhaust and postnatal allergen challenge was conducted in C57Bl/6 mice dosed repeatedly throughout pregnancy to diesel exhaust particles or PBS (Manners et al. 2014). Offspring were challenged with OVA or PBS and assessed for asthma phenotypes. Findings concluded that increased asthma susceptibility following prenatal exposure to diesel exhaust particles was contingent on natural killer cell responses and associated with upregulation of aryl hydrocarbon receptor and oxidative stress-related genes. Early life co-exposure to DEP and house dust mite (HDM) has also been characterized in a mouse model (Acciani et al. 2013). DEP significantly exacerbated response to HDM exposure, even at levels low enough to evoke no independent inflammatory response.

The strains commonly employed in these models, BALB/c and C57Bl/6, have been shown to exhibit differing airway inflammatory responses in a postnatal model of

prolonged exposure to DE from birth to six months of age (Li et al. 2008). In addition, these strains showed varying susceptibility to the development of allergic airway disease when challenged with OVA following prolonged exposure to DE (Li et al. 2009). In summary, these studies continue to reveal that genetic factors play an important interactive role in response to environmental stimuli such as air pollution and allergen sensitization. Thus, in design of our experiment, we decided to compare response in two genetically and phenotypically distinct strains of mice.

1.5 Epigenetic Mechanisms

Recently, environmental epigenetic regulation has been recognized as an important mechanism in asthma development following early life exposure to outdoor air pollution (Begin and Nadeau 2014). Epigenetics encompasses multiple mechanisms by which DNA transcription can be controlled including methylation, hydroxymethylation, histone modifications, and microRNA (miRNA). Typically, methylation is thought to disable or at least reduce transcription by blocking action of transcription factors, whereas hydroxymethylation is considered to be a demethylation intermediate. Hydroxymethylation may also play some other roles in DNA repair, but its specific role is still being elucidated (Shukla et al. 2015). Histone modifications include various post-translational modifications such as acetylation that impact chromatin structure or histone modifier recruitment and can ultimately lead to alterations in gene expression. MicroRNAs are small non-coding RNAs that can act on the transcribed mRNA to, in effect, silence protein expression by cleavage or destabilization methods.

In the ENVIRonmental influence ON early AGEing (ENVIRONAGE) birth cohort, 210 mother-newborn pairs were investigated using a candidate miRNA approach (Tsamou et al. 2016). Specifically, $PM_{2.5}$ and NO_2 exposure were estimated during each trimester and compared to miRNA changes in the placental tissue. A positive association was found between $PM_{2.5}$ exposure during the first trimester and upregulation of miRs-20a and -21. miRs-16, -20a, -21, -146a, and -222 were found to be inversely associated with $PM_{2.5}$ exposure during the second trimester ,and miR-146a was also found to be inversely associated with exposure during the third trimester. Janssen et al. (2013) investigated changes in global DNA methylation in placental tissue from the ENVIRONAGE cohort. On average, $PM_{2.5}$ exposure was considered low, 17.4 $\mu g/m^3$; however, even at this low level, there was an inverse association with global DNA methylation in the placenta. Broken down by trimester, they found the most significant association in the first trimester, particularly during the implantation stage. As with most other toxicant exposures during pregnancy, this indicates a potential window of increased susceptibility.

Based on the literature indicating global demethylation following prenatal exposure to air pollution, investigators have probed specific genes related to DNA methylation including DNMT1, TET1, and TET2. It has been found that exposure to traffic-related air pollution (TRAP) is associated with increased TET1 promoter methylation at a single CpG site in nasal epithelial samples (Somineni et al. 2016). This pattern was also consistent across saliva and peripheral blood mononuclear cells (PBMCs). Further, loss of methylation at the same site and increased global 5-hmC

levels were associated with asthma in children. This differential response indicates a potential for interacting mechanisms in asthma development and TRAP exposure. Breton et al. (2016) examined the association between multiple air pollutants (including $PM_{2.5}$, PM_{10}, and NO_2) and DNA methylation levels in LINE1 and AluYb8 measured in newborn blood spot tests. Findings included an inverse association between exposure to PM_{10} and O_3 pollutants in the first trimester and DNA methylation in LINE1 and a positive association between O_3 exposure during later pregnancy and LINE1 DNA methylation. In another study exploring NO_2 exposure during pregnancy, exposure was found to be associated with altered cord blood DNA methylation in three CpG sites located in mitochondria-related genes (Gruzieva et al. 2017). One of the gene associations was significantly associated with exposure into later childhood. Collectively, these environmental epigenetic studies in exposed populations underscore the lasting impact early life air pollution may have on health effects even years after initial contact with pollutant.

Accumulating epidemiologic and experimental evidence supports the hypothesis that *in utero* air pollution exposure alters epigenetic regulation of genes involved in T cell differentiation (Table 2) (Brand et al. 2012; Tang et al. 2012). In a subset of umbilical cord blood samples collected from a longitudinal cohort of children in New York City, methylation of the acyl-CoA synthetase long chain family member 3 (ACSL3) 5'-CpG island was shown to be significantly associated with high levels of maternal polycyclic aromatic hydrocarbon (PAH) exposure during pregnancy, largely from traffic-related combustion emissions, in children exhibiting asthma symptoms

(Perera et al. 2009a). Although the role of this gene in asthma is not yet elucidated, it could serve as a transplacental exposure marker for future exposure studies. In addition, in this same cohort, maternal PAH exposure was more recently associated with interferon-γ (IFNγ) promoter hypermethylation in cord blood DNA indicating a possible mechanism of Th1 silencing (Tang et al. 2012). In a population of asthmatic children in Fresno, California, increased ambient air pollution exposure was associated with hypermethylation of the forkhead box protein 3 (FOXP3) locus in circulating T cells, impaired Treg cell function, and worsened asthma symptom scores (Nadeau et al. 2010). Hew et al. (2015) went on to link higher PAH exposure to impaired Treg function and increased methylation in the FOXP3 locus in asthmatic children in Fresno, California exposed to high levels of particulate air pollution. In summary, increased Th1 or Treg methylation may underlie the skewing toward Th2/Th17 response in asthmatics.

Exposure (assessment method)	**Genes Affected**	**Reference**
PAHs (personal air monitors)	ACSL3	Perera et al. 2009
PAHs (personal air monitors)	IFNγ	Tang et al. 2012
Ambient air pollution (land use regression model)	FOXP3	Nadeau et al. 2010
PAHs (land use regression model)	FOXP3	Hew et al. 2014

Table 2. Epigenetic Alterations Following Prenatal Exposure to Air Pollution in Humans. ACSL3 (acyl-CoA synthetase long-chain family member 3), IFN-γ (interferon gamma), FOXP3 (forkhead box protein 3).

Evidence of epigenetic reprogramming following prenatal exposure to PM in animal models is scant. However, it is well known that T cell differentiation is controlled through epigenetic mechanisms (Wilson et al. 2009). Liu et al. (2008) showed hypomethylation in IL-4 promoter CpG sites (activation of Th2 response) and hypermethylation of IFNγ promoter CpG sites (silencing of Th1 response) occurred in adult BALB/c mice in a model of postnatal exposure to diesel exhaust particulate and allergen, *Aspergillus fumigatus*. Only one group has investigated epigenetic mechanisms after *in utero* diesel exhaust exposure demonstrating that the preventive effects of endotoxin are dependent on IFNγ histone acetylation (Reiprich et al. 2013). Isolated CD4+ T cells displayed a loss of histone 4 acetylation at the IFNγ promoter following co-exposure to diesel exhaust particles and endotoxin *in utero* and postnatal OVA challenge compared to offspring solely exposed to endotoxin *in utero*. Fedulov and Kobzik (2011) found that epigenetic DNA methylation present in dendritic cells from offspring of allergic dams was critical to confer allergic susceptibility to naïve adult mice.

Investigation into lifestyle factors such as a methyl-rich diet during pregnancy has also revealed possible epigenetic components to allergen sensitization (Hollingsworth et al. 2008). In this group, C57Bl/6J mice were time-mated and given either a high methyl-donor or low methyl-donor diet until weaning. Eight- to twelve-week-old mice were then sensitized and challenged with OVA and assessed for asthmatic response. Animals exposed to high methyl-donor diet prenatally and during early life were more likely to develop more severe allergic airway disease and had

differential methylation in 82 gene-associated loci. Adult mice chronically exposed to house dust mite for 5 weeks were found to have differential methylation patterns, specifically in the transforming growth beta (TGFβ) signaling pathway (Cheng et al. 2014). Overall, these findings provide preliminary evidence that epigenetic regulation is an important mechanism by which prenatal environmental exposures predispose offspring to asthma development.

Despite efforts to unravel health disparities among minority populations, current research neglects to explain the interaction of genetic differences and pertinent environmental factors, like air pollution, during important periods of lung and immune system development. Data in animal models is sparse and somewhat inconsistent. Comparisons can be drawn from tobacco smoke exposure since major constituents of air pollution are derived from similar incomplete combustion processes. Epigenetic studies indicate that prenatal exposure via maternal smoking during pregnancy can differentially regulate methylation patterns in newborn cord blood samples (Joubert et al. 2012). This particular study, conducted in the Norwegian Mother and Child Cohort Study (MoBa), investigated epigenome-wide alterations using the Illumina 450K Beadchip system and illustrated the vast impact that prenatal inhalation exposures can have on the newborn epigenome. Therefore, our overarching goal is to gain insight into epigenetic immunomodulatory mechanisms via a dual-strain animal model of prenatal particulate matter exposure and postnatal allergic airway disease induction. Furthermore, we hope to gain insight into the genetic and socioeconomic disparities of asthma by examining

exposure among a population with high childhood asthma prevalence (compared to the national average) in south Texas.

1.6 Research Objectives

While early life exposure to particulate matter air pollution increases asthma susceptibility, the role of epigenetic mechanisms is not yet fully explored. Moreover, a remaining fundamental challenge is the impact of genetic variation on epigenetic regulation and ultimately the development of asthma following prenatal exposure to air pollution. Thus, there is a critical need to identify epigenetic modifications in the context of genetic variability. In the absence of such information, the potential to predict asthma risk from early life exposures will likely remain limited.

As a first step to investigate mechanisms of asthma pathogenesis, the major research goal of this dissertation was to develop a representative model of prenatal air pollution exposure to probe interacting determinants of increased susceptibility. To lay the foundation for translating the relevance of this model to exposed human populations, we conducted a pilot project to evaluate exposure to particulate air pollution during pregnancy in a high-risk population. Studies in this dissertation focus on developing the proof-of-principle for incorporating genetic variance in an air pollution prenatal exposure models, refining prenatal exposure offspring asthma models, and characterizing exposure in populations through the following specific objectives:

1) Examine changes in DNA methylation across strains following prenatal exposure to particulate air pollution;
2) Characterize offspring responses to allergen challenge in an improved model of prenatal particulate air pollution exposure; and
3) Measure exposure to $PM_{2.5}$ and PAH exposure in pregnant women in pilot study in South Texas in an area with a high prevalence of childhood asthma.

2. STRAIN-DEPENDENT DNA METHYLATION CHANGES FOLLOWING PRENATAL EXPOSURE TO PARTICULATE MATTER: PRELIMINARY STUDY

2.1 Introduction

It is increasingly recognized that early life exposure to particulate matter (PM) air pollution increases asthma susceptibility; however the underlying mechanisms are not yet fully understood. Emerging evidence suggests epigenetic modifications following prenatal PM exposure as a potential means of developmental reprogramming that may predispose offspring to allergic asthma development. Therefore, our initial study objective was to evaluate changes in DNA methylation status in response to prenatal diesel exhaust PM (DEPM), a primary component of particulate air pollution. Previous postnatal PM exposure models have demonstrated alterations in DNA methylation influence adaptive immunity and response to allergens (Liu et al. 2008; Soberanes et al. 2012). While methylation status has yet to be evaluated in prenatal exposure models, epidemiological studies measuring intrauterine or early life exposure to PM-related pollutants, (e.g., polycyclic aromatic hydrocarbons (PAHs)), have revealed DNA methylation changes in peripheral blood cells are correlated with childhood asthma development (Breton et al. 2016; Gruzieva et al. 2017; Hew et al. 2015; Nadeau et al. 2010; Perera et al. 2009a; Tang et al. 2012). Thus, to determine changes in target tissues in response to prenatal PM exposure, we leveraged our murine prenatal exposure model to assess DNA methylation status, specifically in offspring lung tissue.

The status of DNA methylation is frequently determined by measuring 5-methylcytosine (5-mC) levels, which represent methyl groups derived from S-adenosyl-L-methionine covalently attached to the fifth carbon of the cytosine ring (Bird 2002; Umer and Herceg 2013). More recently, the role of 5-hydroxymethylcytosine (5-hmC) has been recognized in epigenetic regulation, and 5-hmC is considered a marker of DNA demethylation. In mammals, enzymes in the ten-eleven translocation methylcytosine dioxygenase (TET) family catalyze the conversion of 5-mC to 5-hmC (Tahiliani et al. 2009). Altered TET1 methylation status and resulting impact on global 5-hmC levels have been implicated in asthmatic patients exposed to traffic-related air pollution (Somineni et al. 2016). Evidence of global hydroxymethylation changes in lung tissue in experimental asthma models further suggests an important role of 5-hmC in asthma pathogenesis (Cheng et al. 2014).

Importantly, asthma predisposition is impacted by genetic factors. Variance in host responses to oxidative stress response pathways therefore may further impact PM-induced asthma. Original mechanistic studies in adult mouse models of DEPM-enhanced allergic asthma capitalized on strain differences in mouse models to reveal BALB/c mice mount a differential response to PM, enhancing subsequent inflammatory response to allergen challenge, compared with C57Bl/6 mice (Li et al. 2009). These differences were attributed to variation in host defense responses to oxidative stress. Since oxidative stress has been shown to affect TET expression and influence DNA methylation status (Niu et al. 2015), we hypothesized epigenetic changes could be differentially impacted across strains with known divergent oxidative stress responses This initial approach of

comparing PM-induced DNA methylation changes in C57Bl/6J and BALB/cJ mice could provide the proof-of-principle for genetically variable epigenetic modifications underlying asthma susceptibility, which would have important implications in genetically diverse human populations.

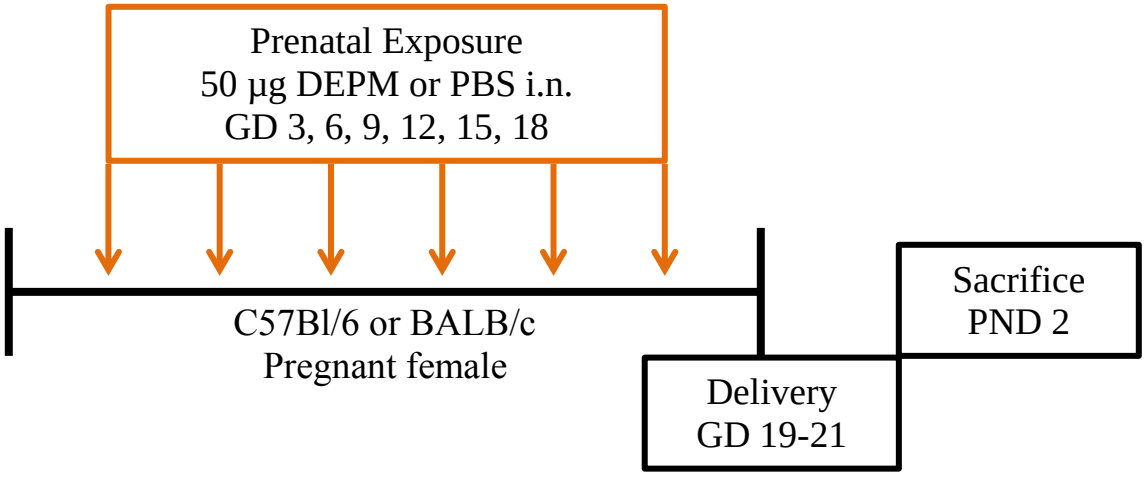
2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Diesel Particulate Matter Standard Reference Material (SRM 2975) was purchased from the National Institute of Standards and Technology (Gaithersburg, Maryland). Phosphate Buffered Saline (PBS) was purchased from Sigma Chemical Co. (St. Louis, Missouri) for use as a control. All other chemicals and reagents used were obtained commercially at the highest available purity.

2.2.2 Animals

Male and female C57Bl/6J & BALB/cJ mice, approximately 8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, Maine) and allowed to acclimate for 1 week under controlled conditions of temperature, humidity and light. Females received 9% fat breeder diet (Harlan, Indianapolis, Indiana) while males received standard rodent chow (4%) (LabDiet, St. Louis, Missouri). Food and water were provided *ad libitum* and fresh diet was provided to animals at least twice per week. Mice were weighed each day of dosing and checked for signs of distress every day throughout the dosing period. All experiments were approved by the Texas A&M



	Treatment	**Number of Dams**	**Number of Pups Per Litter**	**Total Pups**
C57Bl/6	PBS	2	5/2	7
	DEPM	2	4/0	4
BALB/c	PBS	1	6	6
	DEPM	3	8/5/0	13

Figure 4. Experimental Protocol for Prenatal Diesel Exhaust Exposure in C57Bl/6 and BALB/c Mice. Maternal exposure was performed by intranasal (i.n.) dosing of 50 µg diesel exhaust particulate matter (DEPM) or PBS (control) six times during pregnancy on gestational days (GDs) 3, 6, 9, 12, 15, and 18. Following birth, pups were sacrificed at postnatal day 2 (PND 2).

University Animal Care and Use Committee (IACUC# 2014-0354) prior to experimentation.

2.2.3 Overall Study Design and Procedures

Mice were time-mated and checked for the presence of a vaginal plug to define gestational day (GD) 0. Following the same protocol as Manners et al. (2014) that previously demonstrated prenatal DEPM exposure leads to offspring asthma susceptibility, we exposed pregnant dams to 50 µg DEPM in 50 µL phosphate-buffered saline (PBS) or PBS alone via intranasal application (i.n.) (25 µL in each nostril 30 seconds apart) on GDs 3, 6, 9, 12, 15, and 18 while under light isofluorane anesthesia (Figure 4). Dams were allowed to deliver spontaneously. On postnatal day (PND) 2, offspring from exposed or control dams were sacrificed, and tissues including lung, liver, and spleen were collected, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

2.2.4 Analyses

DNA from lung tissue was extracted using either a Qiagen DNeasy Blood & Tissue Kit according to manufacturer's instructions or using a slightly modified method based on Lambert et al. (2000). Briefly, lung tissue was excised and ~5 mg was cut and transferred to a 1.5 ml microcentrifuge tube containing TKM buffer. The cells were homogenized with a 200 µl filter-tip pipette; 400 µl TKM buffer and 37.5 µl SDS 10% were added and vortexed. The tube was incubated at 55° C from 5 min. Proteins were

precipitated by adding 200 µl of saturated NaCl, vortexing, and centrifugation for 5 min. An aliquot of the supernatant was transferred into a fresh microcentrifuge tube containing 900 µl 100% ethanol. The DNA was pelleted by centrifugation for 3 min and washed in 70% ethanol. Ethanol was removed and the DNA pellet was allowed to dry at room temperature for 3 min, then dissolved in 100 µl 10mM Tris HCl pH8 for 10 min at 55° C. DNA concentration was read using a Qubit (Life Technologies, Eugene, Oregon) following manufacturer's instructions.

Lung samples were analyzed for global methylation and hydroxymethylation using enzyme-linked immunosorbent assays (ELISAs) (Zymo Research, Irvine, California) with detection antibodies for 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC). Results were confirmed in a subset of samples using a selected reaction monitoring mass spectrometry (MS) method at Zymo Research Corp.

Sex-typing was performed using modified PCR methods from Clapcote & Roder (2005) employing the Jarid1F and Jarid1R primers, which bind to Jarid1c and Jarid1d, respectively, corresponding to the X and Y chromosomes. Electrophoresis was run using a Novex XCell SureLock system with the PowerEase500 as electrical source and detection using GreenGlo Safe DNA Dye (Dennville Scientific Inc., Holliston, Massachusetts). Gels were imaged using a LI-COR Oddysey Fc imaging system (LI-COR Biosciences, Lincoln, Nebraska). Upon analysis, if two bands were present, the animal was classified as male, whereas if only one band was present, the animal was determined to be female. Adult animals of known sex were used as controls (Figure 5).

Figure 5. DNA Gel Image from Electrophoresis to Determine Offspring Sex. Primers utilized include Jarid1F and Jarid1R correspond to regions on the X and Y chromosomes, respectively. Therefore, if a sample had two bands, it was determined to be a male and if only one band was found to be present, it was determined to be a female. Known male and female controls were run on each gel for consistency. From left to right: 50 bp ladder, M control, F control, F, F, M, F, M, M, M, M, F.

2.2.5 Statistical Analysis

Statistical analysis was run using SigmaPlot statistical software (Systat Software, Inc., San Jose, California). Groups were compared using a t-test and declared significantly different where $p \leq 0.05$. Where values did not pass normality testing, a Mann-Whitney Test was used instead.

2.3 Results

Following breeding, at least one litter per treatment group per strain was obtained with a minimum of four animals per group. Initial results using ELISA to detect global methylation and hydroxymethylation data indicated no differences between treatment groups (Figure 6 A&B). However, due to the large deviation in the methylation data, as well as biological basis of sex differences in methylation, we were interested in differentiating between male and female offspring. Following PCR-based sex-typing, we observed a reduced amount of global methylation in lung tissue from female offspring compared to males from the same treatment group (Figure 6C). Male and female levels of hydroxymethylation were not found to differ (Figure 6D).

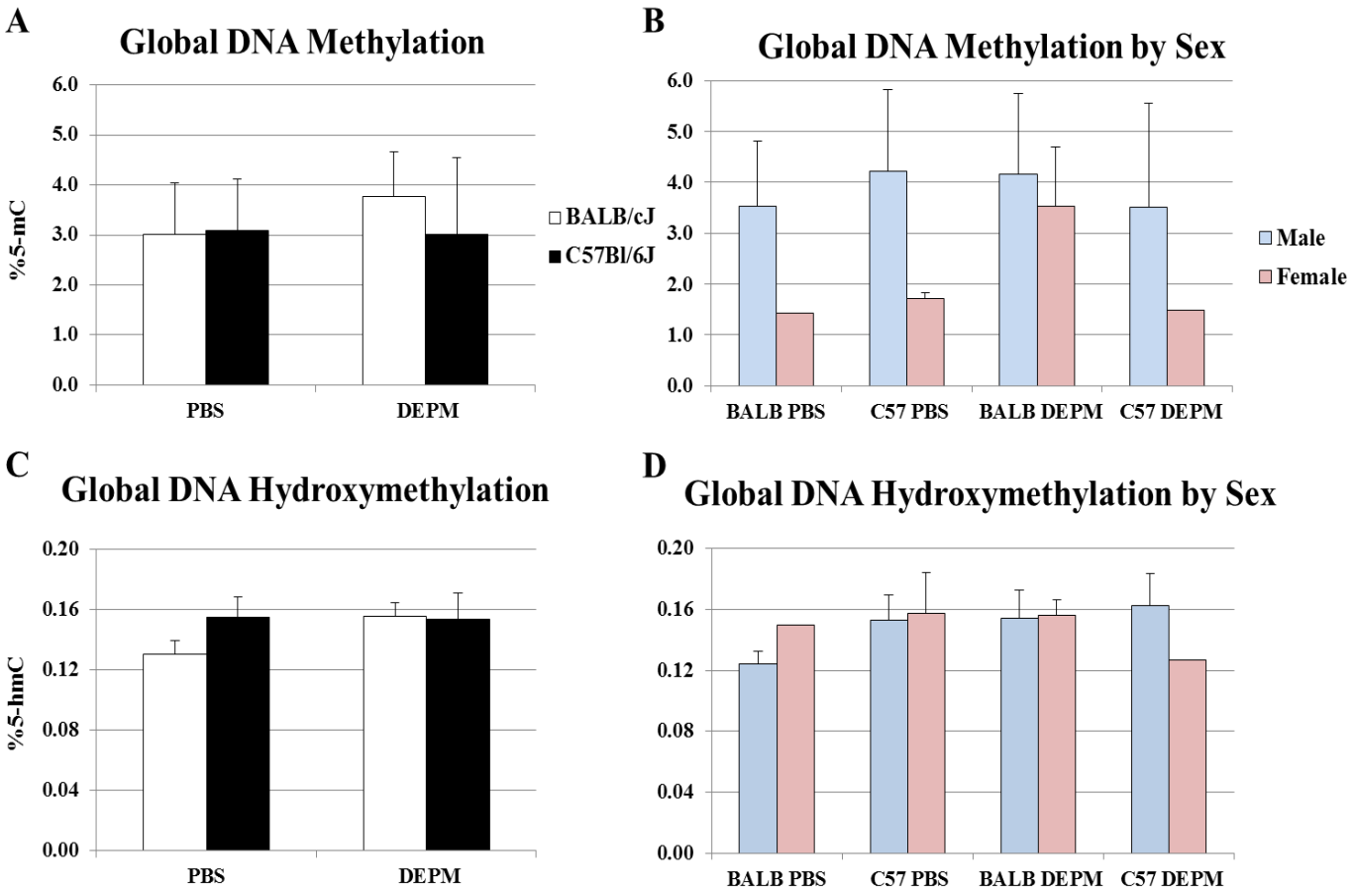


Figure 6. DNA Methylation and Hydroxymethylation Status in Offspring Lung Tissue. (A) DNA methylation data as analyzed by ELISA, using detection antibodies for 5-methylcytosine (5-mC), separated by strain and (B) sex. (C) DNA hydroxymethylation data as analyzed by ELISA, using 5-hydroxymethylcytosine (5-hmC) antibodies, separated by strain and (D) sex. Shown as mean +/- standard error.

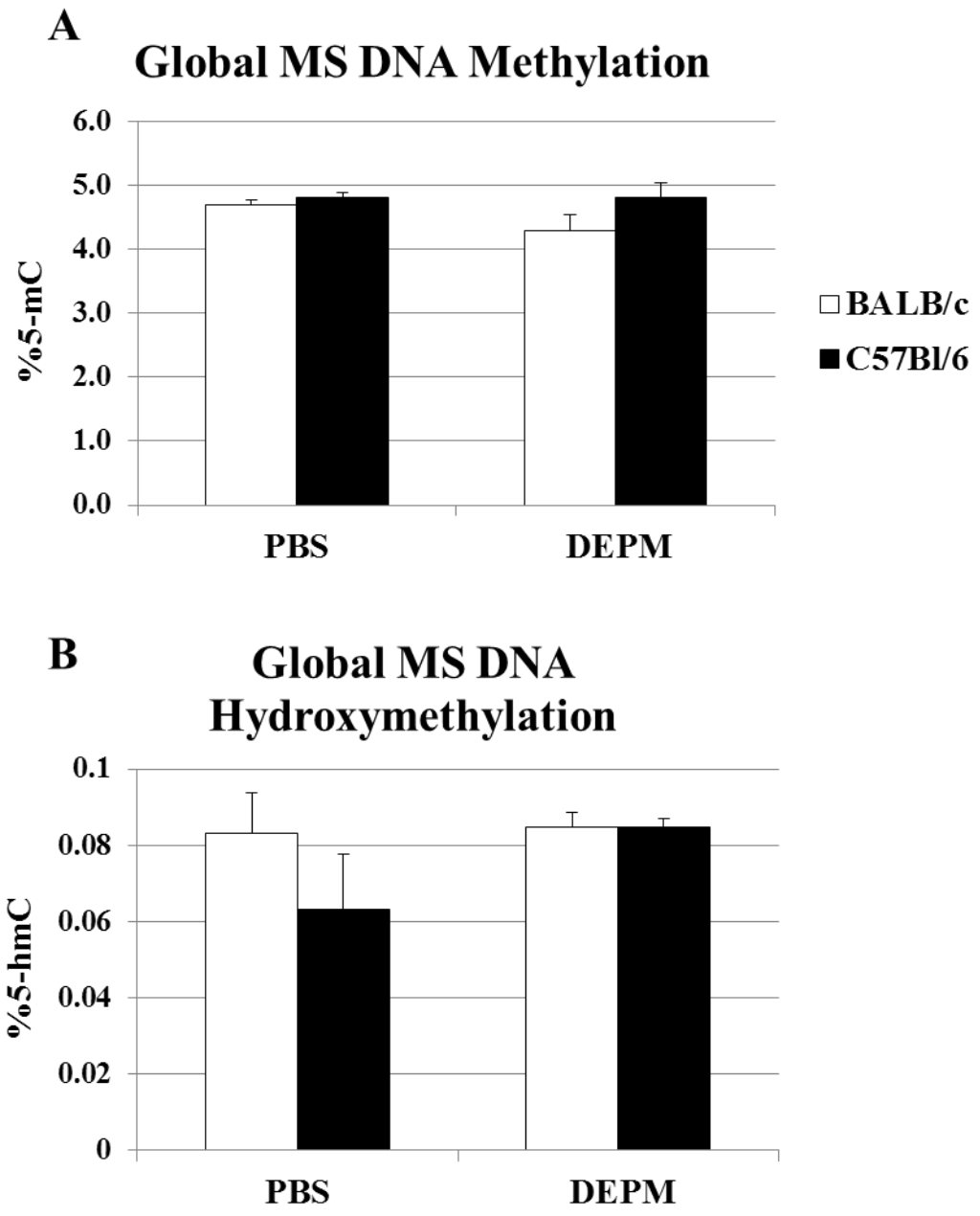


Figure 7. Global Percent Methylation and Hydroxymethylation Mass Spectrometry Data. A subset of samples were sent to Zymo Research Corp. to measure total 5-mC or 5-hmC using a selected reaction monitoring (SRM)-based mass spectrometry method. (A) Percent global methylation (5-mC) and (B) hydroxymethylation (5-hmC) shown as mean +/- standard error.

A subset of samples were sent to Zymo Research Corp. to measure total methylation (5-mC) or hydroxymethylation (5-hmC) using a highly quantitative mass spectrometry method. Average 5-mC and 5-hmC levels within this subset were not significantly different between treatment groups; however, C57Bl/6J mice prenatally exposed to DEPM had increased trend of percent global hydroxymethylation compared to PBS controls whereas BALB/c mice prenatally exposed to DEPM had decreased trend of percent global methylation compared to PBS controls (Figure 7).

2.4 Discussion

Results from this pilot study indicate a possible strain- and sex-specific alteration in lung tissue DNA methylation following intrauterine DEPM exposure. Findings from our initial ELISA analysis demonstrated no significant differences in global methylation or hydroxymethylation. 5-hmC levels in BALB/c pups exposed to DEPM *in utero* appeared slightly higher (albeit non-significant). Notably, sex played a role in 5-mC levels of offspring lung tissue; however, this contrast was not as apparent in the 5-hmC data. In the subset sent for mass spectrometry analysis, we noted a decrease in 5-mC levels in DEPM-exposed BALB/c pups. Levels of 5-hmC were not significantly different in this group, and the trending increase in %5-hmC was not as evident. Our initial finding of decreased methylation following prenatal PM exposure is consistent with previous literature (Janssen et al. 2013) indicating a lower degree of placental global DNA methylation in association with particulate air pollution exposure in early pregnancy, measured in a large birth cohort.

Due to the difference in sensitivity between ELISA and MS methods, it is unsurprising that we might see differences in our data following MS analysis. Nevertheless, the ELISA methylation data provided important initial information indicating possible differences in global methylation based on offspring sex. Since pups were sacrificed at two days-of-age, visual sex determination, a method that can be extremely inaccurate at this early time point, was not utilized. Initial clustering of the methylation data into two distinct groups prompted us to determine offspring sex using a PCR-based method determining X and Y chromosomes. After data were analyzed by sex, clear differences were observed. Therefore, we recommend in future studies to account for offspring sex distributions in power calculations to ensure adequate numbers of males and females are available for analysis.

A major limitation of our current experiment was the lack of robust numbers of offspring to evaluate due to the loss of pregnancies in dams exposed throughout gestation. Since we lost many time-mated pregnancies in both the DEPM and control groups, we hypothesized the use of anesthesia itself for dosing may have impacted offspring outcomes. Moreover, this approach of dosing via an inhaled bolus of DEPM represents an unrealistic human exposure scenario. Therefore, results from this pilot study helped to inform our future studies by removing maternal anesthesia and utilizing a more representative inhalation exposure during gestation rather than the intranasal dosing method.

In summary, findings from this preliminary study have important value since data reveal the impact of sex and strain on DNA methylation, an important epigenetic

modification. Furthermore, observed effects in BALB/c mice suggest epigenetic vulnerability in this strain, which has been shown to be at increased risk of allergic asthma development following postnatal DEPM exposure compared with C57Bl/6 mice (Li et al. 2009). These data support future research focused on the linkage between genetic differences in PM-induced oxidative stress responses and variable epigenetic changes that may reprogram offspring lung and immune system development. Future studies should also focus on gene-specific methylation patterns in lung tissue associated with *in utero* particulate exposure. The opportunity to probe these differences across strain, sex, and even tissue type using our prenatal exposure model therefore supports its role as a platform to inform translation studies reporting offspring DNA methylation changes in surrogate tissues, such as placenta or circulating blood cells.

3. STRAIN-DEPENDENT INFLAMMATORY RESPONSES TO HOUSE DUST MITE IN AN IMPROVED MOUSE MODEL OF PRENATAL PARTICULATE MATTER EXPOSURE

3.1 Introduction

Maternal exposure to air pollution during pregnancy has been associated with a significantly increased risk and severity of asthma in offspring. Asthma affects more children than any other chronic disease. While it is widely recognized that early life exposure to particulate matter (PM) air pollution plays a role in asthma pathogenesis, the mechanisms are not fully understood. Data support genetic background (both gender and ethnicity) influence childhood asthma incidence. During childhood, males are more likely to have asthma than females; however, after puberty, this trend switches to a higher prevalence of asthma in adult females (CDC, Center for Disease Control and Prevention 2015). In regards to interactions between environmental factors and gender-specific risk, epidemiologic evidence shows timing of air pollution exposure during pregnancy influences asthma outcomes differently in boys and girls (Hsu et al. 2015). In addition to gender, different ethnic groups have different asthma prevalence rates. For instance, in 2014, the reported incidence of childhood asthma among white non-Hispanics was 7.6% whereas for those of Puerto Rican descent, had the highest incidence of 16.5% (CDC, Center for Disease Control and Prevention 2015). Studies investigating genetic and environmental factors, which may explain these disparities, are relatively unexplored, particularly in experimental models that have the additional

benefit discerning underlying mechanisms. Thus, the need for an animal model to better understand the gaps in knowledge regarding asthma development following prenatal exposure to air pollution is clear.

The few animal exposure models developed to date have relied on diesel exhaust PM as the primary maternal exposure. Although diesel exhaust is a major PM component of traffic-related air pollutants, expecting mothers are routinely exposed to complex PM mixtures of air pollutants from industrial sources and gasoline-powered vehicle emissions. Results from our preliminary study also indicated an adverse effect of maternal anesthesia (often employed during intranasal DEPM dosing) on offspring viability. Therefore, the main goal of this objective was to develop a model of inhalation exposure to air pollution during pregnancy creating aerosolized PM mixtures more representative of human exposures. The establishment of this model will provide a platform to explore the mechanisms underlying prenatal air pollution exposure and asthma susceptibility. To probe respiratory and inflammatory responses impacted by prenatal PM exposure, we incorporated offspring postnatal allergen challenge using house dust mite, a relevant human allergen. Based on findings from our pilot work and previous adult exposure models (Raemdonck et al. 2016; Tomlinson et al. 2010), we repeated our approach comparing BALB/c and C57Bl/6 strains, to determine the phenotypic differences across varying genetic backgrounds.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Diesel Particulate Matter Standard Reference Material (SRM 2975) was purchased from the National Institute of Standards and Technology (Gaithersburg, Maryland). House Dust Mite (*Dermatophagoides farina*, Lot 220744) greater than 99% purity was purchased from GREER (Lenoir, NC). Phosphate Buffered Saline was purchased from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals and reagents used were obtained commercially at the highest available purity.

3.2.2 Animals

Male and female C57Bl/6J and BALB/cJ 8-week-old mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice were housed in a climate controlled room with 12/12h light/dark cycle. Females received 9% fat breeder diet (Harlan, Indianapolis, Indiana) while males received standard rodent chow (4%) (LabDiet, St. Louis, Missouri). Food and water were provided *ad libitum*. Breeder male weights were taken prior to first breeding, and breeder female weights were monitored daily throughout the study period. Mice were time-mated; the presence of a vaginal plug defined gestational day (GD) 0. The experimental protocol was reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (IACUC# 2015-0279).

3.2.3 Study Design and Procedures for Maternal Inhalation Exposure

Beginning on GD-0, dams were placed into whole body exposure chambers and exposed to either a representative particulate matter pollutant mixture (PM) or filtered air (FA) for 6 hours/day until GD18 (Figure 8). Exposure chambers consisted of a 12" x 8" x 32" stainless steel box with 4 inner compartments and a ¼" clear cast acrylic lid (Figure 9A&B). The compact whole-body modular cages allowed for exposure of multiple animals, providing animal welfare while minimizing PM losses by deposition. The transparent top permitted direct observation of the animals during the exposure experiments, and the perforated cast acrylic removable floor suspended from the base of the pan maintained a clean environment for the animals. Air was continuously pumped through the chamber by stainless steel aerosol distribution lines attached to the lid and out return lines on the bottom. Particulate matter 2.5 (composition described in Figure 9C) was generated from an aqueous solution utilizing a commercial constant output atomizer (TSI 3076). PM was conditioned, a process involving water vapor removal in a multi-tube Nafion drier, organic vapor removal in a denuder filled with Spectrum XB-17 reactive adsorbent, and charge neutralization by a Po-210 bipolar diffusion charger. The concentration and size distribution of PM in the chambers were constantly monitored with a Scanning Mobility Particle Sizer (SMPS) system and adjusted by changing the corresponding PM dilution ratios at the source. The SMPS operates with a sheath flow of 6.5 liters per minute and a sample flow of 1 liter per minute. The mass concentration of accumulation mode PM was maintained near 100 $\mu g/m^3$ corresponding to a total number concentration of about 105 particles/cm^3 for 150 nm geometric mean diameter

particles. This concentration is frequently observed in Beijing and is about a factor of 10 higher than annual $PM_{2.5}$ average observed in Houston, Texas. Following exposure on GD 18, mice were removed to individual housing and allowed to deliver spontaneously. Maternal blood samples were collected via submandibular bleed in a subset of pregnant dams on GD18.

3.2.4 Offspring Allergen Challenge

Offspring were maintained on a 9% fat breeder diet (Harlan, Indianapolis, Indiana) and monitored daily. To determine offspring response to allergen challenge following prenatal exposure, we followed a well-characterized protocol entailing chronic dosing with house dust mite (HDM) (Saravia et al. 2014). Starting on postnatal day (PND) 3, pups were briefly anesthetized with 3% isofluorane in oxygen and exposed to either 10 µg HDM in 10 µL PBS or 10 µL PBS alone intranasally (5 µL in each nostril 30 seconds apart) on PNDs 3, 5, 7, 10, 12, and 14 (Figure 8). On PNDs 17, 19, 21, 24, 26, and 28, pups were again briefly anesthetized and exposed to either 15 µg HDM in 15 µL PBS or 15 µL PBS alone intranasally (7.5 µL in each nostril 30 seconds apart). Animals were weighed and measured for length on each exposure day while still under light anesthesia. Absolute control groups were similarly bred, allowed to deliver spontaneously, and weighed and measured; however, neither dams nor pups received any experimental treatment.

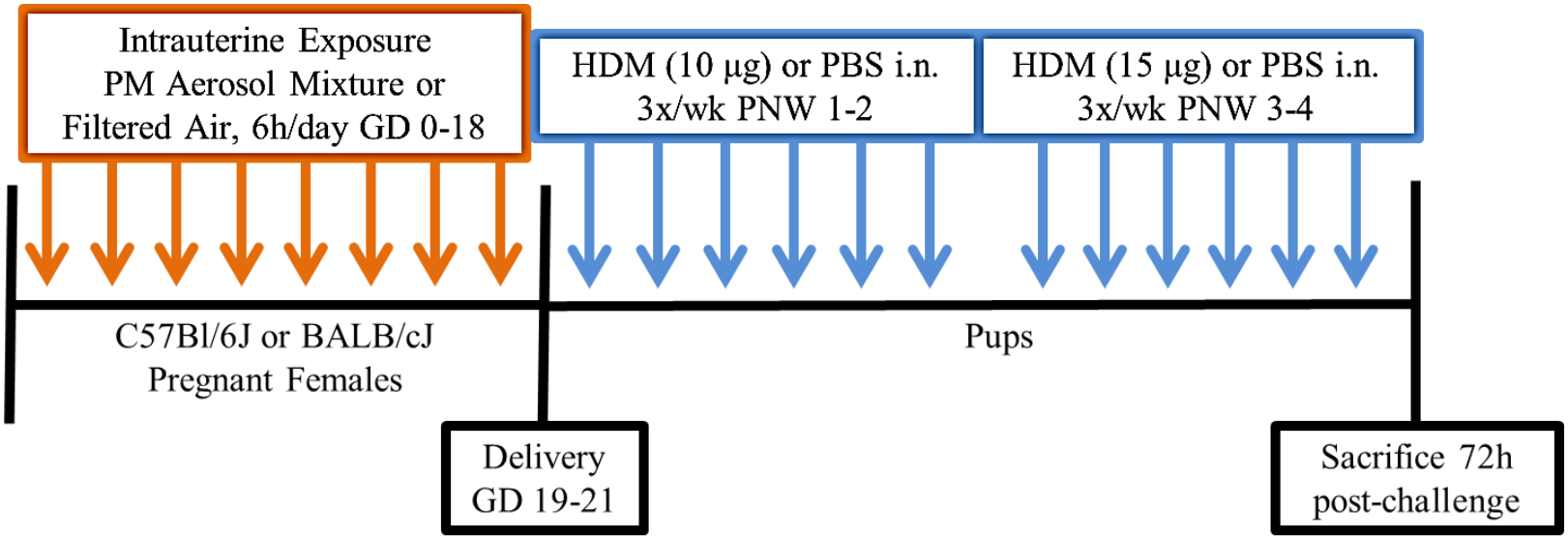


Figure 8. Experimental Protocol for Intrauterine PM Exposure and Early Postnatal Allergen Challenge in C57Bl/6 and BALB/c Mice. Maternal exposure was performed in custom-built rodent inhalation chambers. PM concentrations averaged 101.94 µg/m3 from gestational day (GD) 0-18. Following birth, during postnatal weeks (PNW) 1-4, we challenged offspring intranasally (i.n.) with house dust mite extract (Greer Labs, Lenoir, NC). The house dust mite (HDM) model represents a clinically relevant allergen and widely accepted asthma model previously utilized in a neonatal mouse model.

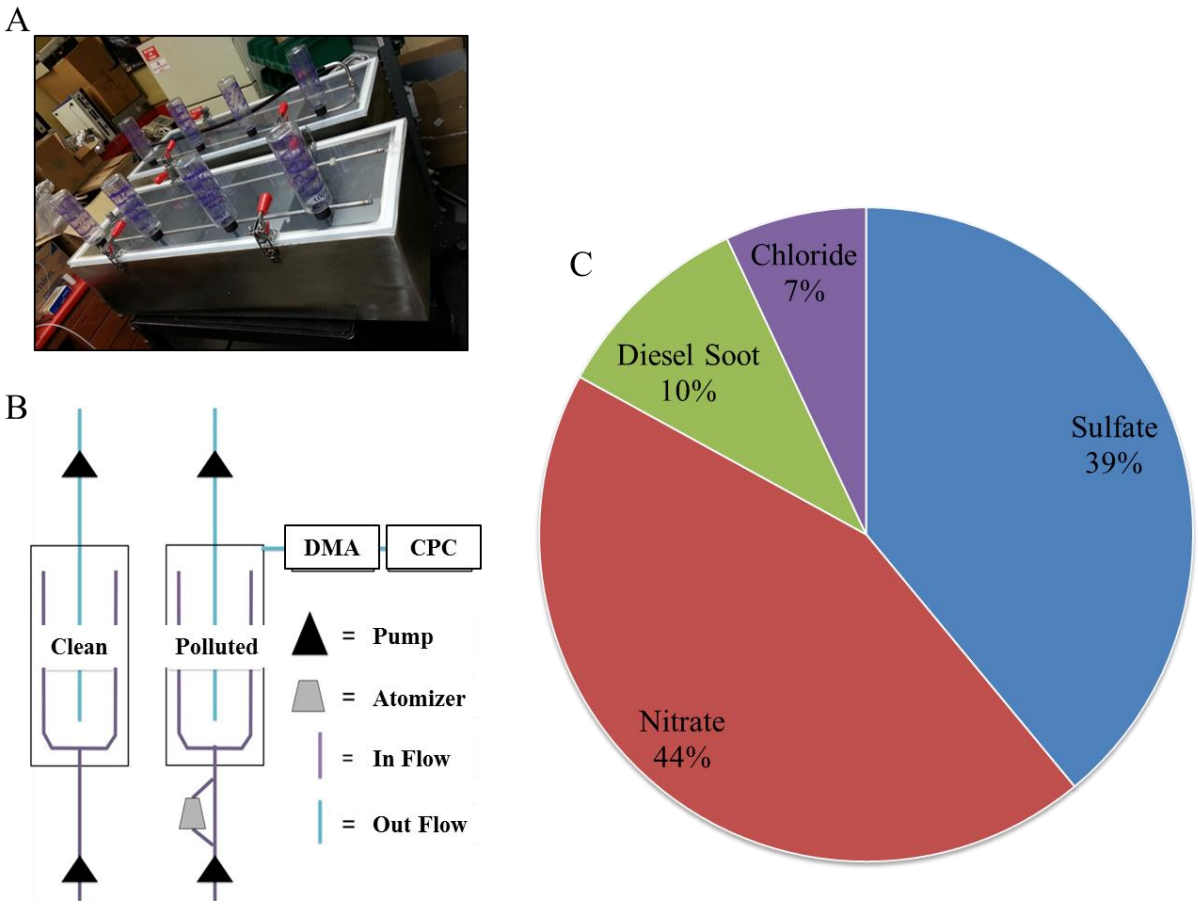


Figure 9. Inhalation Exposure to PM. (A) Photo of our exposure chambers. (B) Schematic of our exposure chambers and monitoring system. DMA (differential mobility analyzer); Condensation particle counter (CPC). (C) Particle chemical compositions represent mass compositions measured in polluted urban air. Nitrate and sulfate mass fractions, generated from ammonium nitrate and ammonium sulfate; chloride mass fraction generated from potassium chloride; diesel soot generated from diesel exhaust PM (NIST, SRM 2975).

At least 1 male and 1 female from each litter were sacrificed on PND31 (72 hours following the final allergen challenge). Animals were sacrificed and underwent a tracheotomy immediately following confirmation of death. Bronchoalveolar lavage (BAL) fluid was collected in EDTA tubes by tracheal cannulation and washing the lungs with 1.0 ml sterile PBS. BALF was kept on ice until analysis of airway inflammation. The left lung was then excised and inflated to a constant pressure of 25 cm with 10% formalin. Fixed lung tissue was then processed for routine histopathology. The right lung was immediately frozen in liquid nitrogen and stored at -80°C. Blood was collected into K_2EDTA microtainer tubes (Becton Dickson, Franklin Lakes, New Jersey), separated within 8 hours of collection and stored at -80°C until analysis. Additional tissues were weighed and collected for histology or saved for future molecular biology analyses. These included brain, nasal epithelia, olfactory mucosa, spleen, liver, kidney, gonads, gonadal fat pad, skeletal muscle, and long bones of both legs. A portion of the remaining offspring were weaned and shipped to the University of Tennessee Health Science Center to undergo airway hyperresponsiveness testing at 8 weeks of age. The remaining animals were allowed to mature to 13 weeks of age and sacrificed at that time.

3.2.5 Analyses

Maternal blood samples were collected in a subset of pregnant dams on GD18 following final exposure and serum cytokines were analyzed using a Milliplex MAP Mouse Cytokine/Chemokine Kit from Millipore (Millipore Corporation, Billerica, MA). The following cytokines were analyzed: eotaxin, G-CSF, GM-CSF, IFN, IL-1α, IL-1β,

IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF. Circulating cytokines and chemokines were also analyzed using this kit in serum from a subset of pups at the 4 week time point. 8-isoprostanes were analyzed in maternal serum by ELISA kit from Cayman Chemical (Ann Arbor, MI).

BAL samples were analyzed at the Texas Veterinary Medical Diagnostic Laboratory (TVMDL). Total number of cells in BAL fluid was counted with a hemocytometer. Differential counts of leukocytes were performed using cytospin smear slides by technicians blinded to treatment group. Cells were classified as lymphocytes, macrophages, eosinophils, or neutrophils based on standard morphology.

For measurement of airway hyperresponsiveness, mice were anesthetized and evaluated using FlexiVent forced oscillation maneuvers (Scireq, Montreal, QC, Canada) as previously reported (Saravia et al. 2014). Resistance was measured in response to increasing doses of methacholine (0, 12.5, 25, and 50 mg/mL).

3.2.6 Statistical Analysis

Statistical analysis was run using SigmaPlot statistical software (Systat Software, Inc., San Jose, California). Groups were compared using a t-test and declared significantly different where $p \leq 0.05$. Where values did not pass normality testing, a Mann-Whitney Rank Sum Test was used.

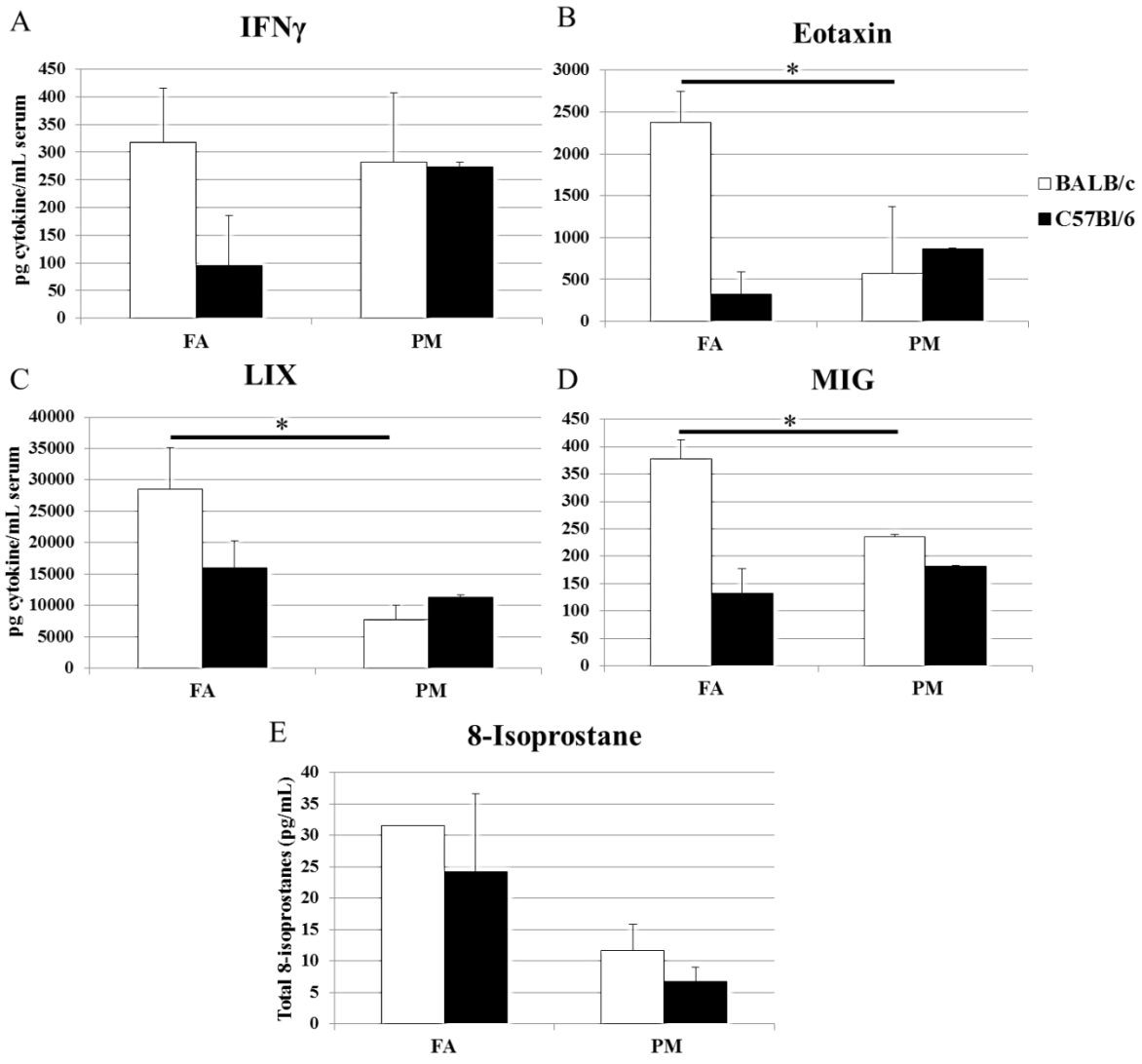


Figure 10. Maternal Serum Cytokines. (A) IFNγ, (B) Eotaxin, (C) LIX aka CXCL5, and (D) MIG aka CXCL9 analyzed by multiplex cytokine panel, (E) 8-Isoprostane analyzed by ELISA kit. Values shown as mean +/- standard error *$p<0.05$.

3.3 Results

Final maternal exposure values had a mean of 101.94 μg/m^3 with a standard error of 0.0784. This corresponds to a mean 24-hour daily dose of 25 μg/m^3. Maternal weight gain during exposure was not significantly different between treatments. In addition, litter sizes were not significantly different between treatment groups. The following cytokines were detectable in maternal serum samples: eotaxin, G-CSF, IFN, IL-1a, IL-5, IP-10, LIX and MIG. An increasing trend is seen for IFNγ in C57Bl/6 mice prenatally exposed to PM (Figure 10A). Interestingly, eotaxin, an eosinophil recruitment factor, was significantly decreased in BALB/c dams exposed to PM compared to FA controls (Figure 10B). LIX, i.e., CXCL5, that acts as a chemoattractant for neutrophils, and MIG, i.e., CXCL9, that acts as a T cell chemoattractant, were also significantly decreased in BALB/c mice exposed to PM compared to FA controls (Figure 10C&D). Since maternal serum was collected immediately following long-term chronic exposure, these alterations could point to the reduced reactivity of the immune system in PM-exposed animals. This reduction in inflammatory response is reiterated in the maternal serum 8-isoprostane data which also shows a decreasing trend in both strains of the systemic oxidative stress marker (Figure 10E).

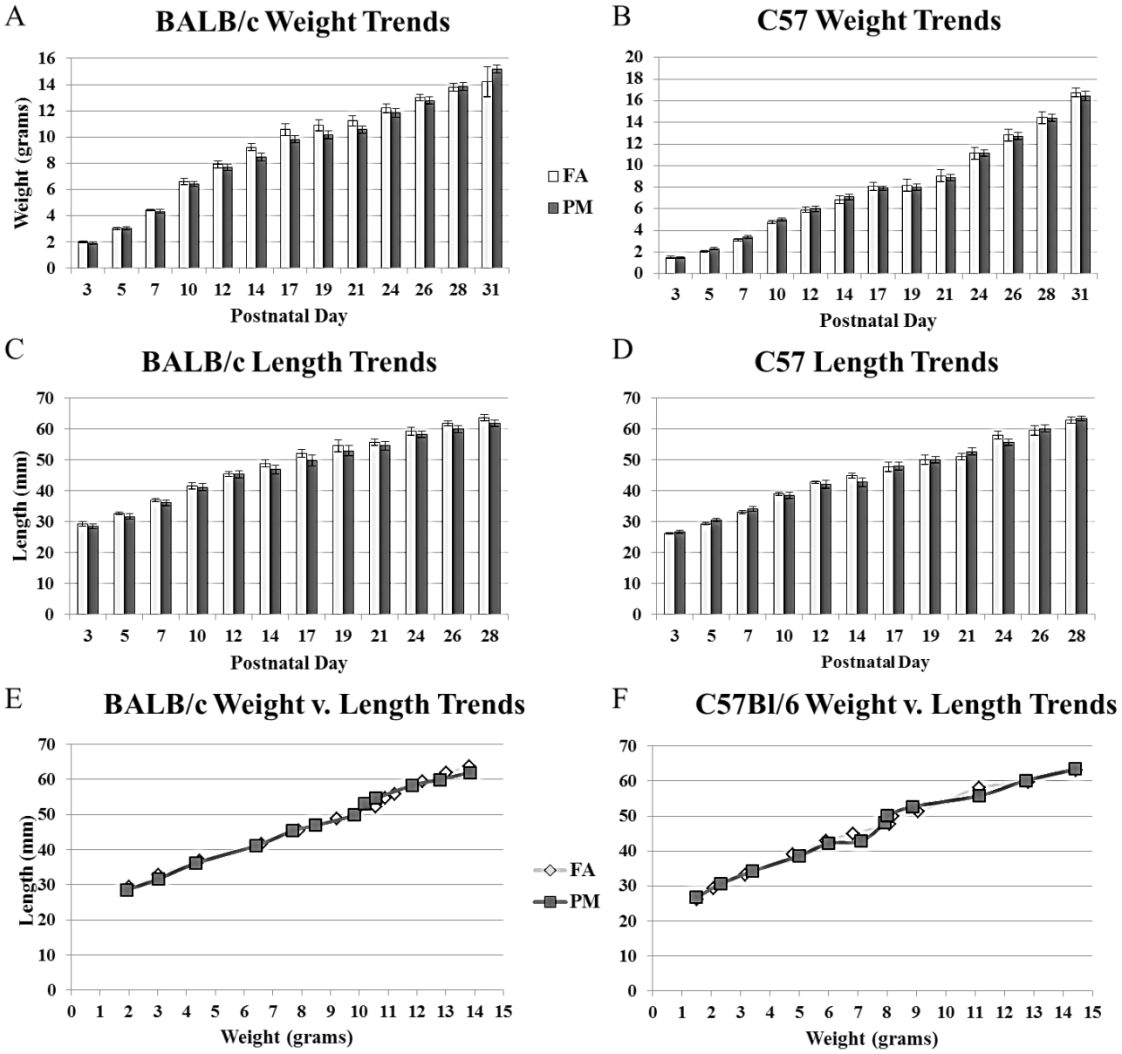


Figure 11. Weight and Length Trends. (A) BALB/c and (B) C57Bl/6 weights and (C) BALB/c and (D) C57Bl/6 lengths from birth until sacrifice (PND31). Plotted as weight v. length trends for (E) BALB/c and (F) C57Bl/6 by maternal treatment group. Values shown as mean +/- standard error.

Exposures in the current study were not sufficient to induce pup weight or length alterations between treatment groups in either BALB/c or C57Bl/6 mice (Figure 11). Normalized organ weights revealed differences between filtered air and particulate matter-exposed C57Bl/6 heart weights, particularly in female pups and in male testes (Figure 12). There is some indication in the literature that exposure to diesel exhaust *in utero* may have detrimental effects on male gonadal development (Takeda et al. 2004). We await histology results to determine whether there is a morphological basis for this difference in normalized organ weights.

Although no overt birth size outcomes were detected in the current study, responses in the lung following postnatal allergen challenge were marked. Airway hyperresponsiveness (AHR) to methacholine was significantly increased in BALB/c mice exposed to house dust mite vs. PBS ($p=0.0357$) (Figure 13). We lacked the statistical power to determine difference between PM and FA prenatal exposure. C57Bl/6 offspring challenged with HDM did not exhibit increased airway resistance compared to the PBS control group. This reiterates strain differences in asthma susceptibility demonstrated in other asthma models (Li et al. 2009).

White blood cells recovered in the BAL were consistently increased in offspring from the FA-HDM group compared to FA-PBS groups in both strains. This indicated the ability of HDM to produce airway inflammation. We originally hypothesized we would see an increased inflammatory response in HDM-challenged offspring exposed *in utero* to PM; however, we consistently observed a reduction in airway inflammation, based on total cell counts in BAL in the PM-HDM groups. (Figure 14). We did not observe

differences in PM-PBS vs. PM-HDM in either strain, whereas, FA-HDM BAL cell counts were significantly increased in C57BL/6 mice (vs. FA-PBS) ($p=0.005$). 6 weeks after the final allergen challenge, a subset of remaining animals were sacrificed to assess inflammatory response maintenance. White blood cell infiltrate in the BAL decreased in all treatment groups (Figure 15 A) and differential cell counts indicated minimal levels of neutrophils, lymphocytes, and eosinophils (Figure 15 B&C).

Systemic immune response was measured by quantification of cytokines in pup serum collected at the time of sacrifice (4 weeks-of-age). In BALB/c mice, animals exposed to PM-HDM had significantly higher levels of IFNγ, IL-1β, IL-4, and IL-5 compared to the PM-PBS group (Figure 16 A, B, D & E). Conversely, PM-HDM-treated mice had significantly lower levels of circulating IL-3 compared to the PM-PBS group (Figure 16 C). Significant differences were not observed between BALB/c mice exposed to FA-HDM vs. FA-PBS. Additionally, there were no significant differences in these cytokine levels in C57Bl/6 mice, although a similar trend in was noted in increased IL-4 and IL-5 levels and decreased IL-3 levels in the PM-HDM-treated mice. Significant changes in cytokine levels in C57Bl/6 mice included reduction in systemic IL-9 and MIP-1α (i.e., CCL3) in PM-HDM mice compared to PM-PBS controls (Figure 16 F & I). In comparison to FA-HDM-treated mice, PM-exposed-HDM-treated mice had significantly higher IL-10 levels, a cytokine thought to suppress Th1 type immune response, and significantly lower MCP-1 (i.e., CCL2) levels (Figure 16 G & H).

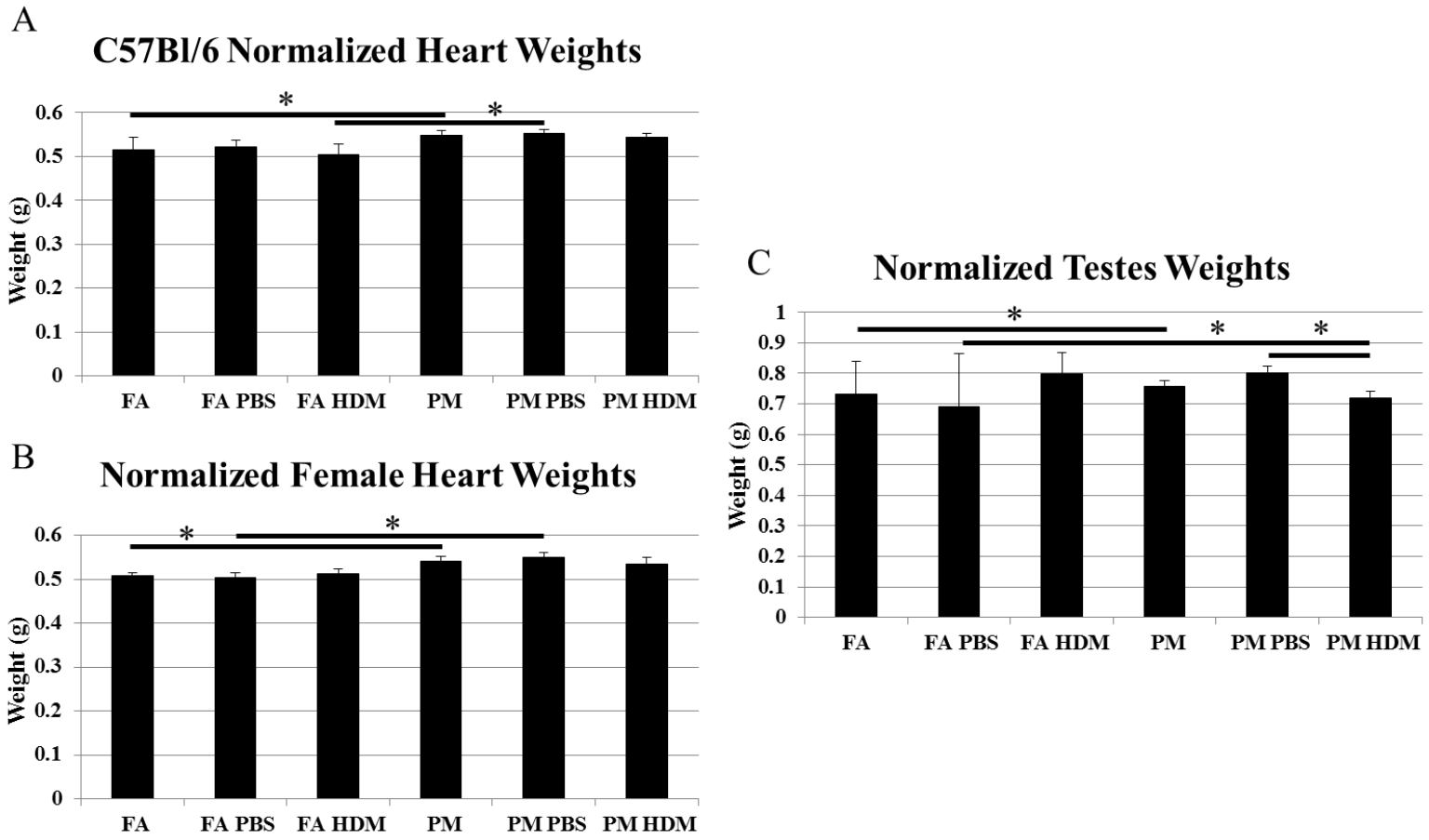


Figure 12. Normalized C57Bl/6 Selected Organ Weights. (A) Normalized heart weights by treatment and (B) sex, (C) and normalized testes weights. Values shown as mean +/- standard error. *$p<0.05$.

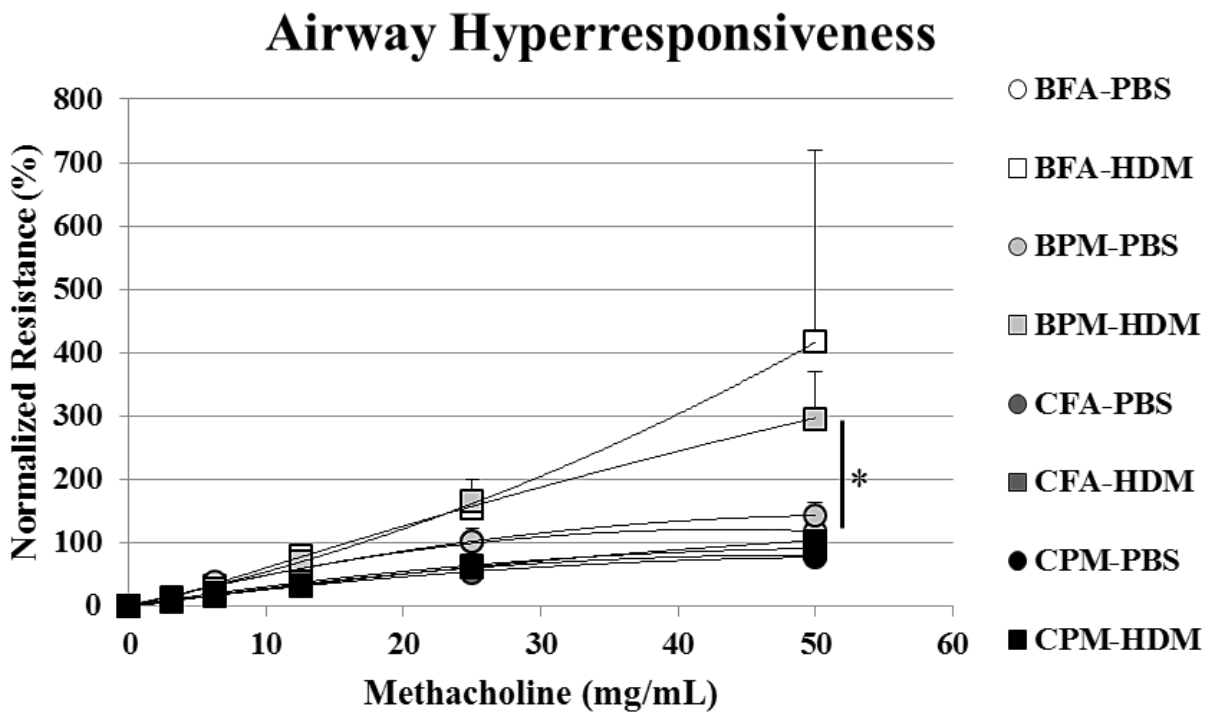


Figure 13. Airway Hyperresponsiveness to Increasing Levels of Methacholine. Resistance was evaluated using FlexiVent forced oscillation maneuvers (Scireq, Montreal, QC, Canada) in response to the following doses of methacholine: 0, 12.5, 25, and 50 mg/mL. Values shown as mean +/- standard error. *$p<0.05$ (BFA-PBS v. BFA-HDM $p=0.0357$).

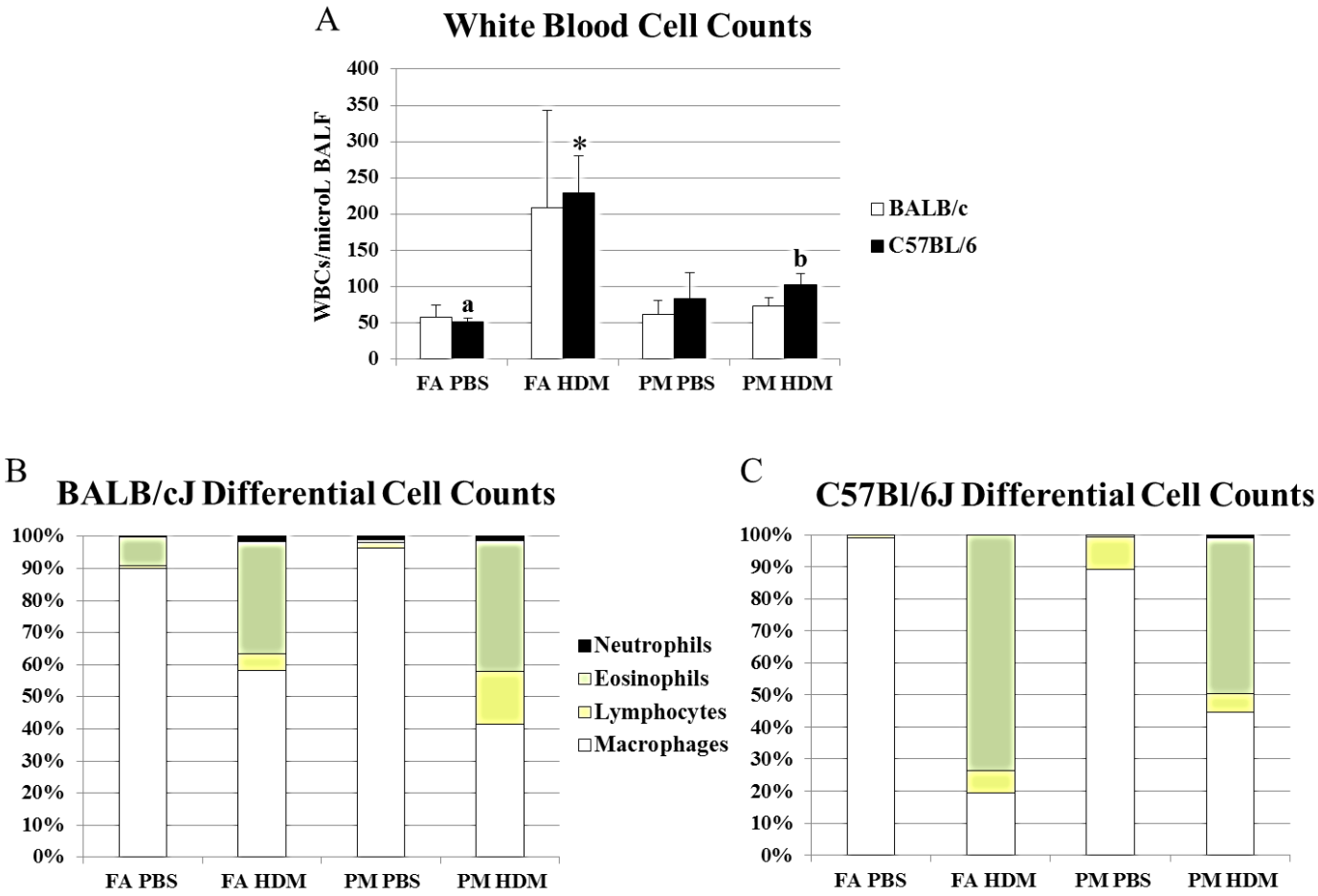


Figure 14. 4 Week Bronchoalveolar Lavage Cell Counts. (A) White blood cell counts (amount per µL BALF), and differential cell counts in (B) BALB/c and (C) C57Bl/6 treatment groups. Values shown as mean +/- standard error. "a" indicates significant difference from "b" where $p<0.05$ using a Mann-Whitney rank sum test. *indicates significant difference from all other groups within strain.

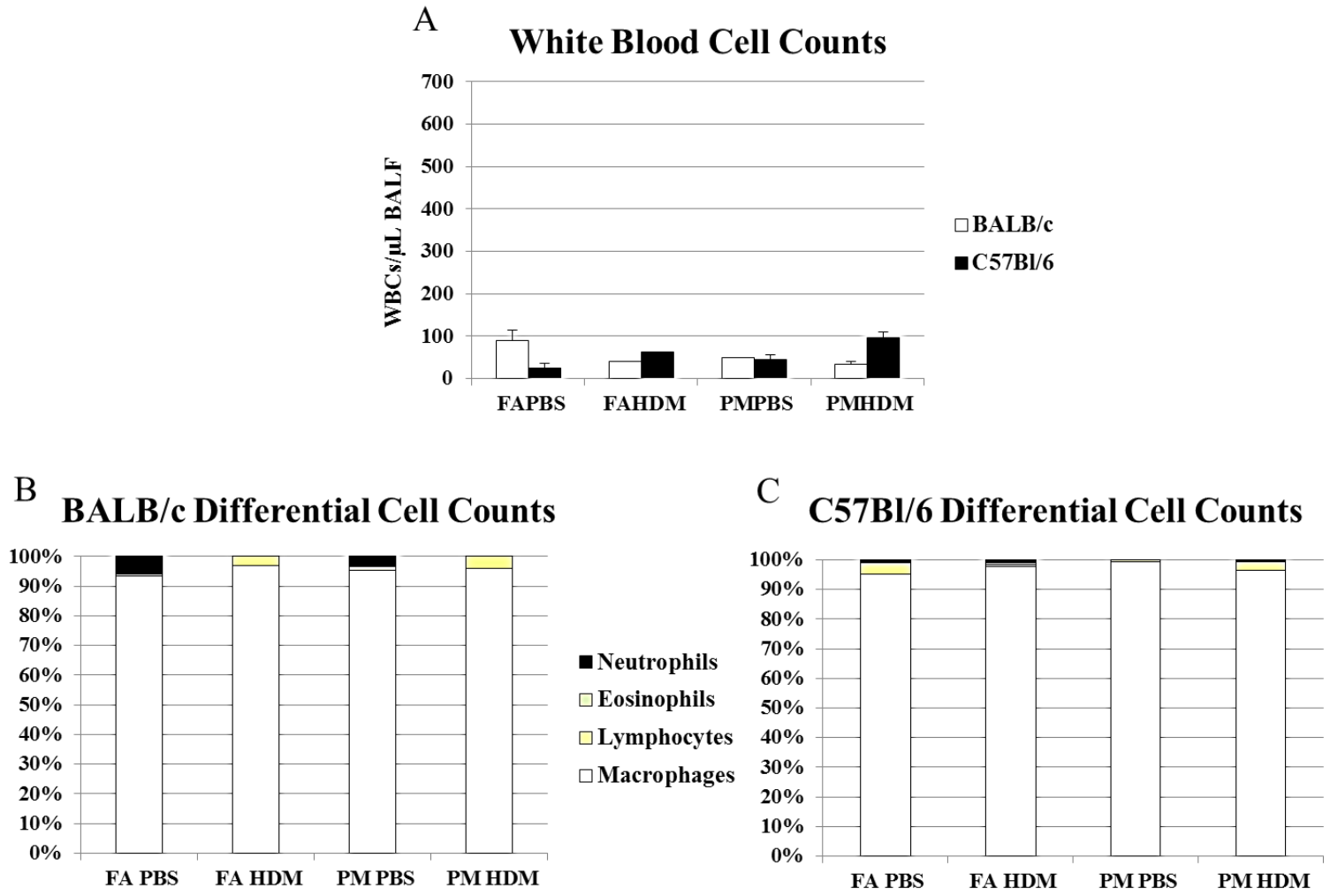


Figure 15. 12 Week Bronchoalveolar Lavage Cell Counts. (A) White blood cell counts (amount per µL BALF), and differential cell counts in (B) BALB/c and (C) C57Bl/6 treatment groups. Values shown as mean +/- standard error.

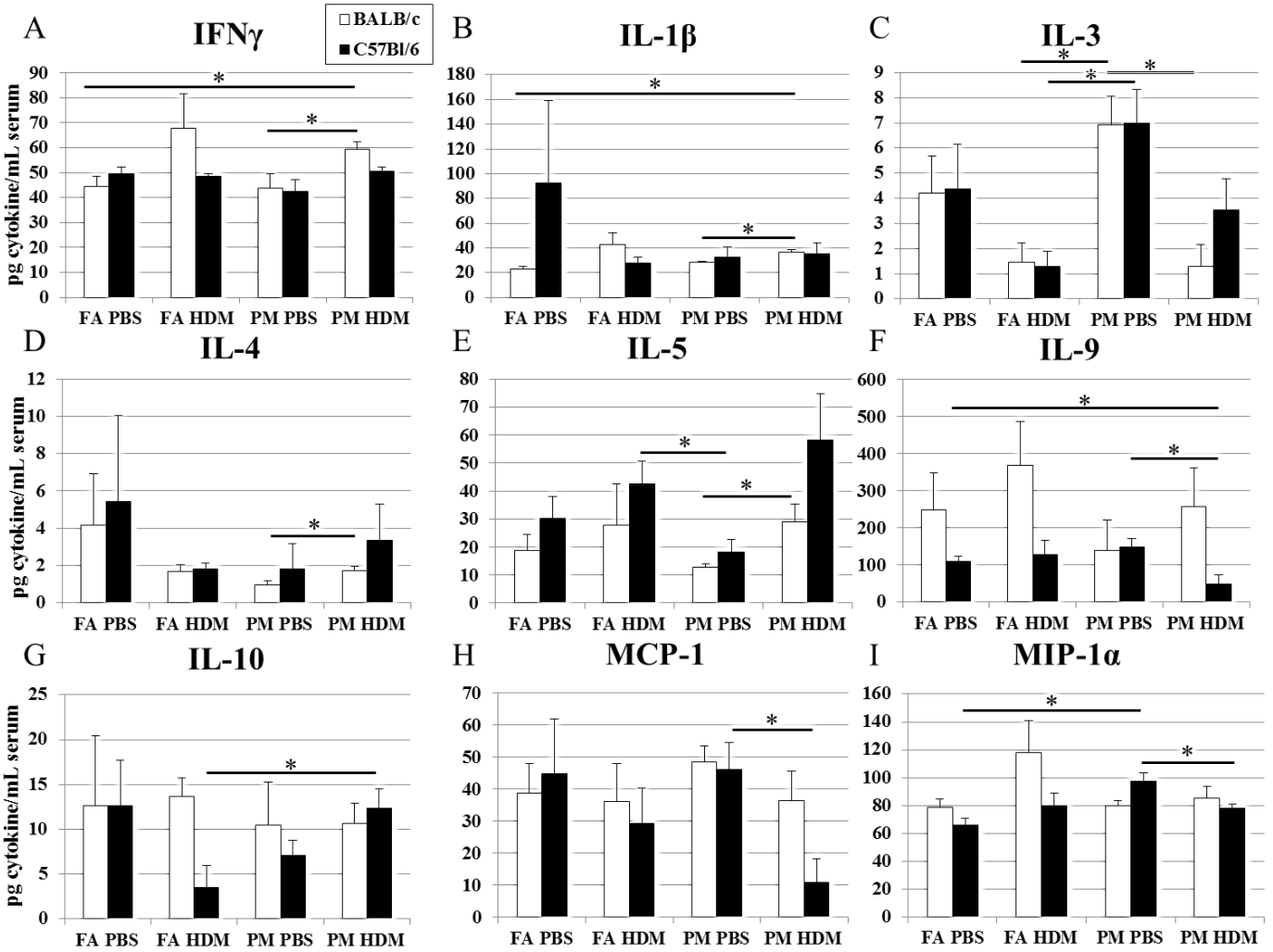


Figure 16. Offspring Serum Cytokines. (A) IFNγ, (B) IL-1β, (C) IL-3, (D) IL-4, (E) IL-5, (F) IL-9, (G) IL-10, (H) MCP-1, (I) M-CSF, and (J) MIP-1α analyzed by multiplex cytokine panel. Values shown as mean +/- standard error. *p<0.05.

3.4 Discussion

Collectively, data from our novel prenatal exposure model show offspring exposed to PM *in utero* and challenged with HDM do not develop as robust airway inflammatory response compared to filtered air-HDM exposed mice. This indicates an early immunosuppressive environment in the lung. Although contrary to other mouse exposure models using intranasal DEPM exposure with offspring ovalbumin challenge, these data are consistent with previous data from a complementary model of PM-HDM exposed infant mice (Saravia et al. 2014). Saravia et al. demonstrated a mechanism where PM exposure during the neonatal period leads to a dampened immune response to house dust mite by increasing regulatory T cells. Since mouse lung and immune system development occurs throughout gestation as well as the early postnatal period, we anticipate immune suppression may act through similar mechanisms.

Inflammation levels in the lungs of our mice reduced after 6 weeks of no exposure. This effect is likely the result of the timing of exposures in this model. Pollutants were administered throughout pregnancy and allergen was administered immediately following birth which in the mouse is equivalent to the third trimester of human gestation. Based on previous literature, we conjecture that a repeat allergen exposure at a later time point would reveal an exacerbated response in older offspring. Upon rechallenge, almost 7 weeks after initial allergen exposure, Saravia et al. (2014) observed significant elevation in allergic response including increased Th2, Th17 and Treg cells. Animals also displayed AHR and increased peribronchial inflammation after adult allergen exposure.

In systemic inflammation data from BALB/c mice, we observed indications of increased inflammation, decreased T cell growth and differentiation and increase in Th2 cell differentiation markers in animals exposed to both PM and HDM. This tracks with information from previous studies but does not explain the observed immunosuppression. In C57Bl/6 systemic cytokines, messages are more mixed. Observations indicate reduction in T cell growth and differentiation cytokine IL-3 but increase in IL-5, a typical Th2 cytokine, in animals exposed to HDM. IL-9 is thought to be a factor in bronchial hyperresponsiveness, however, we see decreased IL-9 in PM HDM groups and no significant differences in AHR. IL-10 is elevated in animals exposed to both PM and HDM compared to those only exposed to HDM, indicating a potential suppression of Th1 cell differentiation in these animals. CCL2 and CCL3 were decreased systemically following HDM exposure in animals prenatally exposed to PM. Due to the fact that these are chemokines, the systemic levels may not adequately reflect a gradient if one exists in this system.

Differences in our model compared to findings of other studies may be due to timing of pollutant exposure during what is estimated to be equivalent to the human 1st and 2nd trimester periods and allergen exposure during early neonatal development. An asset to the current model includes the purity of our house dust mite allergen of greater than 99% with minimal to no lipopolysaccharide or beta-glucan contamination, and therefore no risk of adjuvant-type activity from unwanted constituents. Time of sacrifice at an early pre-pubertal life stage may also have impacted the observed outcomes. A potential window of susceptibility to upper respiratory infection may have been revealed

by this investigation based on the immunosuppressive effect elicited by prenatal exposure to PM. Histological analysis will aid in further characterization of these effects.

In summary, our model provides a novel platform to explore mechanisms of immunosuppression and perhaps delayed asthma susceptibility over time and across strain. Innovative aspects of this model include inhalation exposure to a relevant mixture of air pollutants at a level commonly observed in highly populous cities and postnatal exposure to the germane allergen, highly purified house dust mite, in two strains of mice with uniquely developed immune response phenotypes. Using this model, we can probe population differences on a larger scale or delve into the mechanistic aspects of exposure in a single strain.

4. PRENATAL EXPOSURE TO PARTICULATE MATTER: PRELIMINARY EXPOSURE ASSESSMENT IN SOUTH TEXAS POPULATION AT HIGH RISK FOR ASTHMA

4.1 Introduction

In this study, we characterized the personal exposure to fine particulate matter ($PM_{2.5}$) and polycyclic aromatic hydrocarbons (PAHs) in pregnant women in Hidalgo County, where childhood asthma prevalence and hospitalization rates are among the highest in the state of Texas. In Texas Public Health Region 11, which encompasses the lower Rio Grande Valley, including Hidalgo County, current childhood asthma prevalence rates are reported at 8.6% compared to the overall rate in Texas, which is 7.8% (Wickerham and Bhakta 2013). Additionally, in a recent report, the age-adjusted hospitalization rates due to asthma were significantly higher in Hidalgo County than all of Texas (Wickerham 2014). The contribution of particulate air pollution on asthma exacerbation and development in children has yet to be investigated in this high-risk population, despite the known links between air pollution and asthma.

Hidalgo County, located on the Texas-Mexico border, has a population of 842,300 and a population density of 190/km² (Census Bureau 2016). Typically, sparsely populated regions are thought to be less polluted; however, the McAllen-Edinburg-Mission region, the most populated region in Hidalgo County, is one of the fastest-growing counties in the U.S., with a 19% increase in just 5 years (Zuniga et al. 2011). Residents of Hidalgo County primarily live in urban settings (93%), and the poverty rate

(41%) is 2.3 times the estimated statewide poverty rate (Zuniga et al. 2011). In order to determine if prenatal exposure to air pollution may be a risk factor for asthma development in children, our main goal was to characterize particulate air pollution exposure in pregnant women living in this region.

Techniques to characterize air pollution exposure in human epidemiologic studies have relied upon various methods including: 1) personal monitoring, 2) regional monitoring (i.e., local ambient air monitoring), and 3) modeling exposure (e.g., through land use regression models or more recently geographic information systems (GIS) models), which may incorporate ambient air monitoring networks to estimate individual exposure. The preeminent standard for quantifying individual-level exposure is through personal monitoring, utilizing personal environmental monitoring samplers (PEMS); however, these monitors can be costly to use and maintain and bulky for participants to carry around. In the case of measuring exposure during important time periods, such as pregnancy, personal monitoring over short durations provides high spatial accuracy but lacks temporal coverage to quantify exposures over time. Thus, validation of modeling techniques is important in achieving a balance between accuracy and adequate coverage.

A few studies have compared personal monitoring data obtained from PEMS to modeled exposures. Hannam et al. (2013) compared personal exposure to nitrogen oxides (NOx), using personal air monitors worn in the breathing zone, to multiple exposure estimation techniques, including land use regression, kriging, and inverse distance weighted models. Findings indicated that monthly adjusted exposure interpolation techniques incorporating modeled background concentrations and inverse

distance weighting were most correlated with actual measured exposures. Reported r values were still only around 0.6 showing that even these sophisticated models have difficulty predicting true exposure values. Jedrychowski et al. (2005; 2010; 2015) have reported multiple adverse respiratory outcomes in children exposed to air pollution *in utero*. Using personal monitoring employing PEMS to specifically quantify maternal PAH exposure in a prospective birth cohort in Krakow, Poland, this research correlated prenatal PAH exposure with increased susceptibility to respiratory infection in infants (Jedrychowski et al. 2005) and reductions in lung function in childhood (Jedrychowski et al. 2010). Utilizing PEMS data from this cohort, Choi et al. (2008) compared personal measurements to modeled exposures developed from indoor and outdoor ambient monitors and personal questionnaires. Modeled results showed high precision and validity for individual-level exposure. Although this approach may reduce the need for personal monitoring, it requires adequate indoor and outdoor monitoring and may not be generalizable to other exposure settings.

In Hidalgo County, ambient fine particulate matter air pollution ($PM_{2.5}$) is measured at one site in the McAllen-Edinburg-Mission metropolitan area using a Continuous Ambient Monitoring Station (CAMS). Between 2010 and 2015, the annual $PM_{2.5}$ concentration ranged between 9.6 and 11.1 $\mu g/m^3$, with only 2 days in the 5 years exceeding the 24-hour $PM_{2.5}$ standard of 35 $\mu g/m^3$ (EPA July 2016; TCEQ 2016). Due to this sparse monitoring network and since indoor sources may also contribute to PM exposure, our approach was to investigate maternal PM exposure in Hidalgo County using personal monitoring (i.e., PEMS). This approach allowed us to quantify $PM_{2.5}$

exposure, make comparisons to the existing CAMS station, and importantly, quantify exposure to PAHs, known toxic constituents of PM.

4.2 Materials and Methods

4.2.1 Participant Recruitment and Sample Collection

Participants (n=17) in their third trimester of pregnancy were recruited from Rio Grande Regional Women's Clinics located throughout Hidalgo County. Inclusion criteria included: residence in Hidalgo County, 21-35 years of age, non-asthmatic, non-diabetic, non-smoking household, singleton pregnancy, and no history of preterm birth. All study procedures were approved by the Texas A&M University Institutional Review Board and written informed consent was obtained from each participant before enrollment into the study. Sampling took place between June 2015 and April 2016. Participants completed three non-consecutive 24-hour measurements within a 4-6 week period to reduce seasonal effects on personal measures. Approximately 24 hours prior to a scheduled prenatal care visit, participants were visited at home by a local community health worker and delivered a lightweight backpack containing air sampling equipment, a global positioning system (GPS) device, and instruments to measure temperature and humidity. Participants answered a questionnaire related to their home, commute, and work environment and background information at the first home visit. Following 24 hours of wearing the backpack, it was returned at the regularly scheduled prenatal visit where participants also provided a urine sample and activity log from the measurement period. At the last appointment, participants also provided a hair sample to evaluate

environmental tobacco smoke exposure. Only one participant did not complete all three sampling days, resulting in 50 total sampling days.

4.2.2 Personal Exposure Assessment

Backpack contents included a personal DataRAM™ (pDR-1200, Thermo Scientific Corp., Waltham, Mass.) along with an external pump, a GPS receiver (BT1000XT, Qstarz International, Taiwan), and a HOBO Temperature and Humidity Data Logger (UX100-003, Onset Computer Corporation, Pocasset, MA, USA). Data were logged at 10-s resolution or higher for all instruments. The air intake inlet, mounted on the backpack's shoulder strap, sampled air at the participant's breathing level via a BGI air pump operated at a total flow rate of 4L per minute. Sampling pumps and inlets were averaged after each run and calibrated prior and post 24hr sampling periods. Air passed through a personal environmental monitor (PEM, MSP Inc.), acting as a single-stage impactor PM2.5 inlet for the subsequent pDR. This light-scattering nephelometer, the pDR, contained a built-in filter to provide calibration for mass concentration estimations. A second line from the BGI pump pulled air in at 1L/min to pass through a 2µm, 37mm polytetrafluoroethylene (PTFE) filter (Pall Corp, Zefluor) which collected particles encountered during the sampling period. PTFE filters were removed, individually placed in clean petri dishes, bagged and kept at -20° C for analysis. XAD-2 sorbent tubes to collect any volatile compounds were in-line following the PTFE filter. XAD-2 tubes were also stored at -20° C until analysis. $PM_{2.5}$ measurements were

corrected for the non-linear instrument response at RH values greater than 60% (Benton-Vitz and Volckens 2008; Soneja et al. 2014).

4.2.3 Laboratory Analyses of Polycyclic Aromatic Hydrocarbons

PTFE filters were extracted prior to analysis utilizing NIOSH method 5515 (Polynuclear Aromatic Hydrocarbons by GC). Briefly, filters were spiked with deuterated internal standard, sonicated in solvent for 15-20 minutes, followed by analysis via gas chromatography tandem mass spectrometry (GC-MS).

4.2.4 Analysis

Means, medians, and quartile ranges were calculated using Microsoft Excel.

4.3 Results

Participant information is summarized in Table 4. All participants identified as Hispanic and a majority of participants were not currently employed. Although individual PAH sample averages (Table 5) are not high, naphthalene was the most highly observed compound in our samples across all sampling rounds. Median overall total PAH exposure level was found to be 17.80 pg/m^3. Total levels of PAHs are orders of magnitude lower than the observed levels of $PM_{2.5}$. The median $PM_{2.5}$ value was 16.17 µg/m^3 with a 75% quartile of 19.51 µg/m^3. At the participant-level (Table 6), large temporal differences can be observed between sampling periods. For instance, while participant number 8 experienced low levels in rounds 1 and 3, the values for round 2

PAH and $PM_{2.5}$ concentrations are much higher, indicative of a possible large exposure on that day. Therefore, a repeated measure approach is warranted in evaluating exposure data for PAHs and $PM_{2.5}$.

4.4 Discussion

Observed differences between sampling days may be due to changes in behavior or activity from day to day including cooking, driving, and amount of time spent indoors. These discrepancies are difficult to account for using exposure modeling techniques, and emphasize the need for personal sampling. If we compare total PAH data from our study to that of other birth cohorts, we find that our levels are comparatively much lower. Only one of our sampling points would fall in the "high PAH group" according to Perera et al. (2009a) with a value of 10.58 ng/m^3. Jedrychowski et al. (2010) demonstrated a median $PM_{2.5}$ exposure level of 35.4 $\mu g/m^3$ among pregnant women living in Krakow, Poland. Only two of our individual sampling measurements exceed that level, and our overall median is almost half that reported in the aforementioned study. Recently, individual PAH levels were quantified in that population (Jedrychowski et al. 2017). Median total PAH concentration was reported to be 19.0 ng/m^3, representing exposures 1000X higher than in our population.

Ethnicity	**N**	**%**
Hispanic	17	100
Education		
< 12 years	7	41
12 years	6	35
> 12 years	1	6
Unknown	3	18
Smoking		
Never	12	71
Before Pregnancy	2	12
Unknown	3	18
Employed		
Yes	2	12
No	15	88

Table 3. Study Participant Demographics. Information from survey conducted at the first home visit.

	Round 1 N=15	Round 2 N=14	Round 3 N=13	Overall
Naphthalene	3.79 (2.70, 5.37)	2.77 (2.12, 4.83)	2.40 (2.10, 2.69)	2.77 (2.4, 3.79)
Acenaphthene	0.27 (0.24, 0.34)	0.28 (0.21, 0.52)	0.39 (0.32, 0.52)	0.28 (0.27, 0.39)
Acenaphthylene	0.10 (0.07, 0.17)	0.08 (0.03, 0.12)	0.10 (0.06, 0.27)	0.10 (0.08, 0.10)
Anthracene	0.08 (0.07, 0.15)	0.08 (0.06, 0.32)	0.08 (0.05, 0.21)	0.08 (0.08, 0.08)
Phenanthrene	1.31 (0.78, 2.16)	0.97 (0.52, 2.84)	0.79 (0.52, 1.26)	0.98 (0.79, 1.31)
Fluorene	0.27 (0.26, 0.34)	0.27 (0.23, 0.53)	0.32 (0.25, 0.39)	0.28 (0.27, 0.32)
Fluoranthene	0.18 (0.16, 0.30)	0.23 (0.17, 0.58)	0.20 (0.15, 0.34)	0.20 (0.18, 0.23)
Benzo(a)anthracene	0.33 (0.26, 0.51)	0.30 (0.21, 0.68)	0.37 (0.29, 0.58)	0.33 (0.30, 0.37)
Chrysene	0.17 (0.13, 0.42)	0.22 (0.11, 0.47)	0.32 (0.26, 0.57)	0.22 (0.17, 0.32)
Pyrene	0.27 (0.22, 0.54)	0.29 (0.23, 0.59)	0.29 (0.16, 0.40)	0.29 (0.27, 0.29)

Table 4. Personal Exposure to Individual Polycyclic Aromatic Hydrocarbon Compounds, Total PAH, and 24 hr Average $PM_{2.5}$. Values (pg/m^3 unless otherwise indicated) from three prenatal monitoring periods (median values with quartile range).

	Round 1 N=15	Round 2 N=14	Round 3 N=13	Overall
Benzo(a)pyrene	0.23 (0.17, 0.30)	0.17 (0.07, 0.37)	0.25 (0.20, 0.63)	0.23 (0.17, 0.25)
Benzo(b)fluoranthene	0.25 (0.10, 0.59)	0.18 (0.11, 0.47)	0.27 (0.20, 0.45)	0.25 (0.18, 0.27)
Benzo(k)fluoranthene	0.06 (0.03, 0.15)	0.12 (0.07, 0.21)	0.22 (0.18, 0.64)	0.12 (0.06, 0.22)
Dibenz(a,h)anthracene	0.05 (0, 0.12)	0.09 (0.04, 0.31)	0.15 (0.07, 0.33)	0.09 (0.05, 0.15)
Benzo(g,h,i)perylene	0.58 (0.40, 1.73)	0.37 (0.19, 0.84)	0.32 (0.20, 0.55)	0.37 (0.32, 0.58)
Indeno[1,2,3-cd]pyrene	0.20 (0.11, 0.44)	0.15 (0.06, 0.38)	0.17 (0.14, 0.51)	0.17 (0.15, 0.20)
Total PAHs (pg/m^3)	26.77 (18.71, 40.88)	17.80 (11.67, 44.23)	12.53 (9.37, 19.05)	17.80 (12.54, 26.77)
$PM_{2.5}$ ($\mu g/m^3$)	11.09 (3.07, 17.39)	16.17 (9.22, 32.46)	19.51 (6.95, 25.4)	16.17 (11.09, 19.51)

Table 4. Continued.

Participant	Total PAHs (pg/m^3)				24 hr $PM_{2.5}$ (µg/m^3)			
	Round 1	Round 2	Round 3	Overall	Round 1	Round 2	Round 3	Overall
1	23.99	50.30	18.56	23.99 (30.95)	2.74	10.86	5.04	5.04 (6.21)
2	40.89	8.92	12.82	12.82 (20.88)	31.06	29.81	34.81	31.06 (31.89)
4	27.16	16.57	9.30	16.57 (17.68)	7.48	10.09	7.1	7.48 (8.22)
5	26.77	538.91	12.54	26.77 (192.74)	13.30	32.41	26.15	26.15 (23.95)
6	10.38	42.20	51.76	42.20 (34.78)	17.04	32.61	27.72	27.72 (25.79)
7	10.05	10.50	11.11	10.50 (10.55)	9.85	25.38	20.66	20.66 (18.63)
8	5.06	10581.78	8.89	8.89 (3531.91)	3.07	114.25	23.42	23.42 (46.91)
10	32.59	7.29	-	19.94 (19.94)	17.39	14.69	-	16.04 (16.04)
11	32.01	15.26	20.50	20.50 (22.59)	11.09	73.45	20.40	20.4 (34.98)

Table 5. Individual Exposure to Total PAH and 24 hr Average $PM_{2.5}$. Values from three prenatal monitoring periods [depicted as median (mean)]. Data for participant numbers 3 & 9 were unavailable for this analysis.

Participant	Total PAHs (pg/m^3)				24 hr $PM_{2.5}$ ($\mu g/m^3$)			
	Round 1	Round 2	Round 3	Overall	Round 1	Round 2	Round 3	Overall
12	491.58	19.03	-	255.306 (255.306)	2.07	6.09	13.10	6.09 (7.09)
13	171.61	22.99	15.84	22.99 (70.15)	1.92	5.63	6.51	5.63 (4.69)
14	20.45	24.75	8.84	20.45 (18.01)	28.18	13.12	25.15	25.15 (22.15)
15	42.23	12.06	9.44	12.06 (21.24)	6.83	6.62	13.91	6.83 (9.12)
16	18.71	-	12.80	15.76 (15.76)	11.21	2.11	4.70	4.70 (6.01)
17	24.68	12.69	12.30	12.69 (16.56)	34.58	17.65	18.61	18.61 (23.61)

Table 5. Continued.

Our data demonstrates that individual exposure levels to PAHs and $PM_{2.5}$ can vary widely day to day but that on average in this population, daily levels do not exceed standards set forth by the EPA. Future research will build on this preliminary exposure assessment to investigate low-level prenatal exposure levels and adverse respiratory risks in the children. Characterization of exposure at the resolution we have achieved can provide insight into why childhood asthma rates are increased in this unique population compared to the Texas average.

5. SUMMARY

Exposure to ambient air pollutants is detrimental to health in multiple ways including cardiovascular disease, lung cancer, neurocognitive impairment, and even metabolic dysfunction. Vulnerable populations including pregnant women and young children are more susceptible to toxicant exposures. Furthermore, exposure during these periods may result in an increase in systemic inflammation and epigenetic alterations leading to disease later in life.

Our first aim hoped to address these differing susceptibilities by examining prenatal intranasal exposure to diesel exhaust particulate, a major component of air pollution, in two strains of mice. We probed epigenetic alterations immediately following birth to establish a baseline response in the BALB/c and C57Bl/6 pups. Results indicate that there are small differences in global methylation between male and female pups following prenatal exposure to DEPM. When global methylation analysis was performed with MS, neither BALB/c mice or C57Bl/6 mice treatment groups were significantly different although a trend of decreased methylation in BALB/c mice was noted. As reflected in human data, this indicates that sex and genetic background play integral roles in response mediation. Implications include a need for environmental regulations to account for those individuals that are most susceptible to insult.

Prenatal exposure to air pollution has also been associated with increases in asthma prevalence. This risk increase has been demonstrated in epidemiologic and animal model data; however, the mechanisms are largely unknown. Evidence generated

by animal models has failed to reach consistency due to difference in strain utilized and level, composition, route, and timing of exposure. A standard method of prenatal air pollutant administration has failed to be established and is indeed a necessary procedure for relevant experiment conduction.

Thus, the secondary aim of this dissertation work was to develop a murine model of prenatal exposure to particulate matter air pollution and incorporate a postnatal chronic allergen challenge to investigate phenotypic response in two strains of mice. Not only did we see differing response between strains but also between sexes. Importantly, we have revealed a potential window of susceptibility to respiratory infection in offspring prenatally exposed to particulate matter air pollution as evidenced by a decrease in immune response following exposure to allergen. This data underlines previous findings indicating an early immunosuppression. It demonstrates that we can model variable response based on genetic background differences in inbred strains of mice. If this work were to continue into a population-based mouse strain such as collaborative cross, we could extrapolate findings to a wider base and investigate the specific traits or genes that may make certain individuals more vulnerable to insult.

If we are to understand how vulnerable populations can be more susceptible to insult, sufficient characterization of exposures is essential. In Hidalgo County, Texas, there is an increased incidence of childhood asthma but the cause is unclear. Particulate matter monitors in the area do not reflect increases in exposure beyond national standards. Increased relative risk may be a result of an already genetically vulnerable

population thereby necessitating thorough interrogation of chemical exposure compositions.

Therefore, we desired to assess exposure to PAHs in a small cohort of pregnant women in McAllen, Texas where there is a high prevalence of childhood asthma. The data reveals low levels of PAH and $PM_{2.5}$ exposure in this population when compared to other PEMs cohort data. We demonstrate that total PAH levels may be magnitudes lower than $PM_{2.5}$ exposure but the PAH component may represent an important cause of oxidative stress. Accurate analysis of pollutant constituents is necessary to fully assess exposure and comprehend potential health effects.

In summary, detailed exposure characterization is an essential beginning to understanding the mechanisms by which prenatal exposure to air pollutants increase risk of childhood asthma development. Animal modeling techniques based on human exposure patterns will allow for closer interrogation of these mechanisms. The current work has laid a foundation upon which to build the knowledge base and investigate the health effects of prenatal exposure to air pollution, particularly effects on allergic asthma.

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