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# The Inflammatory Effects of Natural and Engineered Airborne Particulate Matter in the Lung, and Related Cellular Mechanisms of Immunity

Sophia Tracy Carvalho

**The Inflammatory Effects of Natural and Engineered Airborne Particulate Matter in the Lung, and Related Cellular Mechanisms of Immunity**

By

Sophia Tracy Carvalho

Bachelor of Science Case Western Reserve University, 2011

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Approved by:

Kevan Roberts, Ph.D., Chairperson
Center for Environmental Health Sciences
Biomedical and Pharmaceutical Sciences

Andrij Holian, Ph.D.
Center for Environmental Health Sciences
Biomedical and Pharmaceutical Sciences

Stephen Lodmell, Ph.D.
Division of Biological Sciences

Christopher Migliaccio, Ph.D., Pharm.D.
Center for Environmental Health Sciences
Biomedical and Pharmaceutical Sciences

Mark Pershouse, Ph.D.
Center for Environmental Health Sciences
Biomedical and Pharmaceutical Sciences

Sophia Tracy Carvalho, Ph.D. 2017 Toxicology

**The Inflammatory Effects of Natural and Engineered Airborne Particulate Matter in the Lung, and Related Cellular Mechanisms of Immunity**

Chairperson: Kevan Roberts, Ph.D.

**Abstract**

Asthma, defined as a complex, chronic inflammatory disease of the airways, affects approximately 300 million individuals worldwide and is the single most common chronic disease among children. Airway inflammation is the defining characteristic of asthmatic pathophysiology; and as asthma increases in severity, the airways become more susceptible to environmental insults, including air pollutants. Wildfires and prescribed burns are significant sources of airborne particles, as well as gaseous pollution, which can temporarily increase the overall levels of air pollution over hundreds or thousands of square miles. Multi-walled carbon nanotubes (MWCNT) are increasingly used in a broad range of applications, including medical treatments, construction in the aerospace industry, and electronics manufacture. Accordingly, the following studies were conducted to narrow the gaps in existing knowledge of the health hazards posed by wood smoke (WS) and inhaled MWCNT. To model allergic asthma, the most common form of the disease, we introduced house dust mite (HDM) allergen, an allergy trigger for almost 85% of asthmatics. We found that adult C57BL/6 mice previously sensitized to HDM displayed significantly exacerbated lung inflammation following oropharyngeal MWCNT instillation. This was characterized by elevated levels of EPO and correspondingly increased eosinophil populations, measured by flow cytometry, in the BALF. Th2-associated cytokines traditionally implicated in allergic asthma, such as IL-13 and IL-22, were notably lacking; likewise, no clear alteration in CD4+ or CD8+ T cells was found. Instead, a significant increase in levels of cysteinyl leukotrienes (cys-LT) was detected, which correlated to increases in eosinophilia. This coincidence of augmented eosinophil recruitment and increased cys-LT production was again observed in our study of WS exposure. Adult female mice exposed to WS while pregnant displayed markedly increased eosinophilia when later sensitized to HDM, as evidenced by EPO levels and quantification via flow cytometry, as well as elevated cys-LT levels. Interestingly, the offspring of those WS-exposed mice, when sensitized to HDM in adulthood, responded with dramatically exacerbated inflammation in the same mode. H&E and PAS staining revealed mucus deposition and marked cellular infiltration in the lungs of prenatally exposed offspring following HDM challenge. Pups displayed highly significant differences in EPO levels between WS/HDM and Air/HDM groups at 8 weeks of age; eosinophilia decreased over time, with these differences remaining significant at 16 weeks and being lost by 24 weeks. The numbers of CD4+ and CD8+ T cells were also affected: in dams, WS inflated these populations and HDM decreased them; in pups, the opposite was true. These findings indicate a direct relationship between the biosynthesis of cys-LT and the recruitment of eosinophils in response to MWCNT inhalation, and suggest that a similar mechanism—likely with a greater T cell component—may be responsible for the inflammation arising from exposure to WS.

**Acknowledgments**

I must preface the following with the admission that I owe immense gratitude to a great many more individuals than space will permit me to list by name. I have been blessed with the acquaintance of a number of excellent science teachers, from the days of observing butterfly lifecycles in childhood to fetal pig dissections in high school and the study of *Plasmodium vivax* in college. Without their influence, I would not have developed this abiding love of all things scientific, which led me to graduate school. Similarly, my many classmates and labmates over the years have shamelessly aided and abetted my aspirations to a higher plane of madness—for madness this endless pursuit of knowledge certainly is. Please know that every one of you has impacted my work, and myself, and I thank you for that.

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# Chapter One

## 1.0 An Introduction to Allergic Asthma

Asthma is commonly defined as a complex, chronic inflammatory disease of the airways. It affects approximately 300 million individuals worldwide, including 1-5% of adults in Asia and 10% in Australia, and is the single most common chronic disease among children (1-4). It has been more formally defined by the Global Initiative for Asthma (GINA): "Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation" (5). This definition takes into account not only the classical pathologies of asthma, but also the existence of many phenotypes. The root causes of asthma are varied and incompletely understood, at best; they are believed to include genetic predisposition—derived from over 100 possible susceptibility genes—and numerous environmental factors, and this variability results in high individualization from patient to patient. The major disease forms include allergic asthma, steroid-resistant asthma, and asthma induced by exposure to airborne irritants such as cigarette smoke and diesel exhaust (2). The most common of these is allergic asthma (6). However, all types of asthma share a degree of overlap in symptoms and triggers and must to some extent be considered in connection rather than isolation. Accordingly, the studies discussed in this text will focus on the inflammatory processes of allergic asthma: particularly, the ways in which those processes may be impacted by inhaled particulate pollution.

## 1.1 Characteristics of Asthma

Allergic asthma is primarily a disorder of the conducting airways which leads to airway hyperreactivity (AHR) and variable airflow obstruction (7). Airway inflammation is the defining characteristic of asthmatic pathophysiology; this stems from the release of inflammatory mediators and resultant remodeling of the airway wall. Therefore, as asthma increases in severity, the airway becomes more susceptible to environmental insults, including air pollutants. The airway repair response is also altered, with abnormal secretion of growth factors that induce mucus cell, smooth muscle, and nerve cell proliferation; angiogenesis; and fibrosis. Variations in each of these symptoms account for the myriad asthmatic phenotypes and their differing responses to conventional treatment (7).

Allergic asthma is believed to derive from childhood exposure and sensitization to aeroallergens such as those derived from house dust mites (HDM), cockroaches, animal dander, fungi, and pollens. This usually occurs through selective expansion of T-helper type 2 (Th2) cells (7). Indeed, data from adoptive-transfer studies have revealed that Th2 cells alone are sufficient to elicit the major characteristics of asthma (8), although in reality they are supported by other cell types. Phosphorylation of the transcription factor GATA-3 activates CD4+ Th2 cells, which produce a number of cytokines, including interleukins 4, 5, 9, and 13, and GM-CSF (7). These cytokines in turn initiate a cascade of inflammatory mechanisms: isotype switching by B cells to produce immunoglobulin E (IgE) depends on IL-4 and IL-13; IgE crosslinking activates mast cell production of IL-3, 4, 5, and 13; the recruitment of eosinophils to the airways, maturation, and survival are mediated by IL-3, IL-5, and GM-CSF; the recruitment of basophils relies on IL-3 and GM-CSF; and the survival of Th2 cells themselves requires IL-4 (2, 4, 7).

Eosinophils and type 2 innate lymphoid cells (ILC2s) are also capable of making some of the cytokines commonly associated with the Th2 response, including Il-4, IL-5, and IL-13. Additionally, other cytokines, including IL-17 and IL-22, are often detected in both human asthmatics and murine models; and some human patients display predominantly neutrophilic rather than eosinophilic inflammation. Thus, though Th2 cells and associated cytokines are fundamental components of asthma in many instances, it is also important to consider that the classic paradigm of Th2-driven inflammation does not present a complete picture of this disease (8).

### *1.1.1 Characteristics of Asthma: Airway Hyperreactivity*

AHR describes the tendency of airways to respond disproportionately to nonspecific stimuli, e.g., exercise or abrupt temperature change (8). The mechanisms underlying AHR in asthma are complex, but are at least partially dependent on the presence of the Th2-associated inflammatory cytokines. IL-9 and IL-13-are known to be important to the progression of AHR; mice deficient in these display impaired development of AHR and lung eosinophilia (4, 9). Related inflammatory cells are, of course, also crucial. It has been reported that AHR depends on the recruitment of eosinophils to the bronchial submucosa: blockage of MCP-5, monocytic chemoattractant protein 5, a chemokine which affects eosinophilia in the interstitium but not in the airways, decreases AHR (10). In an OVA-based murine model of asthma, antibody neutralization of a stimulated T-cell chemotactic protein (STCP-1), MDC, was shown to prevent the development of AHR. MDC is a potent chemoattractant when bound to the chemokine receptor CCR4; in the mouse, MDC is produced by alveolar macrophages and smooth muscle cells and is

responsible for the recruitment of monocytes and activated T cells and Th2 cells (10), indicating that AHR relies on more than eosinophilia alone. Nitric oxide signaling has also been implicated in the regulation of AHR in asthma (9). Measuring FeNO (fractional exhaled nitric oxide) in exhaled breath is a simple, non-invasive way to identify IL-13-driven inflammation (11). Release of IL-13 in the airways results in the activation of inducible nitric oxide synthase and therefore increased levels of NO; because of this, asthmatic patients typically exhale higher levels of nitric oxide than do non-asthmatics (9, 11). This may be connected to the activity of S-nitrosoglutathione, or GSNO. An endogenous bronchodilator, GSNO would naturally counteract the bronchoconstriction observed in asthma. However, levels of GSNO are decreased in asthmatic patients; this is believed to directly impact the progression of AHR, as the addition of GSNO reductase has been shown to protect against hyperreactivity (9).

### *1.1.2 Characteristics of Asthma: Airway Remodeling*

Airway remodeling is often defined broadly so as to encompass any alteration in composition, distribution, thickness, mass, volume, and/or structure of the airway wall. These alterations include: changes to the airway epithelium, such as epithelial shedding, goblet cell hyperplasia, and basal membrane thickening; subepithelial fibrosis of peribronchial interstitial tissue; hyperplasia or hypertrophy of airway smooth muscle cells; increased neurite sprouting; and alterations of the bronchial vasculature, such as barrier dysfunction and angiogenesis. These symptoms can be separated into two categories: physiological and pathological. Physiological remodeling encompasses any structural change of the sort which occurs during normal lung development, growth, or healing after

acute injury or short-term inflammation. Pathological remodeling includes any structural change that occurs due to abnormal development or chronic inflammation or injury and results in persistent alteration to lung function (5).

### *1.1.3 Characteristics of Asthma: Cellular Inflammation*

Eosinophils: Eosinophils play a pivotal role in a number of allergic inflammatory processes, including those underlying asthma (12). Indeed, the presence of eosinophils in the airways of those suffering from allergic asthma has been well documented (13, 14) and is commonly used as a biomarker of the disease during diagnosis (15). It has been proposed that the presence of infiltrating inflammatory cells, particularly CD4+ T cells and eosinophils, should be considered the defining characteristic of airway remodeling in asthma (5, 13).

The eosinophil has been implicated in host resistance to parasites, particularly helminthes, as well as in antimicrobial activities against bacterial, viral, and protozoan pathogens; they have also been identified as mediators of hypersensitivity diseases (12). Eosinophils are granulocytes derived from multipotent hematopoietic stem cells in the bone marrow. Development from these precursors is dependent on certain growth factors and cytokines, particularly IL-3, IL-5, and GM-CSF (16, 17). Of these, IL-5 is the cytokine most specifically targeted to eosinophil maturation (18). In the presence of high systemic levels of IL-5, mice display depletion of bone marrow eosinophils and a corresponding increase in circulating eosinophils, suggesting that the release of these cells from the bone marrow into the blood is stimulated by IL-5. Further, mice engineered to overexpress IL-5 show pronounced eosinophilia in the blood, spleen, and bone marrow (16, 18), while IL-

5 deficiency prevents eosinophilia, and mice engineered to be deficient in eosinophils themselves show stunted airway remodeling (8).

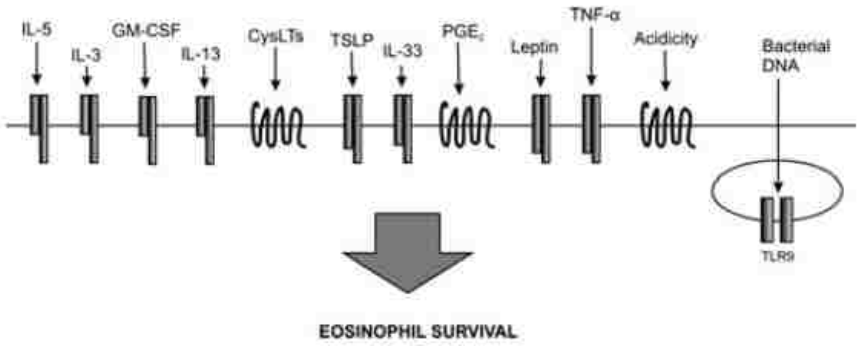



*Fig. 1.1:* Factors responsible or possibly responsible for the survival of eosinophils in inflamed airways, including IL-3, IL-5, and GM-CSF. (Adapted from (19).)

Eosinophils produce a variety of cytokines and growth factors. These are released in small amounts while eosinophils are in a resting state, and they are induced in greater volume during inflammatory conditions. Stimulation by eosinophilic recognition of cytokines, immunoglobulins, or complement can induce the secretion of proinflammatory cytokines including: IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, TGF-α, and TGF-β; chemokines such as RANTES and eotaxin-1; and lipid mediators, such as platelet activating factor and the cysteinyl leukotriene $LTC_4$ (14, 16).

As granulocytes, eosinophils are able to secrete an array of cytotoxic proteins. These include EPO, or eosinophil peroxidase, which is an important experimental marker of eosinophil activity. EPO is localized to the granule matrix and makes up roughly 25% of an individual granule's protein mass. EPO is an enzyme used to catalyze the oxidation of halides, pseudohalides, and nitric oxide to form reactive oxygen and nitrogen species.

These then oxidize nucleophilic targets, thereby promoting oxidative stress and cell death via apoptosis or necrosis (16).

Airway Epithelial Cells: The Innate Immune Response (IIR) is a rapid, non-pathogen-specific intracellular inflammatory response produced by detection of a pathogen signature by sentinel airway cells that patrol for non-self antigens (6). These sentinels include airway epithelial cells, alveolar macrophages, and dendritic cells (DCs) acting as specialized antigen-presenting cells (APCs). With IIR activated, a host of immune cells undergo induced changes resulting in phenotypes which favor the production of inflammatory cytokines and protective mucosal interferons. These factors contain the spread of any pathogens and regulate the magnitude of ensuing adaptive immune activity (6).

Sentinel cells use pattern recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs), such as dsRNA, lipoteichoic acids, mannans, or flagellins (6). PRRs can be divided into two major classes: membrane-resident Toll-like receptors (TLRs) and cytoplasmic Retinoic Acid inducible Gene (RIG)-I-like RNA helicases (RLHs). RLHs are most useful in the detection of invading dsRNA within cells, whereas TLRs are surface-localized and able to bind diverse PAMPs, including the asthma trigger LPS (6).

The current body of evidence indicates a major role for epithelial cells in initiating and sustaining pulmonary IIR. Epithelial cells produce CC, CXC, and C-class chemokines, which trigger acute inflammation. CXC chemokines are involved in the recruitment of leukocytes to the interstitial spaces and airway lumen, from whence activated leukocytes can then migrate to the site of infection and induce phagocytosis and antigen uptake (6).

Several types of lymphoid cells, including eosinophils, basophils, and mast cells, are recruited to affected airways immediately after allergen challenge. These generate inflammatory mediators, such as histamines, cysteinyl leukotrienes, and proinflammatory cytokines IL-4, 5, 10, 13 and tumor necrosis factor (TNF) (2).

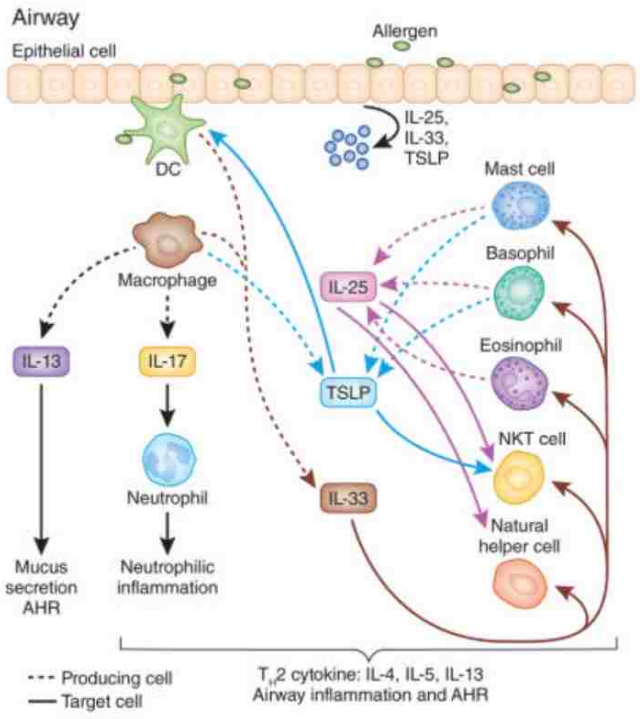



*Fig. 1.2:* Pathways of innate immunity involved in allergic asthma. IL-25, IL-33, and TSLP induce Th2-associated cytokine production by "natural helper cells," now known as ILC2 cells, in the absence of Th2 cells. NKT cells are stimulated to produce IL-13, thereby promoting AHR and airway remodeling. (Adapted from (2).)

CD4+ T cells: The involvement of CD4+ T helper cells in allergic asthma has been well-documented (20-25). T helper cells differentiate into distinct subsets based on their microenvironment and cellular interactions; these phenotypes include Th1, Th2, and Th17,

which are distinguished by their differing immune functions and cytokine production. Th1 cells produce IFNγ and mediate cellular immunity; Th2 cells produce IL-4, IL-5, and IL-13, which mediate humoral immunity, immunoglobulin production by B cells, and allergic responses. Th17 cells produce IL-17 and have been associated with chronic inflammation. Through these processes, IIR induces not only acute inflammation but also Th polarization and sensitization in the airways (6).

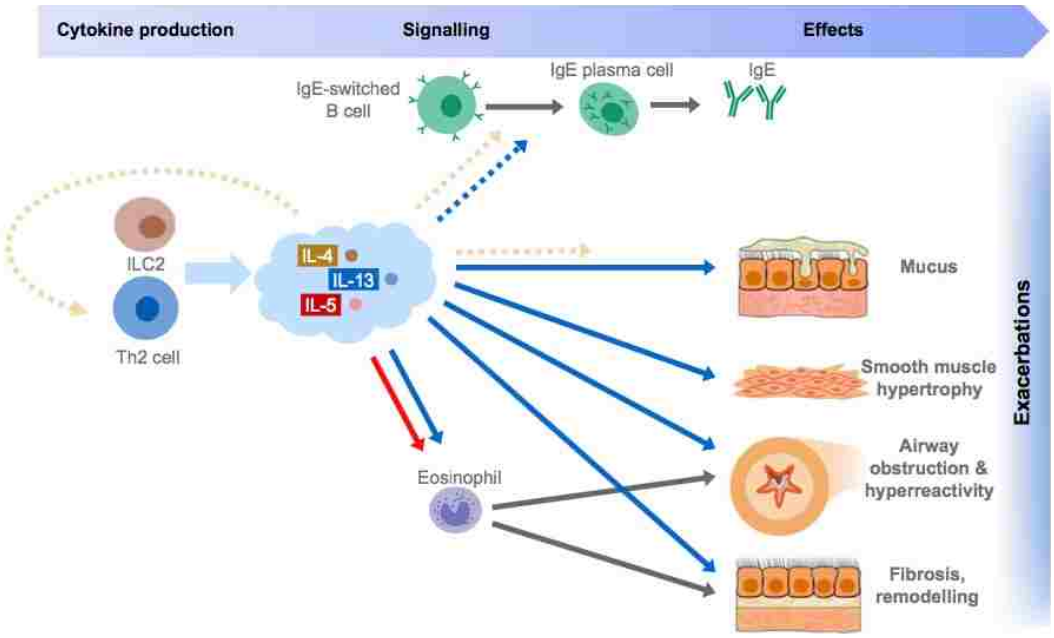



*Fig. 1.3:* An overview of the type-2 inflammatory response to allergens. Th2-associated cytokines produced by ILC2 or Th2 cells drive IgE switching in B cells and recruitment of effector cells such as eosinophils, which promote the hallmark characteristics of allergic asthma: mucus hypersecretion, airway hyperresponsiveness and remodeling. (Adapted from (11).)

Maturation of a naïve T helper cell, dubbed Th0, is prompted by recognition of an antigen by an APC (e.g., a dendritic cell), which presents that antigen to the Th0 cell. At that point, the Th0 may differentiate into any one of a number of possible phenotypes. The phenotype most commonly associated with allergic asthma is the Th2 cell (23). Precisely what process drives differentiation from Th0 to Th2 remains unclear; but it has been shown that IL-4, possibly produced by mast cells or basophils, is necessary to activate STAT6 (signal transducer and activator of transcription 6) and GATA-3 (GATA-binding protein 3) (7). When expressed by activated Th2 cells, these transcription factors regulate the production of several proinflammatory cytokines (26). These are IL-4, which regulates the synthesis of allergen-specific IgE; IL-5, which mediates the recruitment of eosinophils; IL-9, which stimulates the growth and recruitment of mast cells; and IL-13, which promotes AHR and mucus production (23).

Dendritic Cells: Another cell type that is crucial to mediation of airway inflammation is the dendritic cell. It is believed that immature monocytes are recruited to the lung through the action of the chemokine receptors CCR2 and CCR6 and CCL20-MIP-1α signaling arising from IIR activation (27). Mature DCs coordinate the development of adaptive immunity from the acute inflammatory response, in part by inducing Th2 activation (27, 28). The sources of this ability are twofold. Firstly, DC maturation is enhanced by the presence of epithelial-derived thymic stromal lymphopoietin (TSLP) and granulocyte-macrophage colony stimulating factor (GM-CSF), both of which are IIR-inducible cytokine-like molecules produced by lung epithelial cells (27). Secondly, matured DCs express an array of PRRs, TLRs, NOD (nucleotide-binding oligomerization domain)-like

receptors, and C-type lectin receptors, which collectively enable these cells to recognize numerous allergens and present them to T cells (28).

Innate Lymphoid Cells: ILCs are non-T, non-B effector cells that show high levels of cytokine production following activation (29). They were first identified in the intestine and have since been found in the lung as well. ILCs are involved in tissue homeostasis, repair and remodeling, and immunity. Due to their lack of T- and B-cell receptors, ILCs are antigen nonspecific and react rapidly to a vast range of innate signals. These cells have been classified into three distinct subsets based on their cytokine profiles: ILC1, ILC2, and ILC3. All of these subsets share a common progenitor, identified as $Lin^{-}IL\text{-}7R\alpha^{+}Kit^{low}Sca^{low}$; and all ILCs require IL-7 signaling and the action of Id2, a transcriptional repressor that regulates Notch signaling, for proper development (29). Of these subsets, ILC2s are particularly important in the context of asthma.

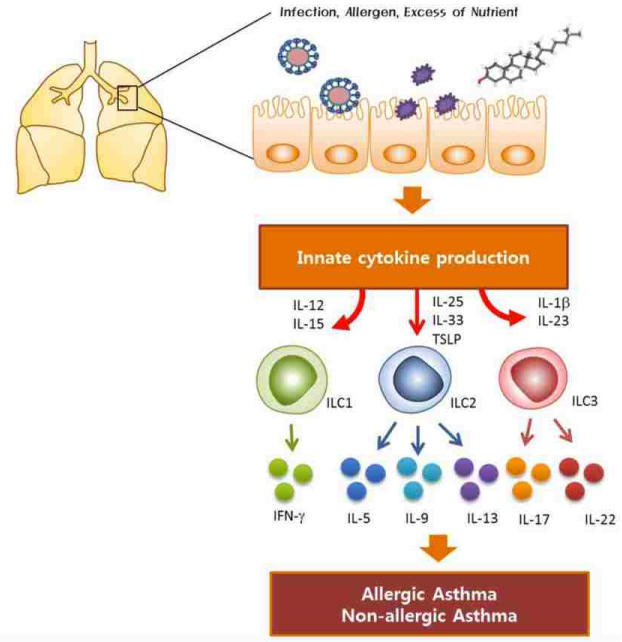



*Fig. 1.4:* The function of innate lymphoid cells in the development of asthma. An inflammatory stimulus, such as HDM allergen, prompts airway epithelial cells to release cytokines that influence the differentiation of ILC subsets. Activated ILC1, 2, and 3 produce an assortment of cytokines

capable of activating other immune cells downstream and intensifying asthmatic symptoms. (Adapted from (29).)

Th2 cells are a major source of some of the cytokines most closely associated with the symptoms of allergic asthma. However, the notion that Th2s are essential to asthmatic development was challenged by the observation that mice lacking a functional adaptive immune system, e.g., *Rag1-/-* mice, nonetheless mount a strong type 2 response to inflammatory stimuli (4). This can be attributed to the activity of ILC2s. Although they lack the specific antigen receptor of Th2 cells, ILC2s are fully capable of producing the type 2 cytokines critical to asthmatic development (4, 30, 31). ILC2s are now regarded as a key source of such cytokines during the earliest stages of allergic response before Th2 cells are recruited to the site of inflammation (30).

ILC2s can impact both innate and adaptive immune processes in asthma. It has been demonstrated that pulmonary ILC2s are capable of significant IL-5 and IL-13 production following stimulation with inhaled allergens, including HDM. IL-5+ and IL-13+ populations of ILC2s have even been measured at levels comparable to IL-5+ and IL-13+ Th2 populations in BALF following HDM allergen challenge (4).

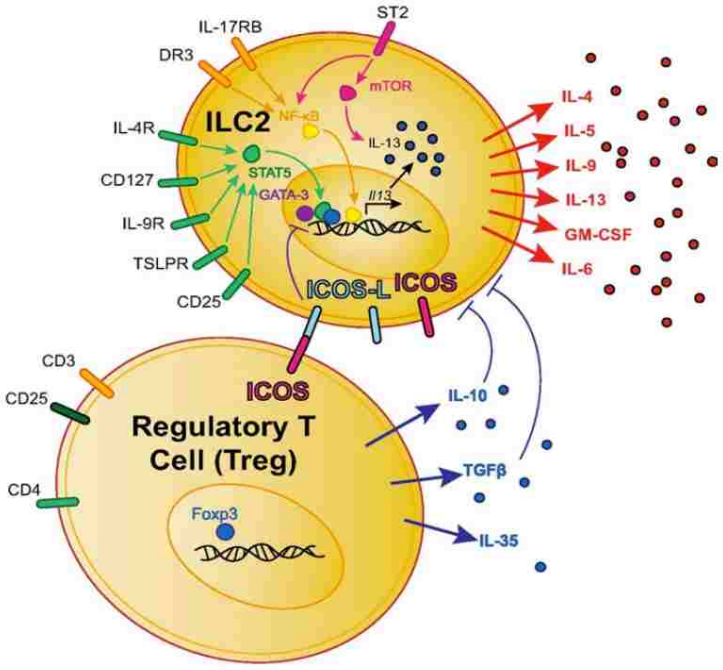



*Fig. 1.5:* Interactions of ILC2s with their environment. Proinflammatory cytokines produced by ILC2s echo those made by Th2s, including IL-4, IL-5, and IL-13. Treg cells are able to exert a suppressive influence through inducible T cell co-stimulator (ICOS). (Adapted from (31).)

While the precise mechanisms of ILC2 activation remain somewhat unclear, it does appear that much like Th2s, ILC2s can be activated by a variety of epithelial-derived cytokines, including IL-25, IL-33, and TSLP (4, 30). ILC2s have further been shown to rely on at least some of the same transcription factors as Th2s, including RORα and GATA3 (30, 31). Prostaglandins and leukotrienes have also been reported to activate ILC2s. All of these signals promote the production of type 2 cytokines and ILC2 expansion (4).

### *1.1.4 Characteristics of Asthma: Production of Cysteinyl Leukotrienes*

Cysteinyl leukotrienes (cys-LT) are small, peptide-conjugated lipids derived from arachidonic acid. They are potent mediators of inflammation throughout the body and are demonstrably important in the pathogenesis of asthma (32, 33). Oxygenation of arachidonic acid by 5-lipoxygenase (5-LO), chaperoned by 5-lipoxygenase-activating protein (FLAP), yields leukotriene $A_4$ ($LTA_4$). $LTA_4$ is then hydrolyzed to form the dihydroxy leukotriene $B_4$ ($LTB_4$) and the cysteinyl leukotrienes $C_4$, $D_4$, and $E_4$ (33). $LTB_4$ interacts with its major receptor, BLT1, to promote the recruitment of inflammatory cells. Cys-LT produce similar effects: by acting upon types 1 and 2 cysteinyl leukotriene receptors (CysLT1, CysLT2) and g-protein-coupled-receptor 99 (GPR99), they activate various kinase cascades (34, 35).

Cells involved in the acute inflammatory response, including macrophages, secrete cys-LT to recruit eosinophils and other leukocytes to sites of infection (36). Some, if not all, of these cell populations—notably eosinophils and alveolar macrophages—display increased biosynthesis of cys-LT in asthmatic patients compared to healthy individuals (32). Through the action of CysLT1, cys-LT induce the classic symptoms of asthma: bronchoconstriction, tissue swelling, and increased mucus secretion. Activation of CysLT2 mediates inflammation, vascular permeability, and fibrosis (37). Furthermore, mice with experimental fibrosis and human patients with idiopathic pulmonary fibrosis show increased cys-LT in their lungs. Cys-LT can also stimulate fibroblasts to multiply and produce the collagen used in fibrotic remodeling. Importantly, inhibition of cys-LT activity

has been shown to decrease both acute inflammation and the development of fibrosis (36).

Cysteinyl leukotrienes can be produced in the lung by mast cells, basophils, alveolar macrophages, dendritic cells, and pulmonary eosinophils; all of these cell types constitutively express the enzymes necessary to synthesize $LTC_4$ from $LTA_4$. Expression of CysLT1 and CysLT2 at both the mRNA and protein levels can be modulated by Th1 and Th2-associated cytokines such as IL-4, IL-5, IL-13, TGF-β, and IFNγ (35). It has previously been shown that airway epithelial cells upregulate expression of these enzymes, i.e., 5-lipoxygenase and $LTC_4$ synthase, in response to lipopolysaccharide. $LTC_4$ is made rapidly in response to cellular activation and is then excreted and converted into $LTD_4$ and $LTE_4$ outside the origin cell (38). $LTE_4$ is the most stable of the cys-LT and thus the most abundant. It is also the most potent leukotriene in *in vivo* systems, highly effective in eliciting eosinophilia, basophilia, and muscular constriction in asthmatic bronchii as well as in increasing airway sensitivity to histamine and vascular permeability (33, 39). $LTE_4$ produces these effects by binding to the recently-discovered GPR99, which is commonly expressed by nonhematopoietic cells and binds $LTE_4$ more strongly than either CysLT1 or CysLT2 (35). Direct inhalation of cys-LT, particularly $LTE_4$, induces heightened bronchoconstriction in asthmatic individuals compared to healthy ones (39).

Accordingly, disruption of cys-LT signaling appears to have a protective effect against related asthmatic symptoms. Mice deficient in $LTC_4$ synthase or CysLT1 are incapable of synthesizing or recognizing cys-LT, respectively. Both mouse strains display significantly impaired eosinophilic inflammation and Th2 responses following exposure to HDM (38). Furthermore, compounds formulated to inhibit the oxidation of 5-LO have proved as effective as selective receptor antagonists, and both classes of drugs are effective

in improving airflow in asthmatic patients and reducing the frequency of asthma attacks (38).

## 1.2 Modeling Allergic Asthma in Mice

### *1.2.1 House Dust Mite*

Mites of the genus *Dermatophagoides*, particularly the *pteronyssinus* and *farinae* species, preferentially colonize human habitats and can thus be found in homes around the world. Perhaps as a result of their ubiquitous presence, house dust mites (HDM) pose a significant allergic hazard; it is estimated that one to two percent of the global population is allergic to HDM (40). Exposure can cause rhinitis, conjunctivitis, and dermatitis. HDM allergens are also potent activators of asthma, and 50-85% of asthmatics are allergic to HDM (1).

Both *D. pteronyssinus* and *farinae* live for approximately ten weeks, requiring three to four weeks to reach reproductive maturity. During the four to six remaining weeks of their lifespan, female mites lay between 40 and 80 eggs each; the resulting population boom enables HDM to colonize the entirety of a house within a single year. Human habitats are particularly beneficial for HDM because they provide ample supplies of organic debris. Keratin can be derived from sloughed human skin, cellulose from textiles, and chitin from fungi and mite carcasses. Keratin is the house dust mite's primary food source, although the mites also eat bacteria, pollen, and fungal mycelia. As part of the digestive process, these food particles are bound to dissociated cells from the gut wall; the digestive enzymes contained in these cells are a major source of HDM allergenicity (40).

Allergy to HDM involves both the mites themselves and their waste. The mites' exoskeletons are composed of the structural polysaccharide chitin, which is a potent adjuvant capable of regulating both innate and adaptive immune responses. Chitin causes eosinophils and basophils to accumulate and stimulates macrophages through surface receptors TLR2 and dectin-1. Mammals do not produce chitin, but chitinases and chitinase-like proteins have been found in human asthmatics and are thought to play important roles in the development of adaptive inflammation and fibrosis (1).

Dust mites' fecal pellets are another source of chitin and also contain proteolytic enzymes derived from the mite's gut. *Dermatophagoides* species synthesize cysteine and serine proteases that, when inhaled by mammals, directly cleave the tight junctions connecting airway epithelial cells. This damage to the epithelium stimulates the release of alarmins, such as IL-33 and TSLP, and damage-associated molecular patterns (DAMPs), namely ATP and uric acid. Alarmins can directly stimulate the secretion of Th2 cytokines, recruitment of eosinophils, and AHR. ATP influences mucin production and recruitment of neutrophils. Uric acid has been shown to be critical to the development of Th2-type allergic inflammation and AHR in mice, and both DAMPs upregulate the recruitment of eosinophils to the damaged airway (1).

Mite allergens vary in size, with smaller particles able to penetrate more deeply into the lung. Larger particles, those 4.7 μm and greater in diameter, are more common and more likely to be inhaled. However, particles as small as 1.1 μm across may also be present in the indoor environment (40) and will be deposited further into the lung.

Clinical evidence suggests that the age at which sensitization to HDM allergen occurs has a large impact on the severity of related inflammatory responses. In a study

which compared the lung function of atopic and nonatopic children in Germany, 90% of the nonatopic children displayed normal lung function and no inflammatory symptoms. Atopic children, however, who had been sensitized to perennial allergens like HDM during their first three years of life, displayed poor lung function; their symptoms worsened when they were re-exposed to those allergens. A British cohort study spanning the first eight years of life found that sensitization to HDM in particular increased the risk of inflammatory respiratory disease in 87% of enrolled individuals. These studies suggest that children are particularly susceptible to the formation of future allergies during organogenesis and early physical development (40).

### *1.2.2 Murine Sensitization to House Dust Mite Allergen*

Mice can be sensitized to ovalbumin (OVA), house dust mite allergens, cockroach allergens, *Aspergillus fumigatus*, or ragweed pollen; some of these approaches may require the addition of adjuvants such as alum or endotoxin. All of these immunizations are capable of eliciting a Th2 response and allergen-specific IgE production. Subsequent exposures to the same allergen following sensitization evokes in murine models all the classical signs of asthma in humans, i.e., AHR, eosinophilia, and goblet cell hyperplasia (2).

The currently accepted murine model of asthma involves HDM allergens. Some researchers focus on specific elements of HDM extract, such as Der p 2, a cysteine protease borne by *Dermatophagoides* species (41). Experimental models utilizing this allergen often include a purified suspension of Der p 2, produced by expression of the protease by *E. coli* and subsequent affinity chromatography and dialysis (41). The sensitization and

challenge protocol has in many cases been adapted from existing protocols for OVA sensitization. These may include whole HDM extract and/or purified Der p 2 in combination with alum to ensure an allergenic response; and the sensitization exposure, whether intranasal or intraperitoneal, is always followed by subsequent challenge exposures a number of days thereafter (41, 42).

While this method is effective in generating an inflammatory response in the mice, it is not an ideal model of asthma. Real-world exposure to *Dermatophagoides* includes an array of allergenic and inflammatory components; utilization of any single substance in isolation will necessarily yield an inaccurate depiction of reality. Neither does reliance on adjuvants, e.g., aluminum hydroxide, mirror *in situ* exposure. Even the traditional model of OVA-induced asthma presents an incomplete picture of true allergic asthma: OVA is not naturally allergenic to humans and is thus limited in clinical relevance; and prolonged exposure of mice to OVA can induce immunological tolerance and reduced responsiveness of the airways (43). Furthermore, the sensitization step of the OVA protocols typically uses dermal, subcutaneous, or peritoneal routes of exposure, rather than introducing the allergen directly to the respiratory tract (43).

In contrast, the model originally devised by Hammad *et al.* utilizes a commercially-prepared extract of whole dust mite (Greer Laboratories), which is injected directly into the trachea. This elicits localized sensitization of the airways, rather than systemic sensitization, and no adjuvant is necessary to achieve the desired inflammatory response (44). This presents a more realistic exposure method and a clinically-relevant—indeed ubiquitous—human allergen, with no additives present to obscure the effects of that

allergen (45). We have since adapted this model to utilize oropharyngeal instillation rather than intratracheal injection, as this more accurately mimics natural inhalation exposures.

## Chapter Two

## 2.0 The Interaction of Multi-Walled Carbon Nanotubes with Allergic Inflammation in the Lung

### 2.1 An Introduction to Multi-Walled Carbon Nanotubes

Multi-walled carbon nanotubes (MWCNT) are a type of engineered nanoparticle (ENP). ENP are defined as being smaller than 100 nm in any one dimension (46). Carbon nanotubes (CNT) are a category of ENP constructed of carbon lattices. These include single-walled carbon nanotubes (SWCNT)—which are simply a single layer of graphite rolled into a hollow tube—and MWCNT. MWCNT, accordingly, are ENP composed of multiple graphitic sheets rolled and nested to form a series of concentric tubes—the outermost bounds of which are <100 nm in either diameter or length. Many CNT are between 0.7 and 10 nm in diameter, and MWCNT typically display interlayer spaces of 0.342-0.375 nm. They may be as long as several mm (47).

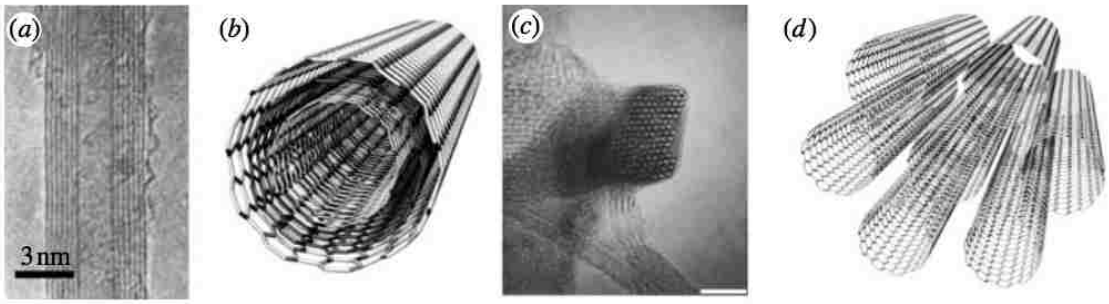



*Fig. 2.1:* Basic carbon nanotube morphology, including TEM images (a, c) and schematic drawings (b, d). Images a and b depict the structure of a multi-walled carbon nanotube; c and d depict a bundle of single-walled carbon nanotubes. (Adapted from (47).)

Because of their highly stable chemical structure and high aspect ratios, MWCNT and similar engineered carbon nanomaterials possess extraordinary thermal conductivity and tensile strength (48). These qualities make MWCNT useful in a wide range of applications, ranging from electronics to medical implants and drug delivery mechanisms (47, 49). However, the same qualities that make MWCNT useful in industrial applications may also make them bioactive. Exposure to MWCNT is of particular interest because of their small size and fibrous structure. The dimensions of some such particles are very similar to those of other mineral fibers, such as asbestos, and animal studies have demonstrated correspondingly similar pulmonary effects, including pulmonary inflammation and rapidly-developing fibrosis (50). 54 animal studies were reviewed by NIOSH. 44 of these reported pulmonary inflammation; 27 observed granuloma formation; and 25 studies gave evidence of fibrosis following nanotube inhalation.

Available data further reveals that carbon nanotubes elicit fibrosis in less time and at lower dosages than ultrafine carbon black or quartz dust (50). The myriad types of nanotubes vary in shape, size, chemical functionalization, and catalyst contamination; these differences make understanding their potential as health hazards a particularly complex problem (50). Characteristics of toxicological interest include length, diameter, aggregation, aspect ratio, rigidity, contamination, and release of ROS (50). Particle retention in the lung is a major concern, given the aspect ratio and rigidity of MWCNT (50). Both SWCNT and MWCNT have been found in the lungs of mice months after pharyngeal aspiration (51). The relative degrees of human risk posed by the many varieties of CNT are critical topics of study.

Carbon nanotubes with high aspect ratios appear to be more dangerous than their shorter counterparts. Of four types of nanotubes injected into the peritoneal cavities of mice, three retained their original lengths and elicited both inflammation and fibrosis. The remaining sample, which was less durable and broke into shorter pieces, induced considerably less inflammation (52).

The toxicity of multi-walled carbon nanotubes can also be affected by the degree of particle aggregation, which in turn is determined by the relative flexibility and aspect ratio of the nanotubes in question (53). MWCNT have a strong tendency to aggregate into bundles, or assemblages. Those assemblages group into clumps, which are mostly non-respirable. Thus, those larger agglomerates must be broken up before experimental inhalation and are less likely to pose a health threat in real-world exposures; smaller assemblages can be more easily respired. Assemblages of all sizes may be electrochemically bonded, and attempts to disperse them can therefore cause nanotube breakage and subsequent formation of even larger agglomerates (48).

## 2.2 Regarding the Nickel Content of MWCNT

Nickel has been identified as the most prevalent contact allergen, and cause of contact hypersensitivity, in the industrialized world (45). Approximately 15% of the global population suffers from contact sensitivity to metals; of these, allergy to nickel is the most common (54). Though originally an occupational hazard of the metal-working industry, since WWII nickel sensitivity has become increasingly common in the general population, largely due to fashion and lifestyle trends (55). An estimated 65 million Europeans have been sensitized to $Ni^{2+}$ and experience allergic contact dermatitis in

response to jewelry, coins, and other belongings made with nickel-containing alloys (45). The precise molecular mechanisms of nickel sensitivity are still being investigated, but the evidence to date suggests a unique sensitization process.

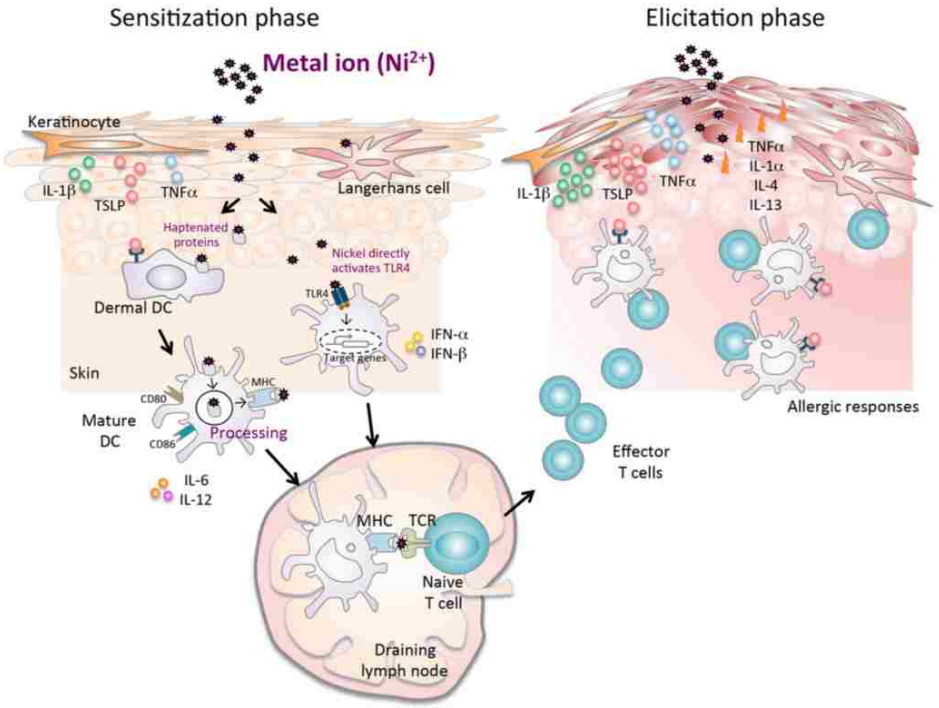



*Fig. 2.2:* Sensitization to nickel (II) ions. Ions stimulate production of inflammatory cytokines by epithelial cells, triggering antigen presentation by APCs to naïve T cells. Subsequent re-exposure induces proliferation of antigen-specific T cells and migration to the site of exposure. (Adapted from (54).)

When nickel (II) ions are released from alloys present in, or in contact with, the body, these ions activate epithelial cells, which produce cytokines such as IL-Iβ and TNF-α. Those cytokines are recognized by antigen-presenting cells (APCs), including DCs,

which then migrate to the draining lymph nodes and present the ionic allergen to naïve CD4+ T cells. Upon repeated exposure to the nickel allergen, activated antigen-specific T cells enter the bloodstream and stimulate the visible signs of hypersensitivity at the site of exposure (54).

Unlike other allergens, which act only indirectly on Toll-like receptors, nickel triggers PRR signaling directly by activating NF-κB, IRF3, and p38/MAPK/MKK6 signal transduction cascades (56, 57). This makes nickel the only contact allergen to directly trigger NF-κB-dependent activation of DCs. It has been further demonstrated that TLR4 is necessary to the development of nickel sensitivity in humans (56). This mechanism is dependent on non-conserved histidine residues and can therefore not be implemented in mice without some engineering. Most murine models of nickel allergy utilize adjuvants to elicit the desired inflammation, but it is also possible to accurately model nickel allergy by using a transgenic mouse transfected with the human *TLR4* gene (56, 57). It has also been observed that expression of the TSLP receptor is upregulated during nickel-induced inflammation, which suggests a role for TSLP involvement. Inhibition of TSLP synthesis decreased the delayed-type hypersensitivity reactions in mice exhibiting nickel-induced allergy (57).

The nickel content of ENP is thus of immunological concern. A variety of techniques exist for the manufacture of ENP and MWCNT in particular; some of the methods most commonly used to generate industrial quantities of MWCNT rely on chemical-vapor deposition or the catalytic decomposition of hydrocarbons (47). Such methods require the addition of transition metal catalysts to the forming nanomaterial. Though these strategies are aptly suited for large-scale production, the use of catalysts

typically results in decreased purity of the finished product, as metal residues contaminate the carbon lattice and diminish the overall stability (47).

The existing body of evidence suggests that the nickel content is also of toxicological importance. Shvedova *et al*. reported that the degree of metal content in CNT appeared to be a factor in determining the cytotoxicity of that sample. Specifically, higher percentages of iron and nickel catalysts generated greater amounts of hydroxyl radicals and significantly decreased cellular viability *in vitro* and *in vivo* (58, 59). Lam *et al.* observed that while all CNT containing a metal catalyst produced adverse lung effects, whether in small proportion or large, CNT contaminated with nickel in particular were more cytotoxic than others (60). It has also been reported by our own colleagues that nickel-bearing MWCNT elicited significant lung pathology (61). This study utilized IL-1β and IL-18 release as metrics of NLRP3 inflammasome activity, and both of these cytokines were significantly upregulated in the lungs of mice dosed with nickel-contaminated MWCNT. Furthermore, increased release of these cytokines correlated with increased nickel content in the MWCNT used, while direct cytotoxicity did not correlate. This suggests that while nickel content may not be a reliable predictor of ENP toxicity, it is intimately involved with the stimulation of inflammatory responses following introduction of ENP to the lung (61). In the following study, we examined the capacity of nickel-bearing MWCNT to exacerbate existing lung inflammation.

**2.3**

# A role for Cysteinyl Leukotrienes in promoting airway inflammation elicited by multi-walled carbon nanotubes.

Sophia Carvalho[1], Maria Ferrini[1], Zeina Jaffar[1] and Kevan Roberts[1]

**Running Title:** MWCNT induced inflammation

[1] Center for Environmental Health Sciences, Biomedical & Pharmaceutical Sciences, Skaggs Building, University of Montana, Missoula, MT 59812.

Correspondence: Address correspondence and reprint requests to Dr. Kevan Roberts, Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, Skaggs Building, The University of Montana, Missoula, MT 59812. Tel. (406)-243-4034, FAX. (406)-243-2807. E-mail address: kevan.roberts@umontana.edu

### 2.3.1 Abstract:

Multi-walled carbon nanotubes (MWCNT) are used in a broad range of applications, including medical treatments, construction in the aerospace industry, and electronics manufacture. As demand for nanomaterials expands, so does the urgency of establishing an appreciation of the degree of health risk associated with their production and use. Inhalation represents a likely route of exposure; consequently, in this study we investigated whether MWCNT influenced the development of allergic inflammation. MWCNTs introduced into the lungs of mice resulted in a rapid increase in the number of eosinophils present in the airways that was observable within 24h. The inflammatory response elicited was also associated with an increase in the level of cysteinyl leukotrienes (cys-LT) present in the BALF (bronchoalveolar lavage fluid). In contrast, the eosinophil response developed in the absence of detectable amounts of the Th2-associated cytokines IL-4, IL5, or IL-13. Cys-LT were implicated in the inflammatory response since inhibition of cys-LT biosynthesis using the 5-lipoxygenase inhibitor, Zileuton, resulted in marked reduction in the severity of inflammation observed. Moreover, MWCNT instilled into mice with pre-existing allergic lung inflammation, elicited by house dust mite (HDM), markedly exacerbated the level of airway inflammation, which was characterized by a pulmonary eosinophilia, lymphocyte infiltration and raised cys-LT levels. The intensity of the inflammatory response elicited by either MWCNT alone, or in conjunction HDM allergen, correlated with the level of nickel present in the material, since preparations that had higher levels of nickel (FA21: 5.54% Ni by weight) were highly effective at eliciting inflammatory responses while preparations containing lower amounts of nickel (FA04: 2.54% Ni by weight) failed to initiate or exacerbate allergic inflammation. In summary,

MWCNT in the lung were found to promote eosinophilic inflammation and exacerbate existing allergic lung inflammation via the induction of cys-LT biosynthesis. These findings suggest that exposure to airborne MWCNT is likely have adverse health effects in individuals suffering from atopic asthma, and further investigation of the potential therapeutic effects of blocking leukotriene synthesis may prove helpful.

### 2.3.2 Introduction:

The commercial demand for nanoparticle-based materials has expanded rapidly in recent years. More than 190,000 metric tons of nanoparticle-based composites were produced in 2012, and that quantity is projected to exceed 500,000 metric tons by 2019. As the industry grows, the likelihood of workers and consumers being exposed to airborne nanoparticles increases. Thus, assessment of any health risks associated with these materials will become increasingly important. Of the many types of nanoparticles available, multi-walled carbon nanotubes (MWCNT) are among the most useful for industrial and medical applications. MWCNT possess excellent tensile strength and electrical conductivity and can be easily functionalized for medical use (62). However, the same properties that make MWCNT so useful also make them potentially hazardous. By design, MWCNT have high aspect ratios and, while only a few nanometers wide, may be several micrometers long (63). As in the case of asbestos fibers, MWCNT are able to aggregate within the lung interstitium and are not easily degraded by alveolar macrophages. Health effects similar to asbestosis have been reported, including pulmonary fibrosis and granuloma formation, in addition to lung inflammatory responses and cytotoxicity (64, 65). There have been significant advances in our understanding of MWCNT toxicity, yet the cellular and molecular events that underpin its inflammatory capacity remain unclear. In this study we examined the ability of MWCNT to elicit and exacerbate allergic lung inflammation and sought to determine the role of cys-LT in this process.

Allergic asthma is a complex and chronic inflammatory disease of the airways that affects more than 300 million people worldwide. In highly populated regions of the globe, which include both North and South America, almost 85% of asthmatics are allergic to

house dust mite (HDM) (66, 67). Asthma is characterized by airway hyperreactivity, contraction of the smooth muscle, goblet cell hyperplasia, increased mucus production and eosinophil recruitment. Many of these pathological elements are thought to be a consequence of the activation of Th2 cells and their associated cytokines, including IL-4, IL-5, IL-9, and IL-13. In this study, MWCNT entering the airways were shown not only to promote the onset of eosinophilic inflammation but also to exacerbate established allergic lung inflammation. The intensity of these effects was shown to be highly dependent on the level of nickel present in the material since MWCNT with low levels of nickel were poor at promoting inflammatory responses. The eosinophilic inflammation elicited following MWCNT exposure required the action of cys-LT since this process was inhibited by treating mice with a 5-LO inhibitor.

### 2.3.3 Materials and Methods:

*Mice*

C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) (10-16 weeks old) were used throughout this study. Mice were bred under pathogen-free conditions in a barrier facility and experimental animals maintained in micro-isolator cages and treated in accordance with National Institutes of Health guidelines and the American Association of Laboratory Animal Care regulations. Animal experiments were approved by the University of Montana, Institutional Animal Care and Use Committee, IACUC according to National Institute of Health guidelines.

*Media*

Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (CM-RPMI), (Hyclone Fetal Calf Serum, Thermo Scientific), L-glutamine, penicillin & streptomycin, HEPES, Sodium Pyruvate (Gibco Life Technologies) and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO).

*Intratracheal Administration of Multi-Walled Carbon Nanotube Preparations*

The inflammatory response elicited by MWCNT entering the airways of naïve and HDM primed mice was examined. MWCNT containing 5.54% or 2.54% nickel (FA21 and FA04 respectively) were administered to adult wildtype C57BL/6 mice by oropharyngeal aspiration. The MWCNT used in this study were characterized by the Research Triangle Institute as part of the National Toxicology Program (NTP). Nanoparticles used were

originally sourced by the NTP from SI, Inc. and MKI Corp., as described by Girtsman *et al.* (65), kindly donated by Dr. Andrij Holian (University of Montana, Table 1).

| Variety | Nickel content (% total mass) | Diameter (nm) | Length (nm) | Agglomerate size (nm) (average) |
|---|---|---|---|---|
| FA21 | 5.54 | 27 | 5-15 | 429 |
| FA04 | 2.54 | 33 | 5-15 | 682 |

Table 1: Physical characteristics of MWCNT used.

To administer MWCNT (50 μg), 30 μL of MWCNT suspension (1.67 mg/mL) was instilled into C57BL/6 mice via oropharyngeal aspiration as previously described (65). Briefly, mice were anesthetized with inhaled isoflurane, then suspended from piano wire by the upper incisors and MWCNT suspension was pipetted into the trachea. The level of airway inflammation and fibrosis was examined after 24h and 6 days.

*Sensitization of Mice to House Dust Mite Allergen (HDM) Prior to Instillation with MWCNT*

To prime mice to HDM and promote allergic lung inflammation, mice were sensitized to HDM on day 0 by the intranasal administration of 100 μg of HDM allergen (*Dermatophagoides pteronyssinus*, Greer Laboratories) in 30 μl of PBS and then on days 7 and 14 by intranasal treatment with 50 μg of HDM (30μl total volume). HDM allergen preparations used throughout this study contained minimal levels of LPS. Control groups

comprised mice receiving 30 µl of PBS on days 0, 7 and 14. To determine the level of mucosal inflammation, BALF and lung tissue were harvested on day 16, 48h after the last challenge. To examine the proinflammatory properties of MWCNT, mice were additionally challenged with a single 50 µg dose of MWCNT (30 µl of a 1.67 µg/mL suspension) administered oropharyngeally 24h prior to harvest (Figure 2.3).

*Determining the Level of Pulmonary Inflammation*

Mice were euthanized 24 hours post-MWCNT instillation. Lung lavage was performed and whole lung tissues were harvested. BALF was divided for analysis of cell populations via flow cytometry, cysteinyl leukotriene (cys-LT) and cytokine production via EIA and sandwich ELISA, and eosinophil peroxidase (EPO) by colorimetric assay. Lung tissues were reserved for histological examination using H&E and trichrome stains. Bronchoalveolar lavage was performed to collect bronchoalveolar lavage fluid (BALF) for analysis. Eosinophil peroxidase (EPO) levels in the lavage cells were determined by colorimetric analysis using orthophenylene diamine dihydrochloride as detailed previously (68). Cell differential percentages were determined by light microscopic evaluation of Hema3-stained cytospin preparations and expressed as absolute cell numbers. Lung tissue was dispersed by collagenase (Type IV; Sigma-Aldrich), and lung mononuclear cells (LMC) were isolated by Percoll (Sigma-Aldrich) density gradient for functional analysis.

*Measurement of Cytokines*

BALF chemokine or cytokine levels were determined using ELISA for measurement of IL-4, IL-5 (Duoset, R&D Systems) and IL-13 (Quantikine, R&D

Systems), or using the sensitive Mouse V-Plex Pro-Inflammatory Panel 1 assay and Meso Quickplex 120 reader (MesoScale Discovery, MD) for measurement of other cytokines (IL-4, IL-5, TNF-α, IFN-γ and IL-12p70).

*Histological Determination of Peribronchial Inflammation and Collagen Deposition*

Lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin using a Leica ASP 300 tissue processor (Leica, Bannockburn, IL). Microtome sections were cut at 5μm thickness and stained with hematoxylin and eosin (H&E) using a Shandon Varistain 24-4 (Thermo Fisher Scientific). In addition, sections were stained using Gomori's Trichrome (EMD Chemicals, Gibbstown, NJ) for histological analysis using a Thermo Shandon automated stainer. The level of peribronchial inflammation (H&E stained) or collagen deposition (Trichrome stain) was analyzed by microscopy, and the transmitted light images were collected on a Nikon Eclipse 800 microscope equipped with an Olympus DP 26 camera and cellSens software (Version 1.9).

*Measurement of Cysteinyl Leukotrienes*

Prior to assay, the IgG present in the BALF (500 μl) was depleted by incubating with 50 μl of packed protein G-Sepharose beads (GE Healthcare). After centrifugation BALF was assayed for the presence of cysteinyl leukotrienes (Express ELISA, Cayman Chemical, Ann Arbor, MI) and absorbance was read at 405 nm.

*Flow Cytometry*

Cells (LMC, BALF, or splenic cells) were FcγR blocked using 2.4G2 antibody (ATCC) and stained with combinations of the following mouse conjugated mAb (all purchased from BioLegend): allophycocyanin (APC) or FITC anti-CD3, APC/Cy7 anti-CD4, PE anti-CD8a, APC or PE anti-CD11c, PE or APC/Cy7 anti-I-A/I-E, APC/Cy7 anti-Ly6G, APC or APC/Cy7 anti-Ly6C, APC/Cy7 anti-Ly-6G/Ly6C (Gr-1), PE, FITC or Brilliant Violet 421 anti-CD11b, APC or PE anti-F4/80. In addition, PE anti-Siglec-F (BD Biosciences) was used to stain eosinophils. Flow cytometric acquisition was performed on a FACSAria II (BD Biosciences) by 4-color analysis using FACSDiVa software and FlowJo, with a minimum of 50,000 live, single-cell events per sample collected.

*Treatment of Mice with Zileuton to Inhibit MWCNT-Induced Inflammation*

C57BL/6 mice were treated by the oropharyngeal administration of either Zileuton 10 mg/Kg (Tocris Biosciences) or vehicle (0.5% methyl-cellulose/0.2% Tween 80 in water) 3h before administration of 50 μg of FA21. Controls comprised mice treated with carrier alone. The level of inflammation elicited was evaluated by harvesting BALF and monitoring eosinophils and cys-LT levels.

*Statistical Analysis*

Data were analyzed using GraphPad Prism 5.0 (GraphPad, La Jolla, CA). Results involving two variables were analyzed by two-way ANOVA with a Bonferroni post-hoc test. Data comparing two groups were analyzed using an unpaired t test. Figures show combined data from multiple studies or independent repeats (two or more). Data shown are

mean ± SEM. A p value < 0.05 was considered statistically significant. Significance denoted by *, **, or *** is defined as $p<0.05$, $p<0.01$, or $p<0.005$, respectively.

### 2.3.4 Results:

*Characterization of the Inflammatory Response Elicited Following MWCNT Inhalation*

The two different types of MWCNT compared in this study were FA21 (Sun Nano) and FA04 (MK Impex Canada), both of which have been characterized (61) and shown to possess several similar physical characteristics which include similar specified length (5-15 pm) and diameter (27-33 nm). To discern their potential inflammatory properties, particles were instilled directly into the airways of mice and evidence of an inflammatory response was sought. A single instillation of 50 μg of FA21 MWCNT into the airways elicited the rapid development of inflammation after 24 hours (Fig. 2.4). The BALF recovered from C57BL/6 mice receiving FA21 instillations contained significantly raised numbers of eosinophils that were detected by increased levels of cell-associated EPO (Fig. 2.4A) and confirmed by cell differential staining of BALF cells (Fig. 2.4B). Both BALF EPO activity and eosinophil counts remained significantly raised for 6 days after a single instillation of FA21 ($p<0.05$). Increased numbers of neutrophils and macrophages were also observed in the BALF following MWCNT exposure; however, this did not reach significance and no evidence of lymphocyte infiltration was observed. To quantify cellular populations present in the airways after MWCNT exposure, inflammatory cells present in the BALF were also analyzed by flow cytometry (FACS Aria II, BD Biosciences) as previously described (69) (Fig. 2.4C). Eosinophils were identified through a gating strategy in which leukocytes were first selected by FSC and SSC, and CD11b+/F4/80- cells were further characterized by degree of Siglec-F and GR-1 expression. Eosinophils were identified by high expression of Siglec-F and neutrophils by GR-1 expression. Alveolar

macrophages were excluded on the basis of F4/80 staining. This process revealed that 6.43% of the cells recovered from the BALF 24 hours after exposure to FA21 were eosinophils, compared to 0% in the lungs of unexposed mice. While FA21 instillation resulted in the recruitment of only low numbers of CD11b+F4/80-Siglec-F-GR1+ neutrophils in the BALF (Fig. 2.4C), MWCNT instillation did result in a large increase in the number of neutrophils present in the lung tissues (i.e., following dispersion of the tissue with collagenase). Histological analysis of lung tissue using H&E and trichrome staining revealed that FA21-exposed mice developed a pronounced peribronchial and perivascular inflammation compared to control animals (Fig. 2.4D). Trichrome staining of the same lungs reveals negligible deposition of collagen, indicating that fibrogenesis had not yet begun within six days of MWCNT instillation.

*A Role for Cysteinyl Leukotrienes in Promoting MWCNT-Induced Inflammation*

Although FA21 promoted the rapid onset of an eosinophil recruitment to the airways, Th2 cytokines were universally absent from the BALF of FA21-exposed mice, as evidenced by FA21 exposure not resulting in a significant increase of IL-13 levels in the BALF (Fig. 2.5). The chemokine $CX_3CL1$ plays an important role in the recruitment of monocytes and NK cells to the tissues and plays an important role in homeostatic and inflammatory states (70). While $CX_3CL1$ was constitutively present in the BALF of naïve C57BL/6 mice, FA21 instillation resulted in a significant reduction in its levels evident both 1 and 6 days after exposure. IL-22 has previously been shown to inhibit eosinophilic inflammation during allergic inflammation (71, 72). Given that eosinophils were the principal inflammatory cell present in airways of FA21-exposed mice, the presence of

eosinophil chemotactic and survival factors was sought. It has been previously reported that cys-LT can promote eosinophil migration (73) and leukotrienes play an essential role during allergic lung inflammation (74). Interestingly, only low levels of cys-LT were present in the BALF of mice that were instilled with vehicle. In contrast, the oropharyngeal administration of FA21 resulted in the marked increase in the level of cys-LT present 24 h later in the BALF, increasing from 184 pg/mL in vehicle challenged to 751 pg/mL in response to MWCNT exposure (Fig. 2.5). Since eosinophils can themselves produce cys-LT (75), it was important to resolve whether their raised levels in the BALF were simply the result of increased numbers of eosinophils or whether already-present cys-LT were the cause of eosinophil migration into the airway. To examine the latter possibility, the effect of blocking leukotriene biosynthesis was ascertained by treating mice with an inhibitor of 5-lipoxygenase. To this end, mice instilled with FA21 were treated with the 5-lipoxygenase inhibitor, Zileuton (Tocris Biosciences), and the effect on MWCNT-induced inflammation was investigated. Mice were pre-treated with Zileuton (10 mg/Kg) 3h before the oropharyngeal administration of 50 μg of FA21 of vehicle. Twenty-four hours later the level of pulmonary inflammation and level of cys-LT present in BALF were determined. Interestingly, pretreatment of mice with Zileuton reduced the cys-LT levels present in the BALF by 62% ($p<0.01$) (Fig. 2.6A) in mice exposed to MWCNT. The reduction in the level of cys-LT was coincident with a corresponding decrease in the degree of observed eosinophilia (Fig. 2.6B).

*The Exacerbation of HDM-Induced Allergic Lung Inflammation by MWCNT*

At the time points examined, the level of eosinophilic inflammation elicited by FA21 alone was markedly less severe than that typically elicited by known allergens like house dust mite. This is likely a consequence of the low numbers of circulating eosinophils present in a non-allergic animal. To assess the potential of MWCNT to exacerbate airway inflammation in allergen-sensitized animals, we added a single instillation of FA21 to the final day of our murine model of allergic asthma (Fig. 2.6). As the house dust mite is a globally ubiquitous allergen to which as many as 85% of asthmatics react (1), this model uses a series of three doses of HDM over two weeks to sensitize mice and prompt an allergic response. Observed inflammatory responses correlate with the findings of our previous studies (69), with mice sensitized to HDM displaying significantly elevated eosinophilia and cys-LT production compared to sham-treated mice (Fig. 2.7). Interestingly, mice that were sensitized to allergen and further dosed with FA21 showed a marked increase in inflammation compared to mice given only allergen or only FA21. Differential cell counts reveal a clear influx of eosinophils into the airways, most notably in mice exposed to both allergen and MWCNT. Alveolar macrophages and lymphocytes were also present, in much lower numbers (Fig. 2.7A). Specifically, exposure of either naïve mice, or mice treated with HDM allergen, to FA21 did not result in increases in the number of BALF CD4+ or CD8+ T cells (Supplemental Fig 2.11). As before, EPO measurements confirmed the presence of a considerable eosinophil population in mice instilled with MWCNT (Fig. 2.7B). BALF was further analyzed by flow cytometry; this revealed a striking ten-fold increase in the airway eosinophil population of dual-exposed mice over those exposed only to MWCNT (Fig. 2.7C). This inflammatory response can be visualized quite clearly in histological images of lung tissue harvested from such mice just

24 hours after MWCNT instillation (Fig. 2.7D). Dual-exposure also elicited significantly greater degrees of cys-LT biosynthesis than did either single exposure (Fig. 2.7E). This confirms that MWCNT introduced to the lungs is indeed capable of exacerbating allergic inflammation already in progress.

*Correlation between the Degree of Inflammation Elicited and the MWCNT Nickel Content*

To determine whether differences in physical properties could be correlated with the relative pro-inflammatory properties of FA21 and F04, each of these materials was administered and the inflammatory processes compared. Previous analysis of the physical properties of FA21 and FA04 materials revealed that both form agglomerates (agglomeration sizes being 712 nm and 675 nm, respectively) with the major contaminant in both being nickel (comprising 5.54% and 2.54% respectively). As detailed previously (61), instillation of 50 μg of FA21 into the lungs of naïve C57BL/6 mice elicited a strong eosinophilic response evident 24 hours later. However, in marked contrast to FA21, the oropharyngeal instillation of an equivalent amount of FA04 did not result in a pulmonary eosinophilic response that was significantly different from mice treated with vehicle or the untreated controls, as measured by cell-associated BALF EPO activity (Fig. 2.8A). Importantly, mice exposed to FA21 displayed noticeably higher levels of cys-LT level in the BALF (720 ng/mL) compared to mice given FA04 (Fig. 2.8B). Consistent with the eosinophilic response elicited, FA04 instillation failed to raise BALF cys-LT levels significantly above what was found in the vehicle-treated control group (Fig. 2.8B). To further investigate the biological properties of these two materials, their pro-inflammatory properties were compared in mice which had been sensitized and challenged with HDM

allergen. Pulmonary eosinophilia elicited by HDM allergen was over three-fold higher than that caused by FA21 administration alone (Fig. 2.9). In HDM-challenged animals the instillation of FA21 24 hours prior to harvesting BALF resulted in a two-fold exacerbation of the level of pulmonary eosinophils present as determined by cell differential counts and cell-associated EPO activity (Fig. 2.9). In marked contrast, FA04 exposure 24 hours prior to harvest resulted in no significant difference in the level of eosinophilia over that elicited by the HDM allergen alone (Fig. 2.9). The marked differences in observed inflammatory responses elicited correlates with the level of nickel contamination of the two materials (5.54% by weight of FA21 is residual nickel catalyst compared to 2.54% of the mass of FA04). These observations suggest a biologically active role for this contaminant and likely a causative role for nickel in the development of MWCNT-induced pulmonary inflammation.

### 2.3.5 Discussion:

As a consequence of the growth of the nanotechnology industry, there has been a striking increase in the types and quantity of MWCNT currently in production. This trend raises the likelihood of human exposure to these particles and necessitates a better appreciation for their potential impact on human health. The health risks arising from the respiration of MWCNT particles are of concern since this material is fibrous and bears several characteristics displayed by asbestos. MWCNT entering the airways of mice have been shown to elicit several proinflammatory events that originate from the activation of the NLRP3 inflammasome (61) and the induction of IL-6, TNF-α and IL-1β (48) and are associated with the formation of granulomas and development of airway fibrosis (63, 76, 77). MWCNT exposure also causes epithelial damage (48, 61, 78) by a process involving COX-2 (79) and is associated with epigenetic regulatory events arising from changes in global methylation patterns (80).

Our study evaluated the inflammatory and fibrotic responses elicited by inhaled MWCNT particles and investigated whether such exposures exacerbated pre-existing allergic lung inflammation. Strikingly, the entry of MWCNT into the airways of naïve mice elicited the rapid recruitment of CD11b+F4/80-GR-1- eosinophils into this compartment and CD11b+F4/80-GR-1+ neutrophil recruitment to the lung. Instillation of MWCNT was associated with the development of a mild peribronchial and perivascular inflammation and collagen deposition in the lungs of MWCNT-exposed animals, which could be detected by trichrome staining six days after exposure.

While the inflammatory response induced by MWCNT alone was present for several days, this response was only 10% of that observed following inhalation of a known

allergen, HDM. Triggers for eosinophilic inflammation can either be non-allergic or allergic in nature with the former involving ILC2 activation and the latter pathway being dependent on T cells (81). The inflammatory response elicited by FA21 MWCNT revealed no detectable IL-5, IL-13, IL-22, $CX_3CL1$ or IL-33; however, there was a marked increase in the level of cys-LT in the BALF within 24h of a single instillation of MWCNT. Mast cells, eosinophils, basophils, dendritic cells and macrophages are known to be cellular sources of cys-LT.

Leukotrienes are mediators derived from arachidonic acid by the action of 5-lipoxygenase (5-LO) resulting in the generation of $LTA_4$ (73). $LTC_4$ is generated by the conjugation of glutathione to $LTA_4$; the extracellular derivatives of this mediator are subsequently formed by enzymes that generate $LTD_4$, which mediates airway constriction, and ultimately the stable metabolite $LTE_4$ (82). Cys-LT play an important role in the development of allergen-induced airway inflammation and airway hyperresponsiveness (AHR) (83). The induction of cys-LT biosynthesis following inhalation of MWCNT played a central role in the inflammatory process since inhibition of leukotriene production, using a 5-LO inhibitor, Zileuton, markedly suppressed the level of pulmonary eosinophilia. Both leukotriene $B_4$ and cys-LT are important lipid mediators that facilitate eosinophil recruitment in both man and mouse (73, 84). Two receptors for cys-LT have been described, CysLT1 and CysLT2. Cys-LT action via CysLT1 induces bronchoconstriction, tissue swelling, and increased mucus secretion, while activation of CysLT2 mediates inflammation, vascular permeability, and fibrosis (37). Cys-LT upregulate the expression of adhesion molecules by eosinophils (85, 86), leukocyte rolling (87) and chemokine expression (88).

Whether eosinophils present in the airways derive from cells resident in the lung tissue or are recruited from the blood remains unclear. The presence of resident, steady-state eosinophils in normal non-inflamed intestinal and lung mucosa has been described previously (89). A population of lung-resident Siglec$^{int}$CD62L$^{+}$ eosinophils containing a ring-shaped nucleus have been described as being IL-5 independent (90). This raises the possibility that cys-LT play a central role in promoting the migration of steady-state eosinophils into the airways. An additional proinflammatory property of cys-LT is that it synergizes with IL-33 to activate murine type 2 innate lymphoid cells, ILC2s (30, 91). ILC2s secrete IL-5 constitutively and express IL-13 on activation, consequently playing important roles in promoting eosinophil recruitment to the airways and promoting their survival (92, 93) and homeostasis (89). IL-33 has also been linked to the release of IL-13 and subsequent increase in AHR and eosinophilia within 24 hours of MWCNT exposure (94).

Several therapeutic agents, such as Montelukast (Singulair, Merck & Co., Inc.), specifically target CysLT1 while Zileuton (Zyflo) was the first USFDA approved competitive inhibitor of 5-lipoxygenase used for the treatment of atopic asthma (95). Leukotriene (LT) modifiers are anti-inflammatory drugs that are used in combination therapies with first-line asthma-controller medications. This suggests that a proven safe and effective drug can reverse some of the damaging effects arising from human exposure to MWCNT. Since blockade of 5-LO action results in inhibition of eosinophils, it appears that 5-LO and cys-LT could play critical roles in the development of airway inflammation.

Although FA21 MWCNT were effective at promoting eosinophilic inflammation in naïve mice, it was important to compare the magnitude of this response to that elicited

by a known allergen. HDM is a clinically relevant allergen which elicits pulmonary inflammation following inhalation without the need to resort to the use of immunological adjuvants (96). Repeated inhalation of HDM allergen results in the activation of both innate and adaptive immunity characterized by a pronounced $CD4^+$ Th2 response, generation of specific IgE and infiltration of eosinophils into the airway (97), processes that typify the disease process in atopic asthma. In addition, it was important to resolve whether this material exacerbated previously established allergic lung inflammation. This was particularly relevant given the evidence that MWCNT appeared to promote eosinophil recruitment, a response that would be expected to be elevated if the number of circulating eosinophils was raised as during allergic inflammation.

Both ILC2s (93, 98-100) and CD4+ T cells (24, 101) contribute to the inflammatory responses elicited by HDM; however, it is unclear which response was most influenced by the MWCNT. Instillation of FA21 resulted in a two-fold increase in the number of eosinophils entering the airway and a dramatic increase in the intensity of peribronchial and perivascular inflammation. As previously detailed, the exposure of mice to HDM resulted in raised levels of cys-LT present in the BALF; however, the levels of this mediator were doubled by instilling MWCNT into the airways 24h prior to analysis. The observed exacerbation of the inflammatory response suggests that exposure to these materials may have profound health consequences in individuals suffering from atopic asthma. This effect likely stems from the innate ability of MWCNT to elicit cys-LT release and the subsequent contribution of these mediators to airway inflammation (Supplemental Fig. 2.11). The effectiveness of MWCNT in exacerbating existing allergic inflammation may prove more problematic than their ability to initiate eosinophilic inflammation. Since FA21

administration did not result in the augmentation of Th2 cytokines in the BALF, it is most likely that cys-LT directly promote the recruitment of eosinophils.

Dramatically lower eosinophilic responses were elicited following the instillation of FA04 compared to mice given FA21. The former preparation is known to contain significantly less nickel, implying that the difference in the inflammatory capacity of these two MWCNT may be a consequence of their nickel content. In the course of these studies, MWCNT containing low levels of nickel did not promote pulmonary eosinophilia when administered alone nor when administered to mice that had been previously sensitized to HDM allergen. The observation that nickel was essential for eliciting an eosinophilic response in the airway is intriguing and suggests that either the nickel content itself, or changes in physiochemical properties of the particles arising from nickel contamination, had profound effects on several physical attributes of the particles, such as their respective agglomerate sizes (Table 1). Moreover, instillation of FA21, but not FA04, was associated with the release of cys-LT, implying that the presence of nickel may facilitate the release of cys-LT.

Nickel associated with MWCNT has been shown to promote inflammasome activation (61) in murine alveolar macrophages. Critically, activation of the inflammasome promotes leukotriene biosynthesis (102). Nevertheless, it is important to note that the nickel levels in the FA04 preparation were reduced rather than the metal being absent (i.e., FA04 and FA21 preparations contained 2.54% and 5.54% respectively). Consequently, any disparity in inflammatory effects mediated by nickel is likely to derive from differences in the amount of biologically active or released nickel. Critically, nickel has well

characterized proinflammatory properties. Nickel is a frequent cause of contact sensitization in humans and elicitation of type IV delayed-type hypersensitivity (DTH), in part by activating the Toll-like receptor 4 (45). In contrast, nickel sensitization of mice does involve TLR4 although activation of innate immunity is evident in C57BL/6 mice (103) and a critical role for TSLP has been proposed in mice (57).

**Acknowledgements:** We thank Britten Postma, Pam Shaw (FACS Core), Lou Herritt and Diane Brooks (Histology Core) for their valuable technical assistance.

**2.3.6 Figure Legends:**

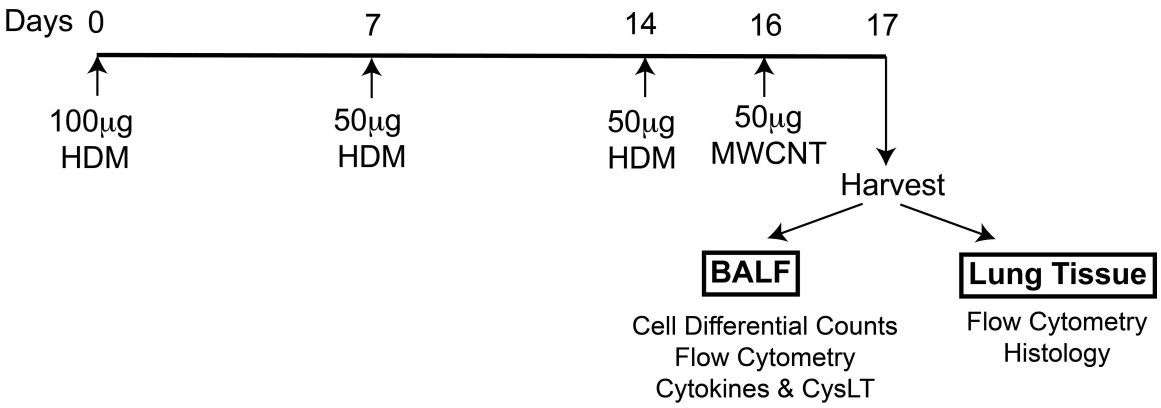



**Figure 2.3**

**Protocol to examine whether MWCNT exposure exacerbates the allergic inflammation elicited by HDM.**

To elicit allergic lung inflammation C57BL/6 mice were given a sensitization dose of HDM (100 μg) on day 0, and followed by two 50 μg challenge doses on days 7 and 14. To examine the proinflammatory properties of MWCNT, mice were additionally challenged with a single 50 μg dose of MWCNT (30 μL of a 1.67 μg/mL suspension) administered oropharyngeally 24h prior to harvest (day 16). Control/sham-treated mice were given PBS in place of HDM and MWCNT. The inflammatory response elicited was examined on day 16 by enumerating inflammatory cells in BALF and lung tissue.

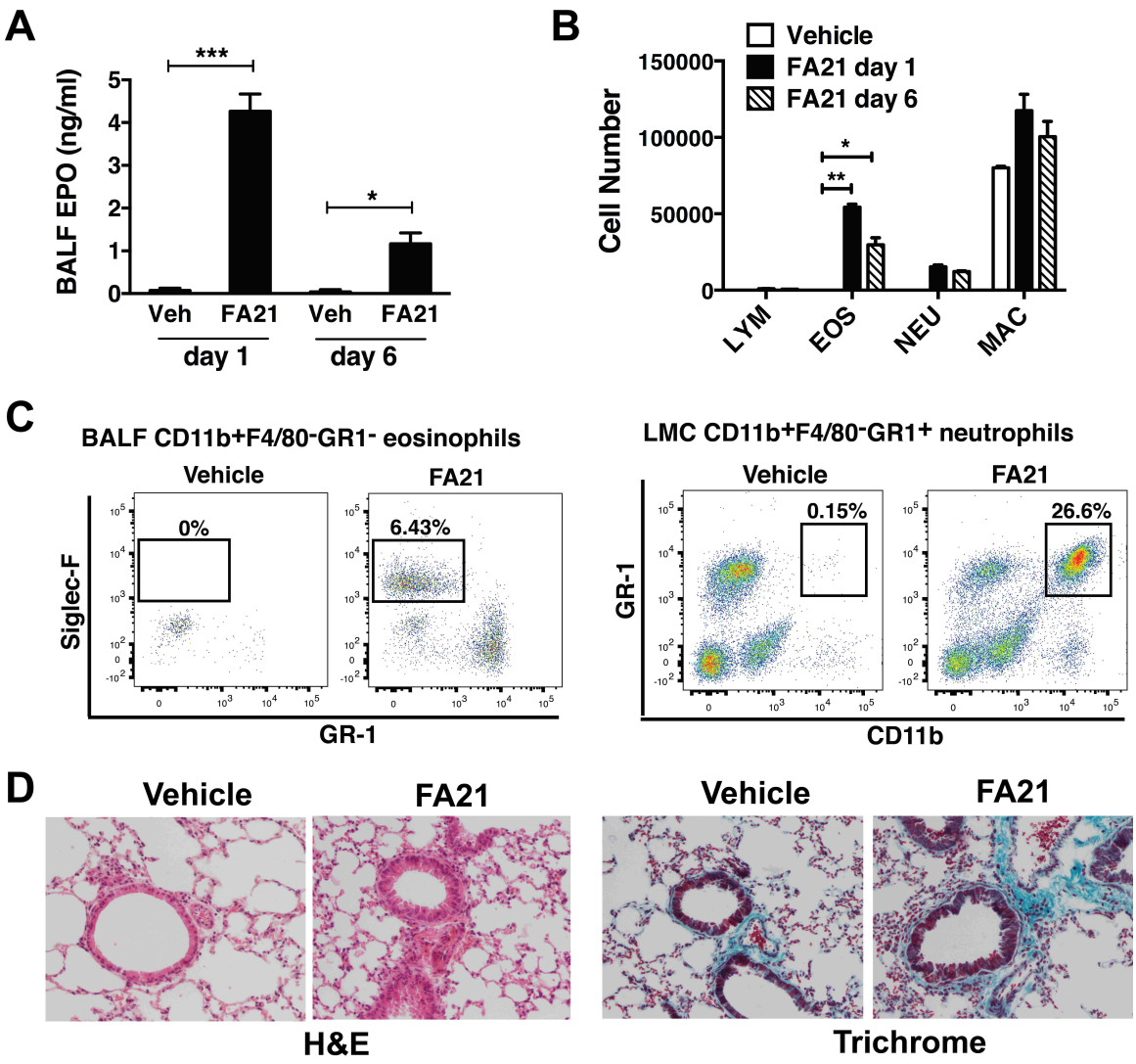



**Figure 2.4**

**Intratracheal administration of MWCNT (FA21) elicited pulmonary eosinophilic inflammation**.

To examine the inflammatory properties of MWCNTs, 50μg of FA21 was administered to C57BL/6 mice by oropharyngeal instillation (30 μL). Controls comprised mice treated with dispersion medium alone (vehicle). To examine the resultant inflammatory response, BALF and lung tissue were collected both one and six days after administration and the cellular composition examined. **(A)** EPO activity expressed by BALF cells was determined

after one or six days by colorimetric assay. EPO levels were notably elevated in mice dosed with FA21 alone compared to sham-treated mice after one day and remained significantly raised over six days (*$p < 0.05$ and ***$p < 0.001$ respectively). **(B)** Differential cell counts one day following MWCNT challenge, measuring the number of lymphocytes (LYM), eosinophils (EOS), neutrophils (NEU) and macrophages (MAC) present in BALF and expressed as the absolute cell number, revealed increased eosinophil recruitment to the airways. **(C)** The immune cells present in BALF from vehicle and FA21 exposed animals (one day) were examined by flow cytometry. Eosinophils were identified as CD11b+F4/80-GR1- cells expressing Siglec-F and neutrophils as CD11b+F4/80-GR1+ cells. In both cases, preliminary gating on FSC and SSC was performed, as described in the Methods. **(D)** Lung sections (5 μm) were stained using either H&E or Mallory trichrome revealing cellular infiltration and minimal collagen deposition, six days after MWCNT exposure. Groups comprised 4 mice ($n = 4$) and results are mean ± SEM *$p <$ 0.05, **$p < 0.01$ and ***$p < 0.001$. Data are representative of 3-4 independent experiments.

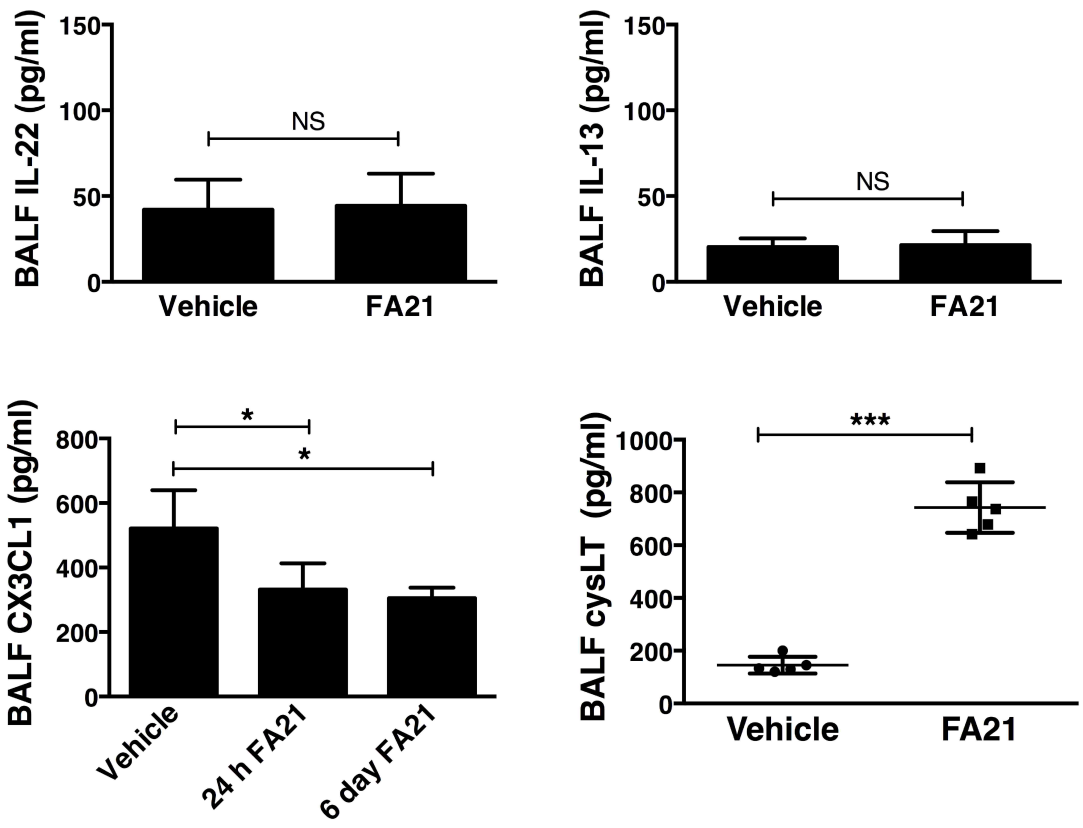



**Figure 2.5**

**The intratracheal administration of MWCNT resulted in the biosynthesis of cysteinyl leukotrienes in the absence of Th2-associated cytokines.**

C57BL/6 mice were challenged by the oropharyngeal instillation of 50μg of FA21 MWCNT or dispersion medium (vehicle alone). BALF from both groups of mice was prepared after 24h and the levels of IL-22, IL13, $CX_3CL1$ and cys-LT determined by ELISA. Cys-LT levels were raised 7-fold after 24h MWCNT exposure, IL-13 and IL-22 levels remained unchanged, while the level of $CX_3CL1$ was significantly decreased. Groups comprised 4 mice (n = 4) and results are mean ± SEM, *$p < 0.05$ and ***$p < 0.001$. NS = not significant. Data are representative of 3-4 independent experiments.

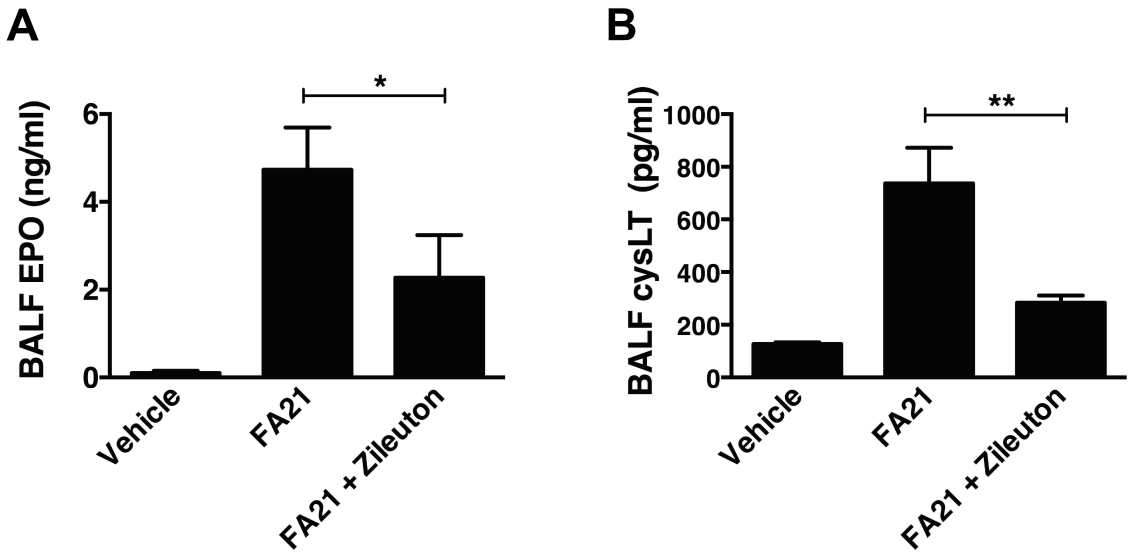



**Figure 2.6**

**Inhibition of cys-LT biosynthesis using a 5-lipoxygenase antagonist also inhibited MWCNT-induced airway inflammation.** To examine whether cys-LT contributed to the inflammation, C57BL/6 mice were treated by the oropharyngeal administration of either Zileuton 10 mg/Kg (Tocris Biosciences) or vehicle (0.5% methyl-cellulose/0.2% Tween 80 in water) 3h before administration of 50 μg of FA21. Controls comprised mice treated with carrier alone. The level of inflammation elicited was evaluated by harvesting BALF and monitoring **(A)** eosinophils by measuring EPO activity by colorimetric assay and **(B)** cys-LT levels. Groups comprised 4 mice (n = 4) and results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$. Data are representative of 3 independent experiments.

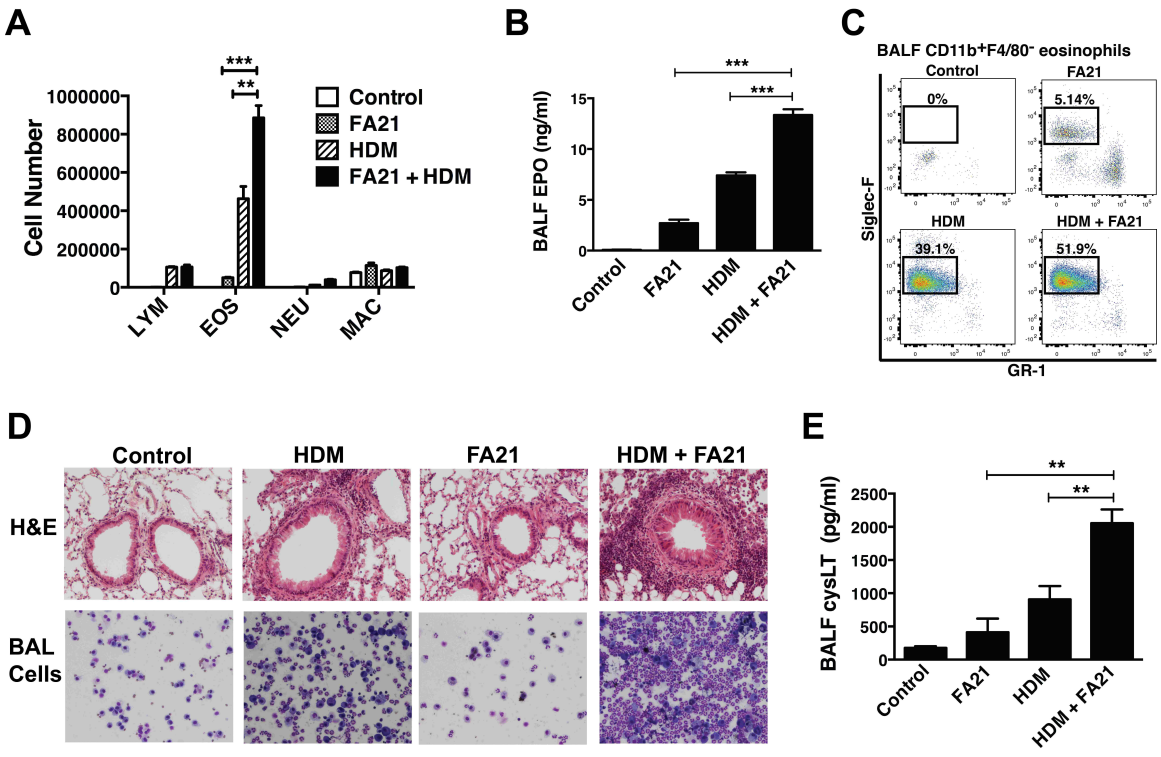



**Figure 2.7**

**Exposure to FA21 exacerbates HDM-induced allergic lung inflammation and promotes cys-LT production.**

The possibility that MWCNT exacerbated allergic inflammation was evaluated in HDM sensitized mice. Allergic lung inflammation to HDM was elicited in C57BL/6 mice by instillation of HDM allergen into the airway (Figure 1). Mice were challenged by MWCNT administered by oropharyngeal instillation of 50 μg of the material on day 15 and the level of inflammatory response elicited was determined on day 16 by examination of lung tissues and BALF. Groups comprised mice treated with either FA21 only, HDM only, or both HDM and FA21. Control/sham-treated mice were given PBS in place of HDM and MWCNT. **(A)** Differential cell counts, measuring the number of lymphocytes (LYM), eosinophils (EOS), neutrophils (NEU) and macrophages (MAC) present in BALF and

expressed as the absolute cell number, revealed increased eosinophil recruitment to the airways. **(B)** EPO activity expressed by BALF cells was determined 24h after MWCNT challenge by colorimetric assay. EPO assays confirm significantly increased eosinophilia in mice exposed to both HDM allergen and FA21 compared to mice given allergen or HDM or FA21 alone. **(C)** Flow cytometry was performed on BALF cells enumerating CD11b+F4/80-Siglef-F+ cells lacking GR1. **(D)** Lung sections (5 μm) were stained for H&E to examine the cellular infiltration. In addition, BALF cells were cytospun onto glass slides and stained using Hema3 prior to microscopic examination, which revealed alveolar macrophages (AM), eosinophils (EOS), and MWCNT agglomerates. **(E)** Cys-LT levels present in BALF were determined by ELISA. Groups comprised 4 mice (n = 4) and results are mean ± SEM $^{**}p < 0.01$ and $^{***}p < 0.001$. Data are representative of 3 independent experiments.

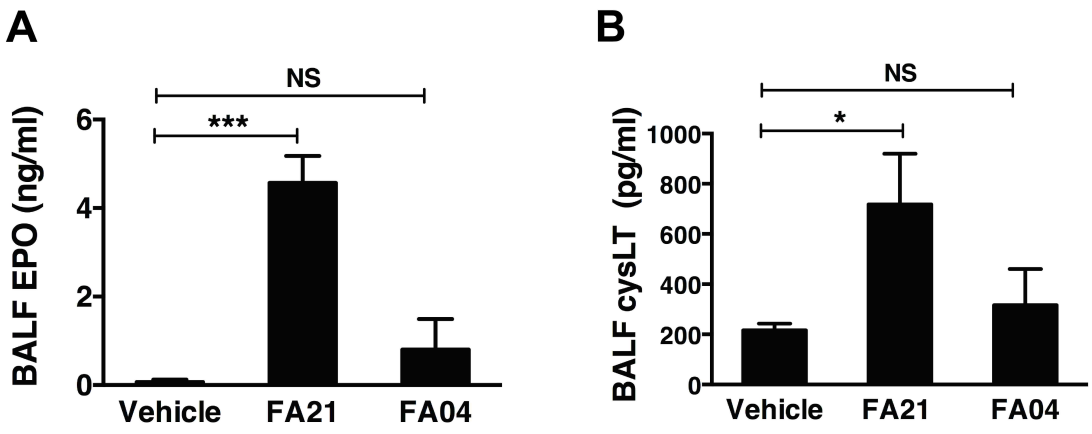



**Figure 2.8**

**The severity of airway eosinophilic inflammation elicited by inhalation of MWCNT correlates with their nickel content.**

The effect of nickel on the inflammatory process was investigated by comparing the airway inflammation elicited by MWCNT with high (FA21) and low (FA04) nickel content. Fifty μg of FA21 or FA04 MWCNT was instilled into the airways of C57BL/6 mice and BALF collected 24 hours later. **(A)** The level of eosinophil infiltration was determined by measuring BALF cell-associated (EPO) using colorimetric analysis. **(B)** The level of cys-LT present in BALF was determined by ELISA. Results are mean ± SEM (n = 3), *$p < 0.05$ and ***$p < 0.001$. Data are representative of 3-4 independent experiments.

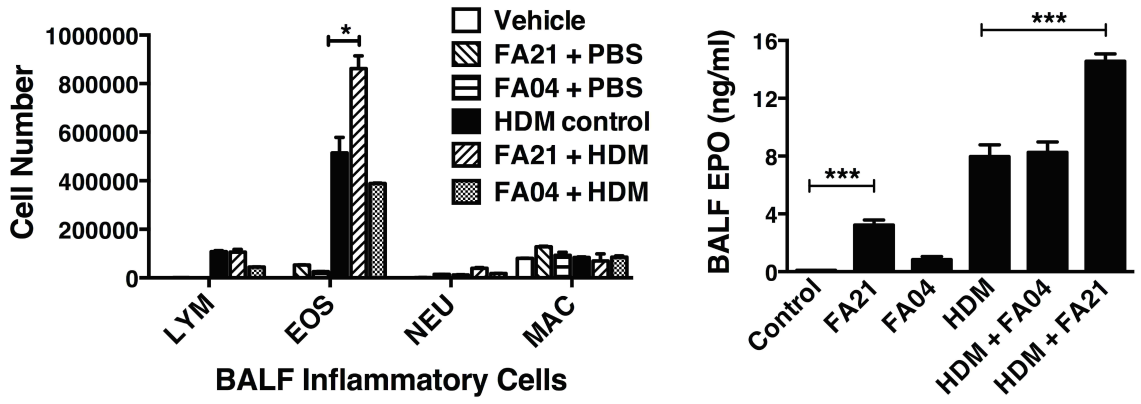
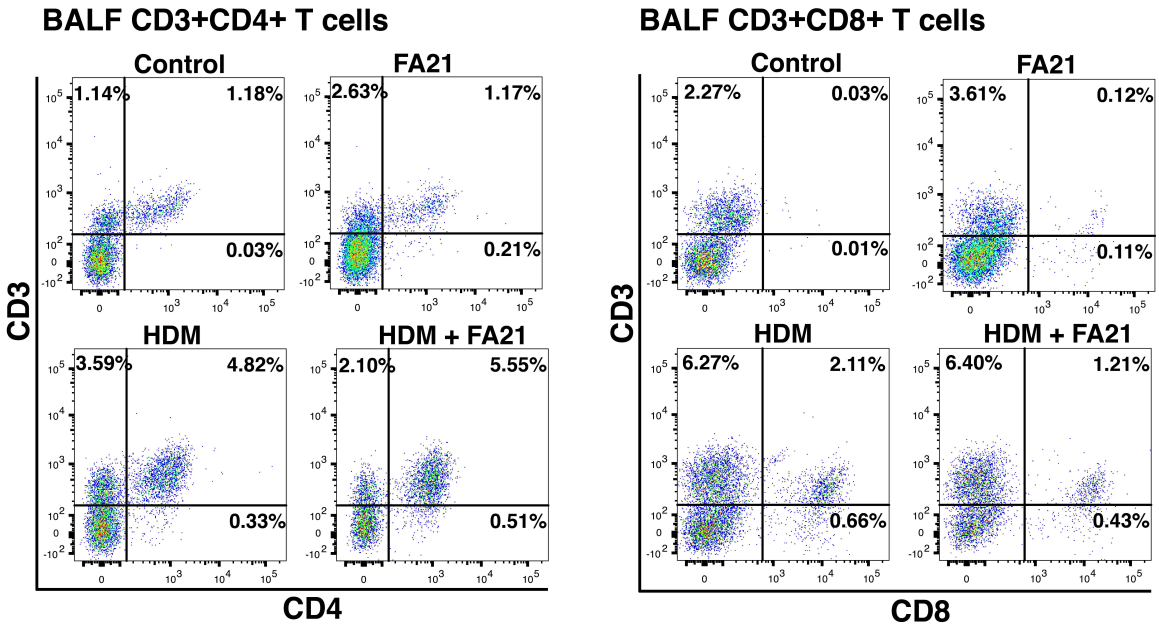



**Figure 2.9**

**The effectiveness of inhaled MWCNT to exacerbate allergic lung inflammation correlates with their nickel content.**

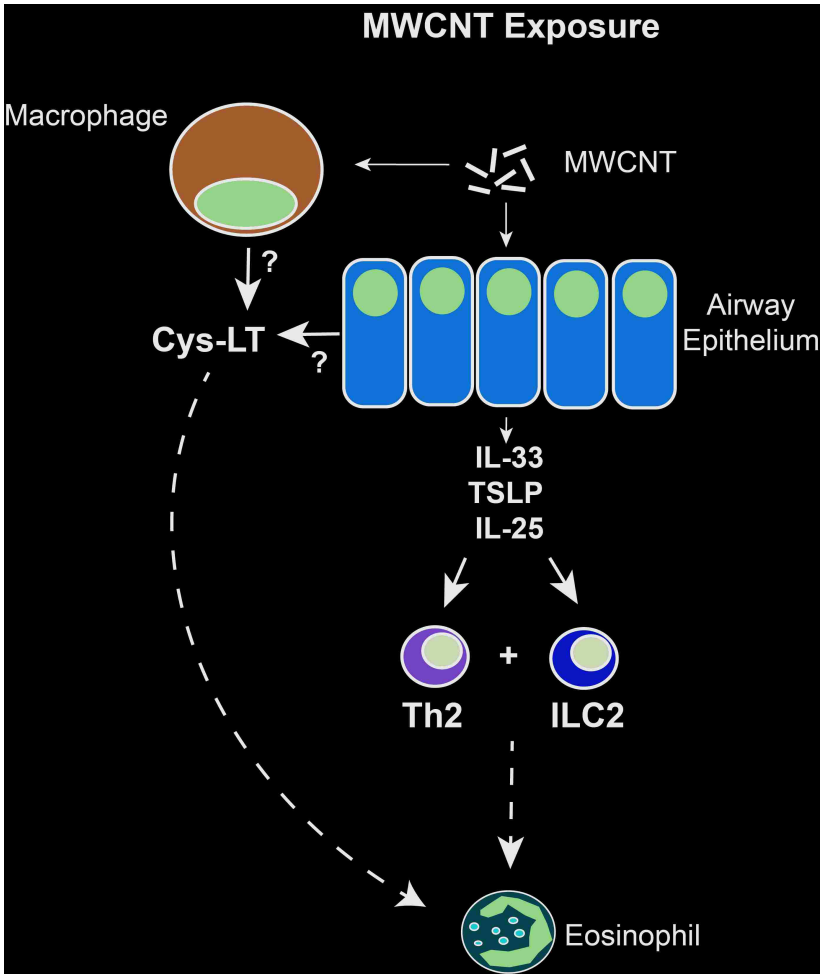
C57BL/6 mice were treated with HDM allergen by oropharyngeal administration of 100 μg HDM allergen on day 0, and 50 μg on days 7 and 14. Controls comprised mice treated with PBS. Both control and HDM sensitized mice were treated with either FA21, FA04 or vehicle and the level of inflammation determined by quantifying the cellular composition of BALF. **(A)** Cell differential counts in the BALF were determined and expressed as absolute numbers (per mouse) of lymphocytes (LYM), eosinophils (EOS), neutrophils (NEU) and macrophages (MAC). Data are representative of 3-4 independent experiments. **(B)** The level of eosinophil infiltration was determined by measuring BALF cell-associated EPO using colorimetric analysis. Results are mean ± SEM (n = 3), *$p < 0.05$ and ***$p < 0.001$. Data are representative of 3-4 independent experiments.




**Supplemental Figure 2.10**

**Flow cytometry reveals no clear connection between CD4+ and CD8+ T lymphocytes and FA21 exposure.**

Sensitization of C57BL/6 mice to HDM resulted in increased populations of CD4+ and CD8+ T cells compared to sham-treated mice; however, addition of FA21 did not reliably elicit either increased or decreased T cell populations.




**Supplemental Figure 2.11**

**Proposed mechanism of inflammation elicited following FA21 exposure.**

Introduction of FA21 to the lung activates airway epithelia or alveolar macrophages to synthesize cys-LT. Active epithelial cells also stimulate differentiation of Th2 and/or ILC2. These work in concert with cys-LT signaling to promote and sustain eosinophilia.

# Chapter Three

## 3.0 The Interaction of Pre- and Postnatal Wood Smoke Exposure with Allergic Inflammation in the Lung

### 3.1 An Introduction to Wood Smoke

#### 3.1.1 Prevalence of Airborne Fine Particulate Matter

Air pollution in the form of fine and ultrafine aerosolized particles is one of the ten leading causes of poor health and premature death, with global mortality attributable to $PM_{2.5}$ inhalation having reached 3.15 million deaths per year by 2010 (104). $PM_{2.5}$ is defined as any airborne particle having an aerodynamic diameter of 2.5 micrometers or less (104, 105). The World Health Organization has stipulated that annual mean concentrations of inhaled $PM_{2.5}$ should be kept below 10 μg/m3 and the daily mean below 25 μg/m3. However, large populations in the Middle East and Asia are exposed to levels of fine particulate far exceeding these guidelines (104). It is further estimated that 30% of the American population is routinely exposed to levels of $PM_{2.5}$ in the ambient air which exceed those outlined as tolerable by the National Ambient Air Quality Standards (NAAQS) (106). Common sources of $PM_{2.5}$ include urban smog produced by vehicles and industry; environmental dust, as in deserts; and organic combustion products, produced by wildfires and the burning of household fuels (104, 105, 107).

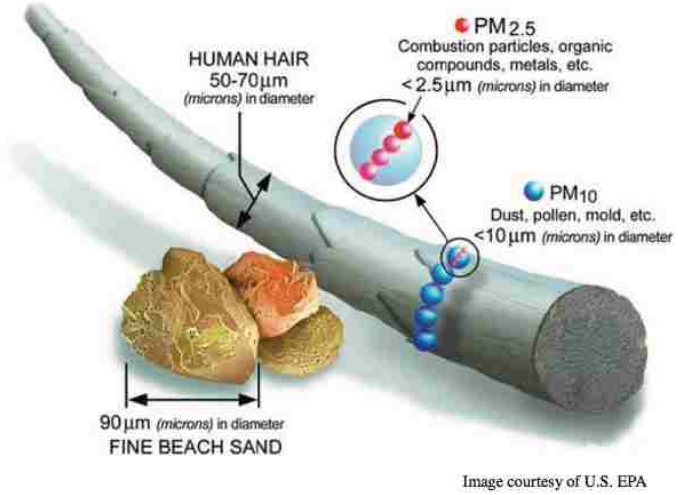



*Figure 3.1*: Fine-fraction particulate matter ($PM_{2.5}$) is many times smaller than visible particles, such as beach sand. (Adapted from (108).)

### 3.1.2 Known Adverse Effects of $PM_{2.5}$

Particulate matter, particularly $PM_{2.5}$, is widely considered hazardous to human health. Particle pathogenicity is determined by size, chemical composition, origin, solubility, and ability to generate reactive oxygen species (105). Smaller particles are deposited deeper within the lung upon inhalation than larger particles, which makes $PM_{2.5}$ notably more dangerous than particulate of greater diameter (105). Exposure to airborne $PM_{2.5}$ has been associated epidemiologically with increased mortality rates, particularly among the elderly, caused by exacerbated asthma, chronic bronchitis, respiratory inflammation and infection, and lung carcinogenesis (105, 109-111). There is also epidemiological and laboratory evidence that such exposure elicits systemic inflammation, and long-term inhalation of $PM_{2.5}$ can be correlated to increased risk of heart disease, cardiac event, insulin resistance, and diabetes (112-114).

A number of potential mechanisms for these adverse effects have been proposed. *In vitro* studies have determined that exposure to $PM_{2.5}$ stimulates release of pro-

inflammatory cytokines such as GM-CSF and IL-8 by bronchial epithelial cells; and prolonged exposure produces visible lung damage, e.g., thickening of bronchioli walls. (110). Additionally, it is thought that one of the major mechanisms of PM carcinogenicity is the induction of oxidative stress, in which exposure to combustion particles triggers the release of reactive oxygen species from alveolar macrophages (115, 116). This arises from the chemical composition of the particles in question: the carbon core of any given combustion particle has a variety of metals and hydrocarbons adhered to it (115, 117). Adhered metals would be sources of ROS, while PAHs would be decomposed into oxygen compounds capable of binding directly to DNA and forming adducts (115). *In vivo* study of urban residents has shown a small but significant increase in oxidative DNA damage associated with exposure to $PM_{2.5}$ containing transition metals, using the biomarker 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) as a metric (118).

### 3.1.3 Significance of Human Exposure to Wood Smoke

The ubiquitous nature of fine particulate air pollution makes this an environmental exposure of global concern. Of particular local concern is the particulate derived from wood smoke, originating both indoors and outdoors. Wildfires and prescribed burns are significant sources of airborne particles, as well as gaseous pollution, that can temporarily increase the overall levels of air pollution over hundreds or thousands of square miles (107, 119). Furthermore, relatively little is known about the health impacts of such planned and unplanned fires on exposed communities (119, 120). There is also a pressing need to examine the adverse health effects of indoor wood smoke. Wood-burning stoves are still quite common, both locally and globally; as of 2014, approximately 2.8 billion people used

solid fuels, including hardwood, for cooking (121). A study of 170 countries estimated that these household fires produced 12% of the global $PM_{2.5}$, which makes cooking fuels a critical point of consideration in the pursuit of improved air quality (121). The impact of wood-burning stoves is especially high during cold weather and in colder climates; at least 11 million homes in the United States use such stoves as heat sources (122).

Rationale for the Study of Prenatal Effects of Wood Smoke Exposure

We have previously investigated the effect of *in utero* cigarette smoke exposure on responses to inhaled allergen later in life. This approach provided an opportunity to not only establish a causative linkage of disease processes but also dissect the possible operative mechanisms (Ferrini, 2017, submitted). During that study, we found that the offspring of pregnant mice exposed to cigarette smoke displayed significantly increased allergic inflammatory responses to HDM. This response included exacerbation of airway hyperreactivity, pulmonary eosinophilia, mucus production, and cys-LT biosynthesis (Appendix 1). Critically, these studies revealed that prenatal exposure to tobacco smoke also resulted in: (i) A higher frequency of pulmonary CD11b+ dendritic cells and CD3+CD4+ T cells compared to air-exposed mice. (ii) A reduction in the number of pulmonary CD3-CD19-NK1.1+CD94+ NK cells.

These findings complement our reported findings that mice exposed to inhaled MWCNT were prone to exacerbated allergic inflammatory responses (Carvalho, 2017, submitted). Moreover, these observations raise the possibility of a common underlying mechanism for the eliciting of inflammatory responses to inhaled carbonaceous particulate material, whether that matter is a complex mixture, such as tobacco smoke, or a much purer

exposure, as in the case of MWCNT. Collectively, these findings form the foundation for the current study on wood smoke exposure. Considering the similar properties of wood smoke and its significance as a health hazard—both globally and locally—we chose to investigate the inflammatory effects of inhaled wood smoke in isolation and in combination with allergic stimulus. As in our MWCNT and cigarette smoke studies, we focused on airway eosinophilia, mucus production, and cys-LT biosynthesis as markers of respiratory inflammation.

**3.2**

**Prenatal wood smoke predisposes mice to exacerbated allergic lung inflammation.**

Sophia Carvalho[1], Maria Ferrini[1], Britten Postma[1], Kevan Roberts[1*] and Zeina Jaffar[1*]

[1]Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, College of Health Professions and Biomedical Sciences, University of Montana, Missoula, Montana, USA.

[*]Corresponding authors: Zeina Jaffar or Kevan Roberts, Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, University of Montana, Missoula, MT 59812. E-mail: zeina.jaffar@umontana.edu or kevan.roberts@umontana.edu

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### 3.2.1 Abstract:

Childhood asthma is strongly linked to atopy and is characterized by a Th2 immune response. Both genetic predisposition and environmental factors play key roles in the development of allergic asthma, particularly during early life. The molecular mechanisms by which such exposures, including air pollutants, can contribute to the development of an asthmatic phenotype are poorly understood. We have previously observed that prenatal exposure of mice to environmental tobacco smoke is associated with an altered innate immune response and enhanced allergen-induced airway inflammation (123) (Ferrini, 2017; submitted). Given that very little is known with regard to whether *in utero* wood smoke exposure can influence the development of asthma, we devised a controlled murine model of prenatal wood smoke exposure to determine the effects on allergic airway disease. We used this model to test the hypothesis that: prenatal wood smoke exposure would compromise innate pulmonary immune responses resulting in exacerbated HDM-induced eosinophilic lung inflammation and increased airway hyperreactivity. In this study, we demonstrate that inhalation of wood smoke by pregnant mice results in altered inflammatory responses in the offspring. The offspring of female mice exposed to wood smoke during pregnancy displayed significantly increased pulmonary inflammation in response to HDM challenge as adults compared to offspring of unexposed mice. Offspring exposed *in utero* experienced heightened eosinophilia and elevated biosynthesis of cysteinyl leukotrienes in the lung, indicating an exacerbated innate immune response to inhaled allergen.

### 3.2.2 Introduction:

Approximately half of the global population relies on solid fuels, particularly biomass such as timber, for cooking and residential heating (124). While this practice is often considered a feature of the developing world, woodstoves are also commonly used in the European Union, the United States, and other first-world nations (125). Woodstoves are in use year-round, but their impact on air quality is particularly severe during cold months (126). In winter, products of wood combustion can account for as much as 90% of fine particulate pollution in both Seattle, Washington and Christchurch, New Zealand (125). Each year, as many as 3.5 million deaths can be attributed to the air pollution generated by solid fuel combustion in homes (124).

Inhalation of airborne particulates is associated with exacerbation of: asthmatic symptoms, allergic responses, pulmonary fibrosis, and lung cancer risk (126). Wood smoke inhalation in particular has been linked to increases in the risk of asthma development and sensitization to airborne allergens in otherwise healthy individuals (127).

The cardinal characteristics of allergic asthma include immune cell recruitment and production of IgE in response to inhaled allergen. Initial exposure sensitizes the individual, and subsequent exposures to the same allergen can produce severe inflammatory reactions. In addition to tissue edema and airway smooth muscle contractions, these reactions are characterized by pulmonary eosinophilia. T cells recruited to inflamed airways secrete IL-5, which is critical to eosinophil recruitment, maturation, and survival; and IL-4, which regulates IgE synthesis. IL-13, another T cell-produced cytokine, has been implicated as a major inducer of the Th2 response and airway hyperreactivity and remodeling seen in allergic asthma. It is also thought to play a role in the recruitment of inflammatory cells

(128). Eosinophil chemotaxis in particular is modulated by lipid mediators such as cysteinyl leukotrienes (cys-LT), which can be produced by dendritic cells, mast cells, or alveolar macrophages (37). Recent experiments in our lab involving cigarette smoke reveal that prenatal exposure results in exacerbated allergic inflammation in the offspring after birth (123). Although other studies in the field have also suggested eosinophil involvement in wood smoke-induced inflammation (127), a detailed characterization of this effect and resolution of the operative mechanisms remain unclear.

Wood smoke is a commonplace exposure worldwide. However, the processes impacted by such exposures and events that make it hazardous remain unresolved. Moreover, the specific dangers of wood particulate inhalation remain to be characterized, highlighting the importance of delineating the proinflammatory events elicited following exposure and making comparisons to other biomass fuels. By elucidating the mechanisms by which the inhalation of wood smoke particulate damages pulmonary tissues and impacts allergic responses, we are able to improve our current understanding of this pollutant's health risk.

### 3.2.3 Materials and Methods:

*Overall Protocol*

The goal of this study was to develop a controlled mouse model of environmental wood smoke exposure and use that model to determine whether prenatal exposure to wood smoke will impact innate immunity and airway inflammation elicited by inhalation of HDM allergen. The overall design of the experiment was as follows: C57BL/6 mating harems (2 dams: 1 sire) were established in our facility and exposed to wood smoke for a period of three weeks. On completion of the smoke exposure mothers and newborn pups were maintained in filtered cages until the administration of HDM allergen into the airways at approximately 8 weeks of age. On day 16 after HDM challenge, lavage and tissue samples were collected and the inflammatory response quantified.

*Wood Smoke Exposure*

Our Inhalation Core Facility at the University of Montana houses a Wood Smoke Exposure System for investigating the health effects from exposure to wood burning emissions. This system is depicted in schematic form in Fig. 3.2. C57BL/6 (wildtype) mice breathed normally in a contained environment supplied with either ambient air or a mixture of ambient air and larch wood smoke pumped in from a wood-burning stove. Wood smoke exposures lasted for three hours each weekday (five consecutive days) for three consecutive weeks, totaling 15 days of exposure at a computer-regulated dosage of 3 $mg/m^3$. This study was designed to model indoor exposures; smoke particulate levels average roughly 30-45 $\mu g/m^3$ in woodstove-heated homes (129). Based on these levels our exposures targeted a fine particulate ($PM_{2.5}$) dose of 3 $mg/m^3$ during gestation of the

dams. Our Center has previously reported (130) that comparison of human exposures and those in mouse models requires consideration of multiple parameters. Without compensating for ventilation rates, lung volume, and body size ("per kg"), identical particulate levels would result in significantly lower amounts of deposition in mice. For example, a two-hour mouse exposure at a concentration of 10 mg/m$^3$ would deposit approximately the same amount as a two-week human exposure at 0.2 mg/m$^3$. This is a level well below ranges (190-400 mg/m$^3$) reported for those residing in wildfire areas (131, 132).

*Induction of Allergic Lung Inflammation*

To assess the long-term effects of prenatal wood smoke exposure on the inflammatory response, an acute allergen exposure protocol was used to induce allergic lung inflammation. Briefly, the offspring of exposed dams were treated with house dust mite (HDM) allergen (*Dermatophagoides pteronyssinus* extract, Greer Laboratories,) or PBS (control) by oropharyngeal instillation over a two-week period. Mice received an initial dose of 100 μg HDM on Day 0 followed by two challenge doses of 50 μg each on Days 7 and 14. Experimental groups of 4-6 mice were used throughout and experiments were performed three times. Blood serum, bronchoalveolar lung lavage fluid (BALF) and lung tissue were collected 48 hours after the final HDM exposure (on day 16). Effects on the cardinal features of asthma were then assessed. Peribronchial inflammation and mucus-producing cells were distinguished via lung histology using H&E and PAS staining. BALF cell differential counts, eosinophil peroxidase levels, Th2 cytokine production, and serum IgE levels were determined as we have described previously (9, 133, 134) using ELISA

and flow cytometry (FACSAria II, BD Biosciences US, San Jose, CA) of inflammatory cells. The effect of wood smoke exposure on allergic inflammatory responses and cys-LT production in the lungs of the dams was also determined and compared with the pups to provide a baseline control. The full exposure protocol, including both wood smoke and HDM components, is depicted in Fig. 3.3 below.

*Intratracheal Administration of House Dust Mite Allergen (HDM)*

To elicit allergic lung inflammation, mice were sensitized to HDM on day 0 by the intranasal administration of 100 μg of HDM allergen (*Dermatophagoides pteronyssinus*, Greer Laboratories) in 30 μl of PBS and then on days 7 and 14 by intranasal treatment with 50 μg of HDM (30μl total volume). HDM allergen preparations used throughout this study contained minimal levels of LPS. Control groups comprised mice receiving 30 μl of PBS on days 0, 7 and 14. To determine the level of mucosal inflammation, BALF and lung tissue were harvested 48h after the last allergen challenge, on day 16.

*Determining the Level of Pulmonary Inflammation*

BALF was divided for analysis of cell populations via flow cytometry, cysteinyl leukotriene (cys-LT) and cytokine production via EIA and sandwich ELISA, and eosinophil peroxidase (EPO) by colorimetric assay. Lung tissues were reserved for histological examination using H&E and PAS. Bronchoalveolar lavage was performed to collect bronchoalveolar lavage fluid (BALF) for analysis. Eosinophil peroxidase (EPO) levels in the lavage cells were determined by colorimetric analysis using orthophenylene diamine dihydrochloride as detailed previously. Cell differential percentages were

determined by light microscopic evaluation of Hema3-stained cytospin preparations and expressed as absolute cell numbers. Lung tissue was dispersed by collagenase (Type IV; Sigma-Aldrich), and lung mononuclear cells (LMC) were isolated by Percoll (Sigma-Aldrich) density gradient for functional analysis.

*Measurement of Cytokines*

BALF chemokine or cytokine levels were determined using ELISA for measurement of IL-4, IL-5 (Duoset, R&D Systems) and IL-13 (Quantikine, R&D Systems), or using the sensitive Mouse V-Plex Pro-Inflammatory Panel 1 assay and Meso Quickplex 120 reader (MesoScale Discovery, MD) for measurement of other cytokines (IL-4, IL-5, TNF-α, IFN-γ and IL-12p70).

*Histological Determination of Peribronchial Inflammation and Goblet Cell Hyperplasia*

Lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin using a Leica ASP 300 tissue processor (Leica, Bannockburn, IL). Microtome sections were cut at 5μm thickness and stained with hematoxylin and eosin (H&E) using a Shandon Varistain 24-4 (Thermo Fisher Scientific). Alternatively, sections were stained using periodic acid-Schiff (PAS) reagent. The level of peribronchial inflammation (H&E stained) or mucus production (PAS stained) was analyzed by microscopy and the transmitted light images were collected on a Nikon Eclipse 800 microscope equipped with an Olympus DP 26 camera and cellSens software (Version 1.9).

*Measurement of Cysteinyl Leukotrienes*

BALF was assayed for the presence of cysteinyl leukotrienes (Express ELISA, Cayman Chemical, Ann Arbor, MI). IgG present in the BALF was depleted prior to assay using Protein G-Sepharose (GE Healthcare Life Sciences). Absorbances were read at 405 nm (on Spectramax 190, Molecular Devices).

*Flow Cytometry*

Cells (LMC, BALF or splenic cells) were FcγR blocked using 2.4G2 antibody (ATCC) and stained with combinations of the following mouse conjugated mAb (all purchased from BioLegend): allophycocyanin (APC) or FITC anti-CD3, APC/Cy7 anti-CD4, PE anti-CD8a, APC or PE anti-CD11c, PE or APC/Cy7 anti-I-A/I-E, APC/Cy7 anti-Ly6G, APC or APC/Cy7 anti-Ly6C, APC/Cy7 anti-Ly-6G/Ly6C (Gr-1), PE, FITC or Brilliant Violet 421 anti-CD11b, APC or PE anti-F4/80. In addition, PE anti-Siglec-F (BD Biosciences) was used to stain eosinophils. Flow cytometric acquisition was performed on a FACSAria II (BD Biosciences) by 4-color analysis using FACSDiVa software and FlowJo, with a minimum of 50,000 live, single-cell events per sample collected.

*Statistical Analysis*

Data were analyzed using GraphPad Prism 5.0 (GraphPad, La Jolla, CA). Results involving two variables were analyzed by two-way ANOVA with a Bonferroni post-hoc test. Data comparing two groups were analyzed using an unpaired t test. Figures show combined data from multiple studies or independent repeats (two or more). Data shown are mean ± SEM. A p value $< 0.05$ was considered statistically significant. Significance denoted by *, **, or *** is defined as $p<0.05$, $p<0.01$, or $p<0.005$, respectively.

### 3.2.4 Results:

*Effect of Maternal Wood Smoke Exposure on Allergic Inflammation in the Offspring*

Previous studies have indicated that maternal exposure to airborne pollutants, including inhalation of harmful aerosols, predisposes offspring to exacerbated airway inflammation (135). However, maternal effects arising from wood smoke exposure have not been detailed previously. In addition, the cellular and molecular mechanisms by which prenatal exposures contribute to the development of asthma phenotypes are poorly understood (136). We assessed whether prenatal wood smoke exposure conferred an exacerbated asthma-like phenotype, characterized by peribronchial inflammation and goblet cell hyperplasia, in later life. This was examined by determining whether maternal exposure to ambient wood smoke affected the allergic responses exhibited by the offspring (i.e., in comparison to the offspring of unexposed dams). To visually assess the extent of inflammation elicited, we conducted histological imaging of whole lung tissue (Fig. 3.4). Histological analysis of lung tissue sections stained with H&E or PAS revealed a marked increase in allergen-induced peribronchial inflammation and mucus production in prenatal wood smoke-exposed 8-week old offspring compared to the air-exposed controls. Our data revealed cellular infiltration, typically forming pockets of inflammatory cells, in the H&E stained sections from mice treated with HDM allergen. Notably, the inflamed regions were often in proximity to both an airway and a blood vessel. PAS staining of tissue sections revealed only low levels of staining of airway epithelial cells in the offspring from both the filtered air and wood smoke-exposed dams not sensitized to allergen. In marked contrast, the airway epithelial cells in mice instilled with HDM allergen showed significant PAS staining. Critically, the offspring of wood smoke-exposed dams that were subsequently

sensitized to HDM allergen displayed dramatically elevated levels of inflammation (and PAS staining) compared to filtered air-exposed controls similarly treated with allergen. In the absence of HDM sensitization and challenge (baseline controls), negligible peribronchial inflammation or mucus production was observed in the lung tissue of both wood smoke or air-exposed offspring. Collectively, these observations reveal that prenatal exposure to wood smoke increases the severity of the eosinophilic influx and mucus hyperproduction elicited by inhaled allergen in later life.

In allergic asthma, it is well-documented that CD4+ T cells are involved in the airway inflammatory process and likely responsible for disease progression (20-25). The size of CD4+ and/or CD8+ T cell populations present in the airways of offspring without allergen challenge did not correlate with either prenatal smoke or air exposure. Notably, four-fold more CD4+ T cells were present in the BALF of pups born to control dams than in the pups of dams exposed to wood smoke (Fig. 3.5a). Similarly, following HDM allergen challenge, there were more CD4+ T cells present in the BALF of pups exposed to filtered air *in utero* compared to mice exposed to wood smoke. This phenomenon was repeated in the CD8+ T cell populations. Very few CD8+ T cells were present in the BALF of mice exposed to wood smoke *in utero* in comparison to the offspring of control dams. Similarly, more than twice as many CD8+ T cells were found in unexposed compared to smoke-exposed pups after allergen challenge (Fig. 3.5b). Taken together, these observations indicate that the allergic responses mounted by mice exposed to wood smoke *in utero* are qualitatively different from the responses displayed by unexposed mice.

*The Augmented Allergic Inflammatory Responses Caused by* in utero *Wood Smoke Exposure are Impermanent*

To determine whether the increased responsiveness to HDM allergen conferred by *in utero* exposure to wood smoke was permanent, we conducted a time-course experiment. In this experiment, the offspring of wood smoke-exposed mice were retained over protracted lengths of time by maintaining in filter-top cages. Offspring were challenged over a two-week period with either HDM or PBS (controls) when the mice reached 6, 14, or 22 weeks of age. EPO levels generated in BALF were assessed by colorimetric analysis. We found that the increase in EPO levels after challenge with HDM allergen was highly significant (***$p<0.001$) in the 8-week-old pups of wood smoke-exposed mice compared to 8-week-old pups of control mice (Fig. 5a). This elevation persisted through the 16-week time-point; although the eosinophilia in WS/HDM offspring had by this time decreased, the difference remained statistically significant (**$p<0.01$) (Fig. 3.6b). By the time the mice were 24 weeks old there was no significant difference detected between EPO levels in Air/HDM mice and WS/HDM mice (Fig. 3.6c). These data reveal that the exacerbated immune response observed in the offspring of smoke-exposed dams is not retained as the mice age.

*The Effect of Exposing Pregnant Mice to Wood Smoke on their Responsiveness to HDM Allergen*

It has been proposed that inhalation of particulate mixtures, such as smoke, has adverse consequences to health (126, 137-141). Epidemiological data has further implied a particularly high risk of such effects in pregnant women (142-144). Since wood smoke

exposure exerted pronounced effects in the offspring, it was important to ascertain whether this exposure also impacted maternal responses to allergen. This could only be done after the dams had delivered and cared for their offspring (i.e., until after weaning, typically 4 weeks after delivery). Four weeks after delivery, dams were challenged with HDM allergen and the level of allergic inflammation that developed in response was determined. Comparisons were made with allergen-challenged filtered air-exposed dams. Challenge of post-delivery females with HDM allergen resulted in increased levels of lung inflammation compared to filtered air control mice.

Firstly, measurement of EPO by colorimetric assay demonstrated a significant increase in the number of eosinophils in the airways of both control and smoke-exposed dams sensitized to HDM allergen compared to PBS controls (Fig. 3.7a). Importantly, there was also significant elevation of eosinophil infiltration in wood smoke-exposed, allergen-sensitized mice compared to filtered air-exposed, allergen-sensitized mice. Secondly, flow cytometry revealed that the frequency of CD11b+/F4-80-/Siglec-F+/GR-1- eosinophils present in the BALF was ten percent higher in mice exposed to both smoke and allergen than in mice exposed only to smoke, and more than twenty percent higher than mice exposed only to allergen (Fig. 3.7b). Thirdly, cysteinyl leukotriene levels (measured by EIA) were elevated in mice exposed to both smoke and allergen, being more than five-fold higher than in mice exposed only to smoke (Fig. 3.7c).

Lastly, to examine whether T cells play a role in the exacerbation of allergic inflammation arising from wood smoke exposure, we examined the number of CD4+ and CD8+ T cells present in the BALF. CD4+ T cells were detected in greater numbers in the airways of dams exposed to wood smoke in than in air controls; however, fewer CD4+ T

cells were counted in dams exposed to both wood smoke and HDM in comparison to those sensitized to HDM without smoke (Fig. 3.8a). CD8+ T cell counts followed the same pattern, with smoke exposure alone elevating the population while the addition of allergen to smoke-exposed dams decreased cell numbers to fewer than those elicited by allergen alone (Fig. 3.8b). This data suggests that direct inhalation of wood smoke may result in elevated CD4+ and CD8+ T cell counts, which are depressed during subsequent immune responses to allergen. Collectively, these observations reveal that inhalation of wood smoke while pregnant exacerbates allergic inflammation in adult females after delivery of offspring.

### 3.2.5 Discussion:

*Health Consequences of Exposure to Airborne Particulate Matter*

Inhalation of airborne particulate matter has been shown to be detrimental to cardiopulmonary and respiratory health (145-147), resulting in increased variability of peak expiratory flow volume (PEF) in asthmatic individuals (148). Also, increased hospital admissions for respiratory and cardiac conditions inclusive of asthma (137, 149), increased risk of bacterial and fungal infection (147, 150), and increased rates of all-cause mortality have all been linked to exposure to airborne particulate matter. Collectively, these observations have led researchers in the field to conclude that such pollution has a significant impact on public health (151). It has also been observed that airborne particulate matter, particularly the fine fraction, contains carcinogens including PCBs, PAHs, and transition metals (108, 147, 152, 153).

Furthermore, maternal exposure to tobacco smoke during pregnancy has been reported to increase the risk of asthmatic development in the child (135). This indicates that there is, beyond the nonspecific risk during gestational development, a capacity for maternal respiratory exposures to have a significant impact on the immune responses of offspring. Significantly, cigarette smoke exposure during pregnancy has been reported to induce epigenetic changes that are responsible for augmented inflammatory responses (Ferrini, 2017; submitted).

Wood smoke is of particular concern as a widespread source of $PM_{2.5}$. The sources of such smoke—woodstoves, wildfires, and scheduled burns—are a global problem. Exposure to the combustion products of household fuels alone impacts nearly 3 billion people worldwide in more than 170 countries (121), including upwards of 11 million households

in the United States (122), and accounts for an estimated 12% of the world's air pollution (121). Inhalation of wood smoke has been strongly associated with the incidence of childhood asthma, asthma morbidity, and severity of asthmatic symptoms (131, 148, 154-157). Evidence suggests that wood smoke exposure can impact lung innate immunity. The primary cellular targets of this change have thus far appeared to be alveolar macrophages and lung epithelial cells (158, 159). This is reasonable, given that these cells have easy access to the airway and that $PM_{2.5}$ particles are able to penetrate deeply into the lung.

*Murine Model of* in utero *Wood Smoke Exposure*

This study revealed that exposure of pregnant females to wood smoke resulted in the generation of offspring that displayed augmented inflammatory responses to inhaled HDM allergen. The events underpinning this effect remain largely unresolved. In part this is because the events elicited by both wood smoke and allergen are likely complex and multifactorial. With respect to HDM allergen, it is known that this allergen both promotes the differentiation of CD4+ Th2 cells and activates ILC2s (24, 101). Both of these events contribute to the development of pulmonary eosinophilia (89, 160, 161) (Fig. 3.9).

In marked contrast to *in utero* cigarette smoke exposure, where the augmented responsiveness in the offspring was stable over the four-month observation period (Ferrini, 2017; submitted), the exacerbated allergen responses caused by *in utero* wood smoke exposure were progressively lost as the offspring aged. It is conceivable that this effect may result from:

(i) Smoke-induced epigenetic gene regulation events that are reversed over time (123, 162). Smoke-induced epigenetic changes in early life could promote ILC2 and/or CD4+ Th2 responses in later life (163-165).

(ii) Smoke-induced changes in the immune cells present in the lungs of the offspring. Increased HDM responses could arise from elevated CD4+ T cells, antigen presenting cells, or ILC2s.

(iii) Smoke-induced retardation of fetal lung development resulting in associative changes in lung mucosal immune function. In this respect, epidemiological studies of human populations have indicated that maternal exposure to $PM_{2.5}$ during pregnancy is positively correlated to preterm birth, low birth weight, and poor respiratory outcomes; exposure is also correlated with intrauterine inflammation, which is a known risk factor for these conditions (142, 143). There is also evidence that chronic maternal inflammation, such as asthma, effects alterations to the placenta and fetus, including low birth weight, preterm birth, and neonatal hospitalization (144). Asthmatic exacerbations are particularly dangerous to the developing fetus and are associated with increased risk of stillbirth by way of maternal alkalosis, reduced levels of maternal oxygen, and increased oxidative stress (144). From these studies, we can deduce that maternal exposure to inflammatory stimuli and subsequent immune responses to these stimuli have a consistently negative impact on the viability of the fetus.

*Effect of Wood Smoke Exposure on Maternal Lung Function*

When considering potential mechanisms responsible for augmented allergen responses in the offspring, the finding that exposure of pregnant dams to wood smoke resulted in augmented respiratory inflammation is important. Specifically, pregnant female mice exposed directly to wood smoke over a period of three weeks displayed significantly increased recruitment of CD11b+/F4/80-GR-1- eosinophils and biosynthesis of cys-LT in the airways following HDM allergen sensitization compared to allergen-sensitized mice that were not exposed to wood smoke. This eosinophilia and cys-LT elevation coincided with increased mucus secretion, as identified through PAS staining of lung tissue. These findings in adult female responses would seem to argue against the possibility that wood smoke effects are solely consequences of retarded fetal lung development (i.e., (iii) above).

*Potential Contributors to Smoke-Induced Exacerbations of Inflammation*

(i) Similarity to MWCNT exposures. An important consideration is that the exacerbation of maternal allergic lung inflammation by wood smoke bears some similarities to exacerbations elicited by the introduction of MWCNT. This may be due to physical similarity between nanoparticles and the $PM_{2.5}$ contained in wood smoke, as both have been previously shown to be of sufficiently minute size to deposit in the bronchial and, on the finer end of the scale, alveolar spaces (108). It has further been observed that some of the combustion products encompassed within the $PM_{2.5}$ metric are themselves naturally-occurring nanoparticles (46) and may thus be reasonably expected to behave similarly to engineered nanoparticles. However, having recently examined the effects of MWCNT on lung inflammation (Carvalho, 2017,

submitted), we theorized that wood smoke particulate might also impact the respiratory immune response through the recruitment of eosinophils and biosynthesis of cys-LT. Metal content should also be considered. Many engineered nanoparticles contain residues of the transition metal catalysts used in manufacture; these residues have been proposed as one source of ENM immune reactivity (Carvalho, 2017, submitted). Toxic metals have also been identified as components of particulate aerosols arising from both wood burning and other sources (152, 153, 166).

(ii) Cys-LT production. It is possible that the increased eosinophilia associated with wood smoke inhalation may be a consequence of elevated cys-LT synthesis, potentially involving metal contaminants. This theory could be tested via the introduction of 5-lipoxygenase (5-LO) inhibitors to the exposure model.

(iii) Allergen-specific CD4+ T cells. Our data also indicated that the wood smoke inhalation by pregnant adult female mice had little effect on the numbers of CD4+ and CD8+ T cells present in the airways. Fewer T cells were found in the BALF of those mice sensitized to allergen than in unsensitized dams, regardless of whether they had previously been exposed to wood smoke or not. The difference in T cell counts between allergen-sensitized mice and their non-sensitized counterparts was less than two-fold. Conversely, the numbers of CD4+ T cells recovered from the pups of smoke-exposed dams increased by nearly ten-fold from control with the introduction of allergen (1933 in WS/HDM vs. 201 in WS/PBS). In comparison in the pups of air-exposed dams, the CD4+ T cell population more than doubled following allergen challenge

(2197 in Air/HDM vs. 888 in Air/PBS). As observed in the dams themselves, these trends persisted in the numbers of CD8+ T cells in the offspring. This is particularly intriguing in the context of allergic asthma, which is commonly characterized by an elevated CD4+ Th2 response. It is worth noting that despite the shared overall trend in CD4+ T cell numbers between air-exposed pups and smoke-exposed pups, the latter group expressed fewer cells in the BALF regardless of allergen sensitization. However, the dramatic expansion of the pups' CD4+ T cell population following sensitization of smoke-exposed offspring to HDM suggests that the maternal smoke exposure has primed the pups' immune systems to respond to allergic stimuli. These observations are supportive of the hypothesis that elevated lung CD4+ Th2 responses underpin the increased pulmonary eosinophilia observed in mice experiencing *in utero* wood smoke exposure.

*Summary*

The data obtained from this study present two novel conclusions. Firstly, we have shown evidence that introduction of wood smoke to the lungs of adult mice results in elevated eosinophilia and cys-LT biosynthesis, and this likely constitutes a shared inflammatory mechanism with respiratory exposure to MWCNT. Secondly, we have demonstrated that the exposure of pregnant females to wood smoke not only elicits respiratory inflammation in the dams themselves, but also impacts the development of T cell-mediated immunity in the offspring in such a way as to distinctly heighten the pups' responsiveness to allergen.

### 3.2.6 Figure Legends

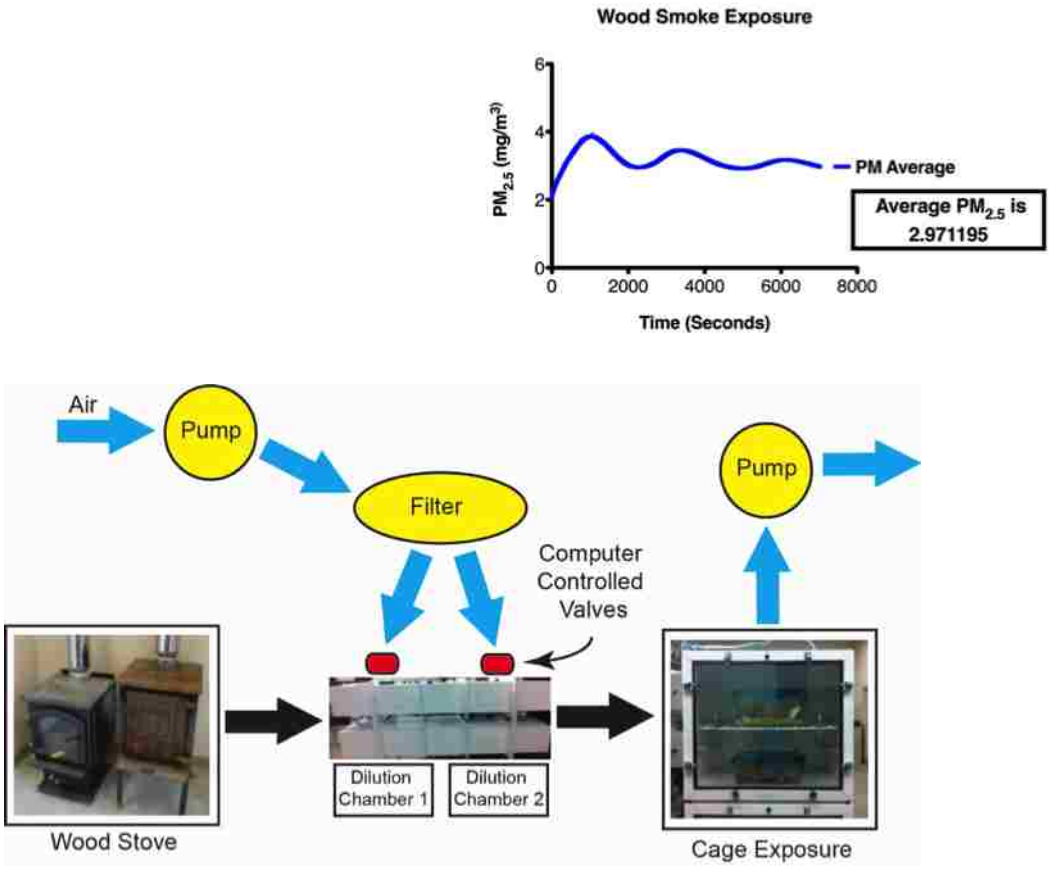



**Figure 3.2**

**Illustration of the wood-burning apparatus and inhalation system.**

Smoke was piped from a wood-burning stove into sequential dilution chambers where it was mixed with filtered outside air before being carried into the exposure chamber. Custom software ensured maintenance of $PM_{2.5}$ levels at approximately 3.0 $mg/m^3$.

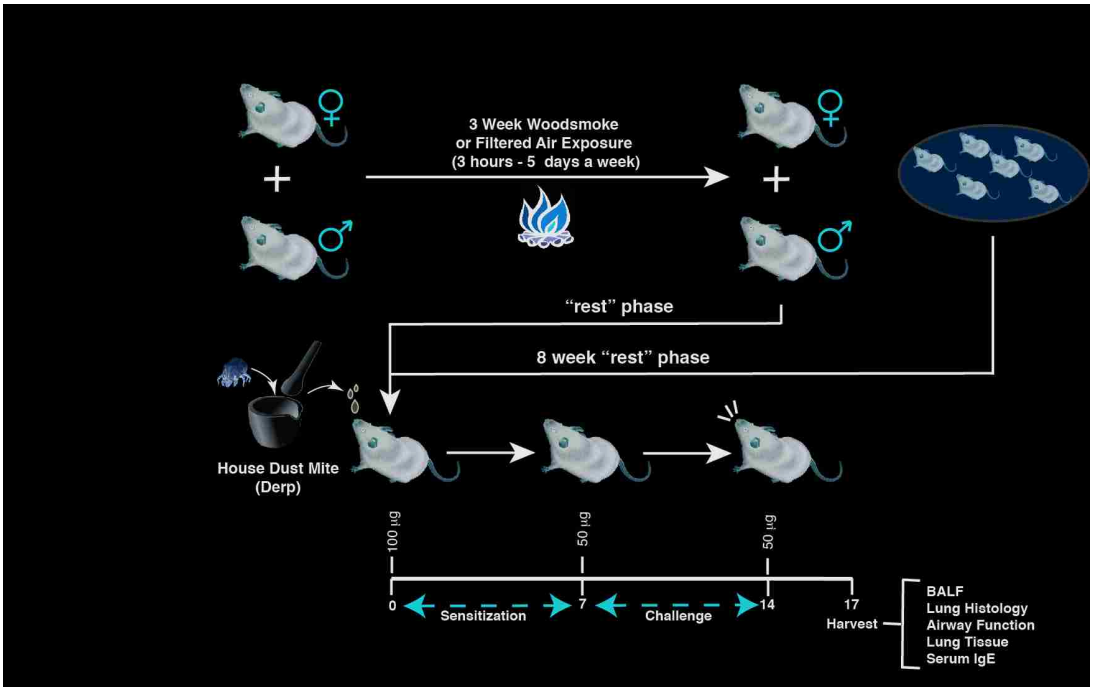



**Figure 3.3**

**Protocol for exposure of dams to inhaled wood smoke and sensitization of pups to HDM via oropharyngeal aspiration.**

Pregnant C57BL/6 mice in homeboxes were placed in the smoke exposure chamber and allowed to respire normally for 3 hours, inhaling either wood smoke or filtered air, each day for 5 days a week for a period of 3 weeks. Following 8 weeks of maturation, the pups of exposed dams were given an initial 100 µg sensitization dose of HDM suspended in PBS on day 0 and two 50 µg challenge doses on day 7 and day 14. Control/sham-treated mice were given filtered air and PBS in place of wood smoke and HDM. All pups were harvested on day 16 after the sensitization dose.

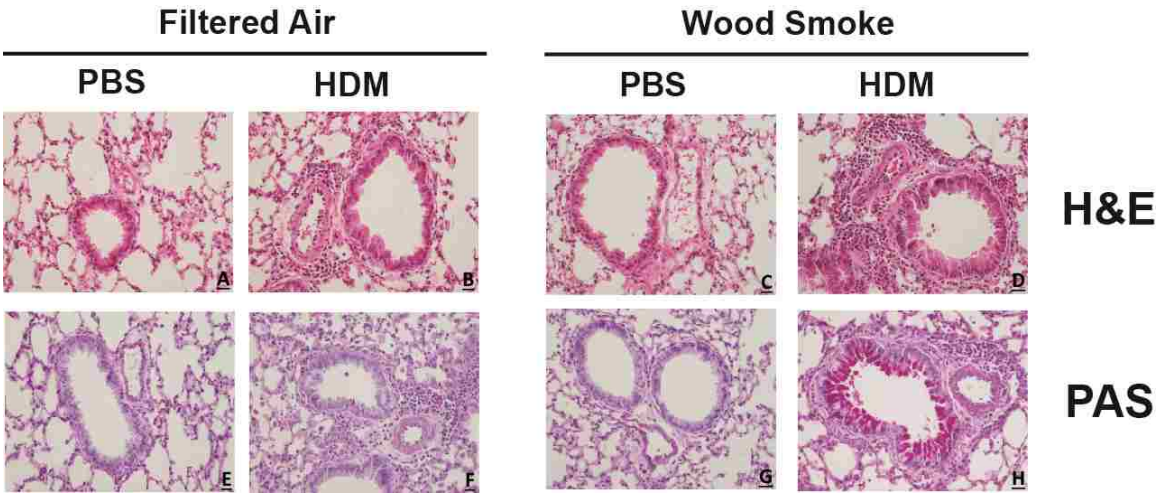
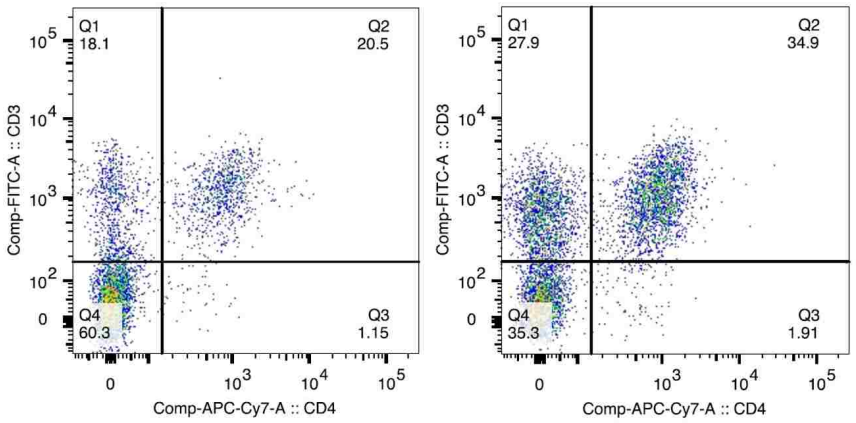



**Figure 3.4**

**Histological imaging of lung tissue from mice prenatally exposed to wood smoke and subsequently challenged with HDM.**

Following euthanasia and harvest, lung tissue was embedded in paraffin and sectioned at 5 μm thickness. Sections were stained with H&E for visualization of respiratory inflammation or with PAS for imaging of mucus production and goblet cell hyperplasia. Sections for each experimental group were taken from 4 representative mice, aged 8 weeks. Control groups: offspring of pregnant females exposed to filtered air instead of wood smoke and PBS instead of HDM; lung sections stained with H&E (a) and PAS (e). Allergen groups: offspring of pregnant females exposed to filtered air instead of wood smoke; pups were later sensitized to HDM allergen as adults. Lung sections stained with H&E (b) and PAS (f). Wood smoke groups: offspring of pregnant females exposed to controlled levels of $PM_{2.5}$ in wood smoke; pups were later dosed with PBS in place of HDM. Lung sections stained with H&E (c) and PAS (g). Dual-exposure groups: offspring of pregnant females exposed to controlled levels of $PM_{2.5}$ in wood smoke; pups were later sensitized to HDM allergen as adults. Lung sections stained with H&E (d) and PAS (h).

**A**

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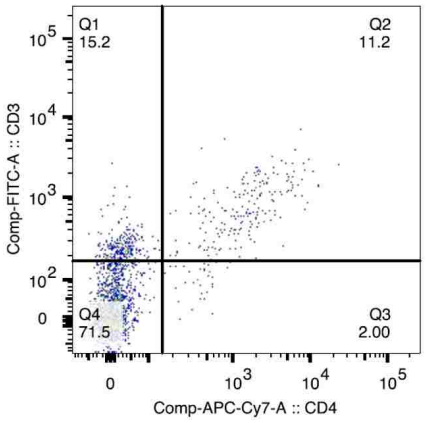
Lymphocytes
4332




T_Cells_AIR_HDM.fcs
Lymphocytes
6295

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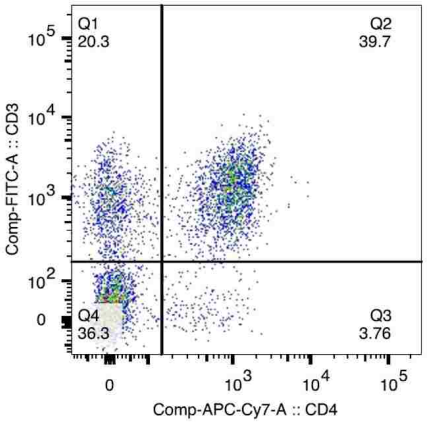


T_Cells_WS_HDM.fcs
Lymphocytes
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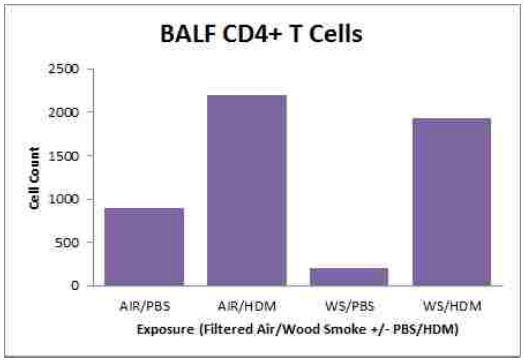
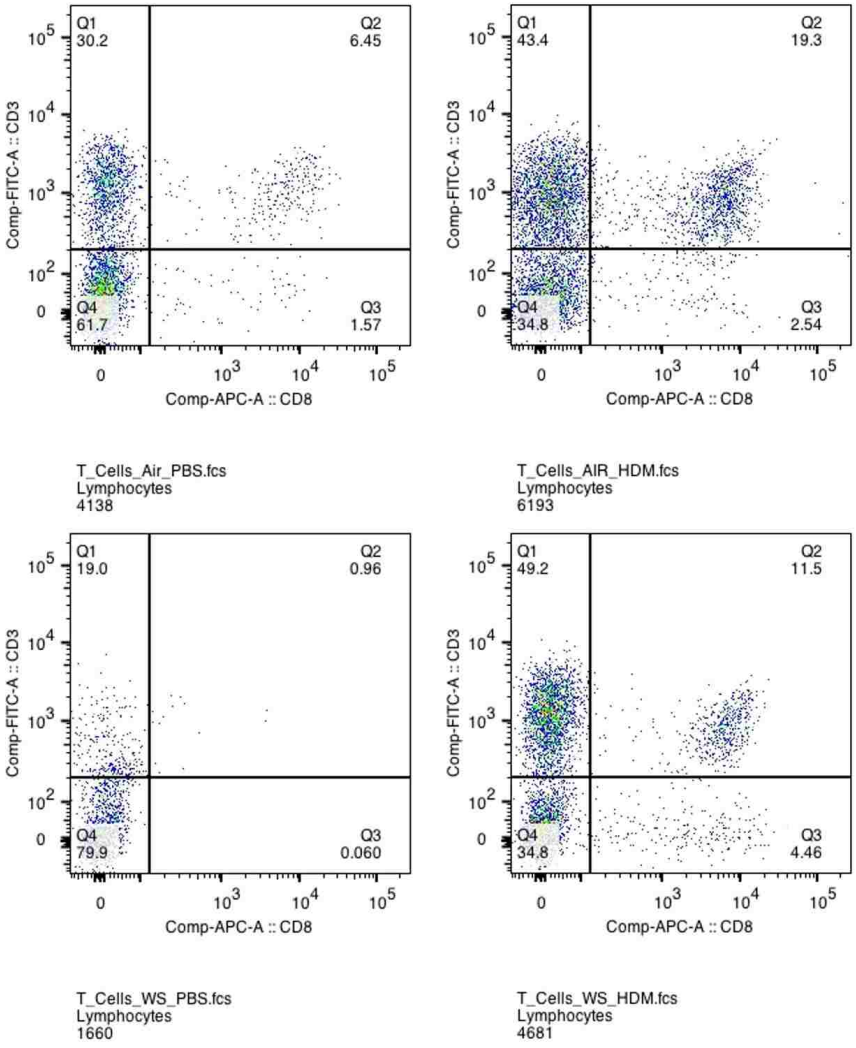


**B**


Q1
30.2
Q2
6.45
Q4
61.7
Q3
1.57
Comp-FITC-A :: CD3
Comp-APC-A :: CD8
T_Cells_Air_PBS.fcs
Lymphocytes
4138
Q1
43.4
Q2
19.3
Q4
34.8
Q3
2.54
T_Cells_AIR_HDM.fcs
Lymphocytes
6193
Q1
19.0
Q2
0.96
Q4
79.9
Q3
0.060
T_Cells_WS_PBS.fcs
Lymphocytes
1660
Q1
49.2
Q2
11.5
Q4
34.8
Q3
4.46
T_Cells_WS_HDM.fcs
Lymphocytes
4681

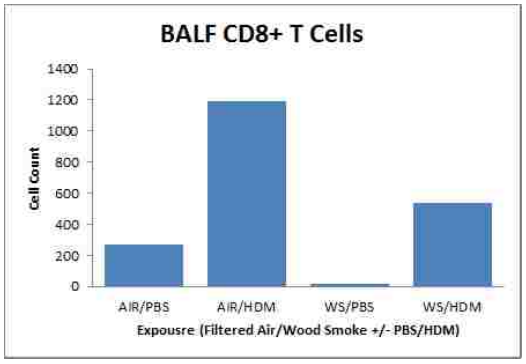

BALF CD8+ T Cells
Cell Count
1400
1200
1000
800
600
400
200
0
AIR/PBS
AIR/HDM
WS/PBS
WS/HDM
Expousre (Filtered Air/Wood Smoke +/- PBS/HDM)

**Figure 3.5**

**Impact of prenatal wood smoke exposure on T cell involvement in allergic responses of adult offspring.**

Dams inhaled either filtered air or controlled levels of $PM_{2.5}$ in wood smoke for 3 hours per day, 5 consecutive days per week, over the course of 3 consecutive weeks. Following birth and natural weaning, pups were maintained for 6 weeks before being sensitized to HDM according to our established model of asthmatic inflammation. Pups (6 per group) were intranasally challenged with HDM allergen or PBS (control). Bronchoalveolar lavage fluid (BALF) was collected for analysis by flow cytometry. Lymphocytes were gated according to size, granularity, and expression of CD3 to identify T lymphocytes. CD4+ (a) and CD8+ (b) T cells were recovered from the airways by bronchoalveolar lavage.

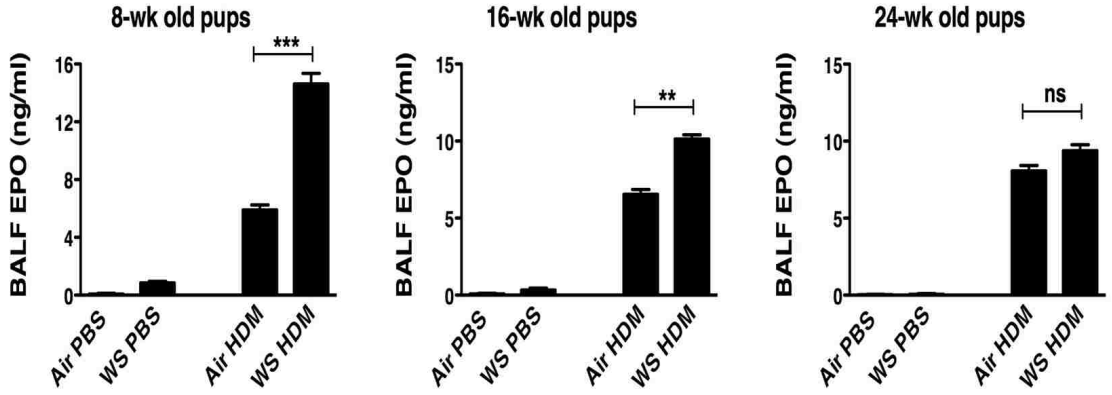
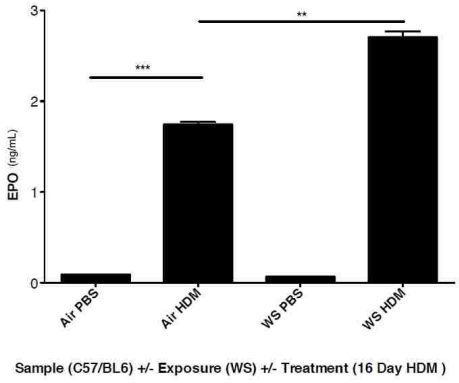


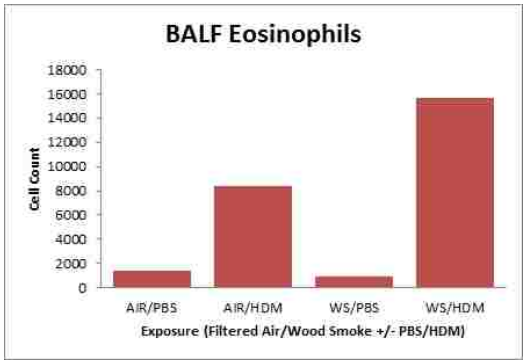

**Figure 3.6**

**Prenatal exposure to wood smoke promotes exaggerated eosinophilia in response to allergen, and the magnitude of this response decreases over time.**

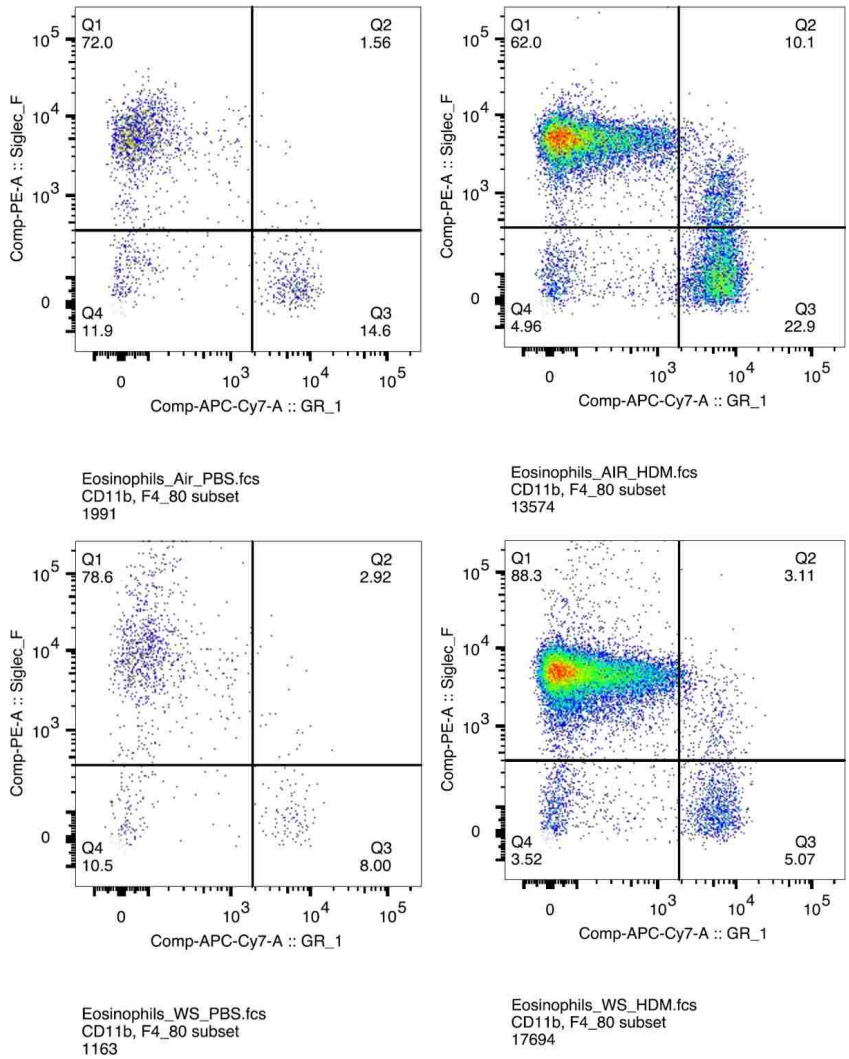
C57BL/6 mice were prenatally exposed to filtered air or wood smoke; they were then sensitized to HDM allergen or dosed with PBS at 6, 14, or 22 weeks of age. EPO levels in the BALF were measured by colorimetric assay. (n=6), *** indicates $p<0.001$; ** indicates $p<0.01$.

**A**


EPO (ng/mL)
Air PBS
Air HDM
WS PBS
WS HDM
Sample (C57/BL6) +/- Exposure (WS) +/- Treatment (16 Day HDM )


BALF Eosinophils
Cell Count
AIR/PBS
AIR/HDM
WS/PBS
WS/HDM
Exposure (Filtered Air/Wood Smoke +/- PBS/HDM)

**B**


Q1 72.0
Q2 1.56
Q4 11.9
Q3 14.6
Comp-PE-A :: Siglec_F
Comp-APC-Cy7-A :: GR_1
Eosinophils_Air_PBS.fcs
CD11b, F4_80 subset
1991
Q1 62.0
Q2 10.1
Q4 4.96
Q3 22.9
Eosinophils_AIR_HDM.fcs
CD11b, F4_80 subset
13574
Q1 78.6
Q2 2.92
Q4 10.5
Q3 8.00
Eosinophils_WS_PBS.fcs
CD11b, F4_80 subset
1163
Q1 88.3
Q2 3.11
Q4 3.52
Q3 5.07
Eosinophils_WS_HDM.fcs
CD11b, F4_80 subset
17694

C

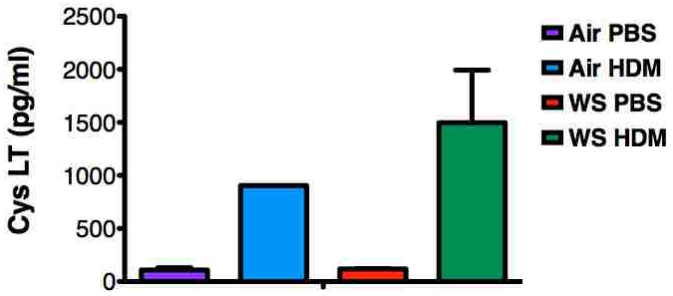
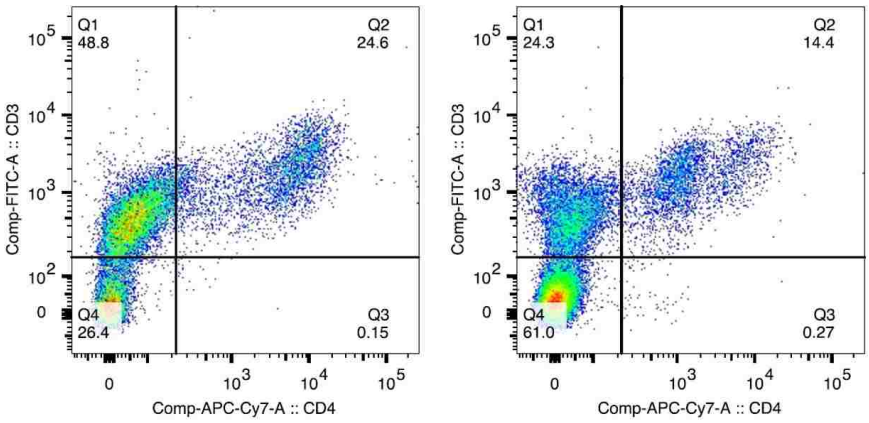



**Figure 3.7**

**Impact of wood smoke exposure on eosinophilia and cysteinyl leukotriene biosynthesis in the airways of adult female mice.**

Mice inhaled either filtered air or controlled levels of $PM_{2.5}$ in wood smoke for 3 hours per day, 5 consecutive days per week, over the course of 3 consecutive weeks. Following birth and natural weaning of pups, dams were sensitized to HDM according to our established model of asthmatic inflammation. Dams (6 per group) were intranasally challenged with HDM allergen or PBS (control). Bronchoalveolar lavage fluid (BALF) was collected for analysis. Relative degrees of eosinophilia were determined by colorimetric assay of EPO recovered from BALF supernatant (a). Portions of supernatant were also analyzed via flow cytometry (b). Leukocytes identified as CD11b+ and F4/80- were further separated according to expression of Siglec-F and GR-1. Those being Siglec-F+ and GR-1- were identified as airway eosinophils. To gauge involvement of cysteinyl leukotrienes in relation to increased eosinophilia, BALF supernatant was further examined by EIA kit, according to manufacturer instructions (c).

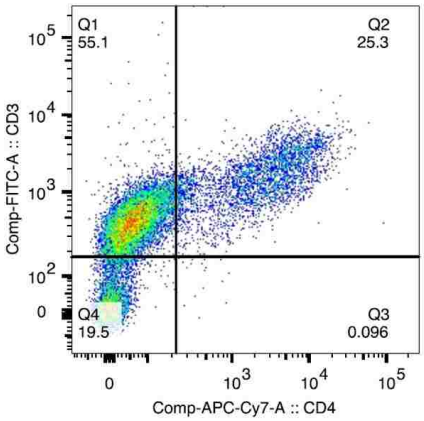
**A**



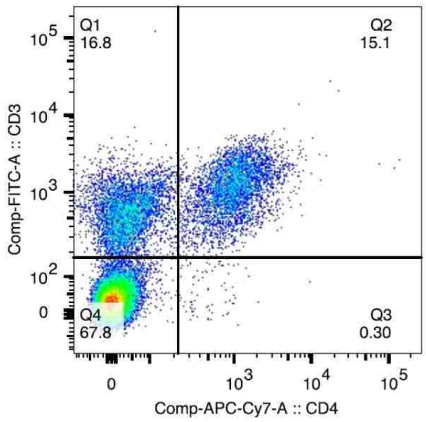

T_Cells_Air_PBS.fcs
Cells
15694



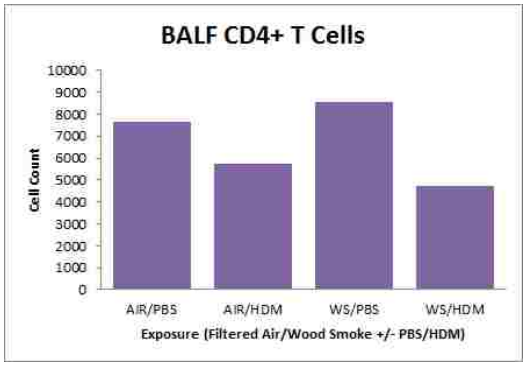

T_Cells_AIR_HDM.fcs
Cells
23589



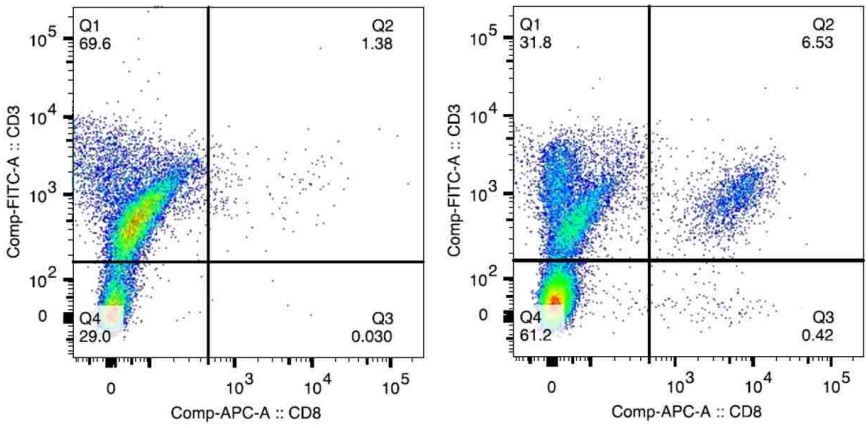

T_Cells_WS_PBS.fcs
Cells
15564




T_Cells_WS_HDM.fcs
Cells
28005



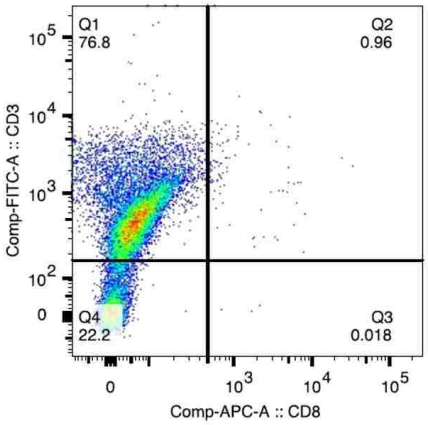
**B**



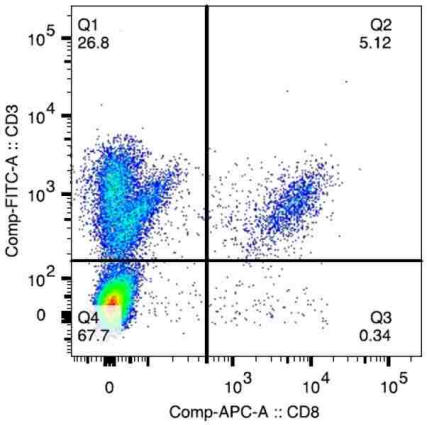

T_Cells_Air_PBS.fcs
Cells
16631




T_Cells_AIR_HDM.fcs
Cells
24668




T_Cells_WS_PBS.fcs
Cells
16461




T_Cells_WS_HDM.fcs
Cells
29053

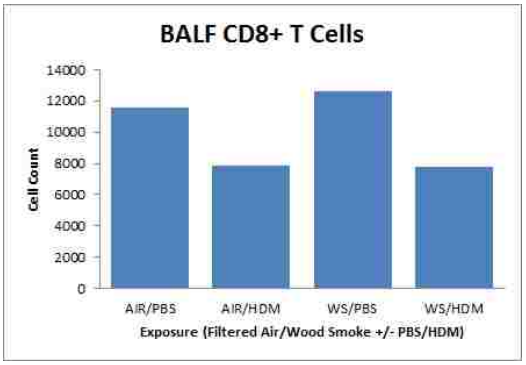


**Figure 3.8**

**Impact of wood smoke exposure on T cell involvement in allergic responses of adult female mice.**

Dams inhaled either filtered air or controlled levels of $PM_{2.5}$ in wood smoke for 3 hours per day, 5 consecutive days per week, over the course of 3 consecutive weeks. Following birth and natural weaning of pups, dams were sensitized to HDM according to our established model of asthmatic inflammation. Dams (6 per group) were intranasally challenged with HDM allergen or PBS (control). Bronchoalveolar lavage fluid (BALF) was collected for analysis by flow cytometry. Lymphocytes were gated according to size, granularity, and expression of CD3 to identify T lymphocytes. CD4+ (a) and CD8+ (b) T cells were recovered from the airways by bronchoalveolar lavage.

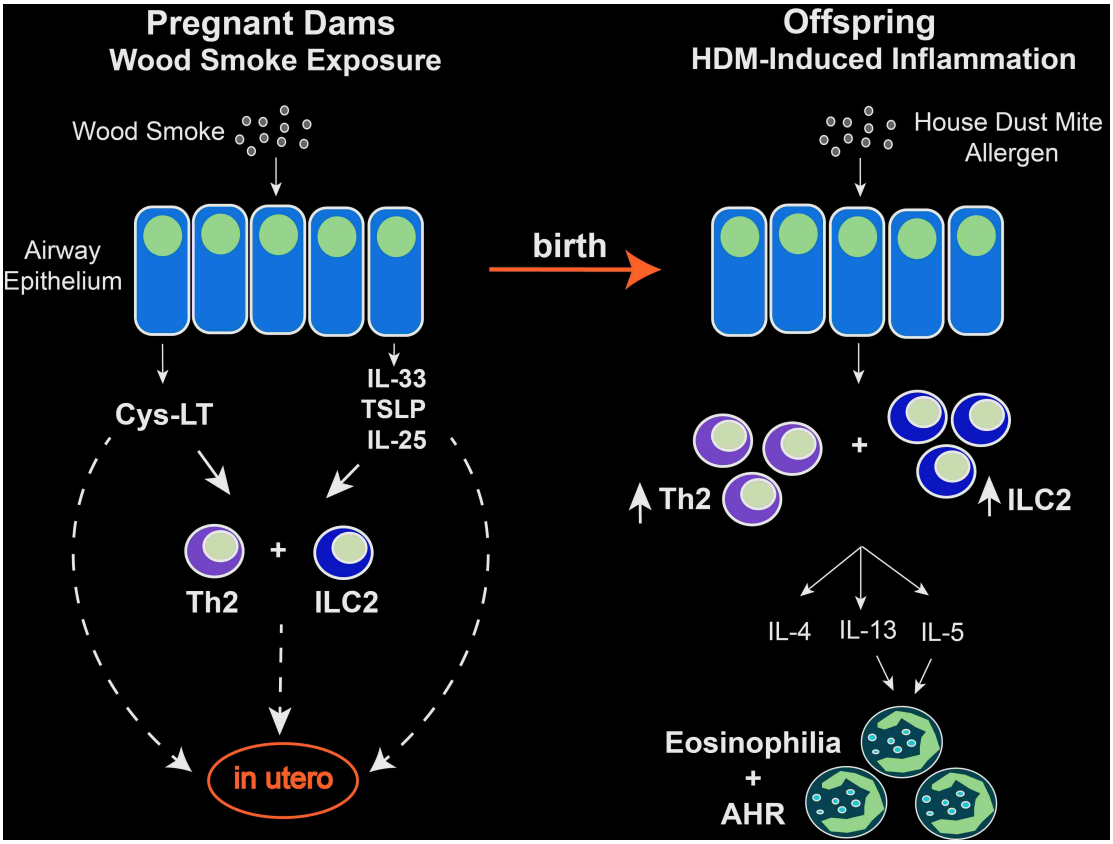



**Fig. 3.9**

**Proposed mechanisms of inflammation exacerbation pursuant to wood smoke exposure.**

Wood smoke inhaled by pregnant dams activates the airway epithelium, which subsequently secretes ILC2-inducing cytokines and potentially cys-LT, which activate Th2 cells. These factors influence the developing fetus. When grown offspring are later sensitized to HDM allergen, their airway epithelial cells respond vigorously and activate Th2 cells and ILC2s. These cells release cytokines conducive to the recruitment of eosinophils to the airways.

# Chapter Four

## 4.0 Overall Conclusions and Future Directions

### 4.1 Conclusions

Allergic asthma is a complex and chronic inflammatory disease of the airways that affects more than 300 million people worldwide. It is of particular concern in highly populated regions of the globe, which include both North and South America, where almost 85% of asthmatics are allergic to house dust mite (66, 67). Asthma is characterized by airway hyperreactivity, contraction of the smooth muscle, goblet cell hyperplasia, increased mucus production, and eosinophil recruitment. While a great deal of research has been performed to determine the cellular and molecular processes underlying these aspects of the disease, much remains to be discovered. One particular need centers on developing a better understanding of the effect of airborne particulate exposures on the severity of asthmatic inflammation.

To address this gap of knowledge, we examined the relationships between the inflammatory processes central to allergic asthma using two model exposures: multi-walled carbon nanotubes and wood smoke. Inhalation of MWCNT is largely an industrial occupational exposure, as materials containing MWCNT are well-suited to numerous applications ranging from electronics to medical implants, but nanoparticles are much less likely to be aerosolized after manufacture (62). Wood smoke, by contrast, is mainly a household exposure. Wood-burning stoves are still quite common, both locally and globally; as of 2014, approximately 2.8 billion people used solid fuels, including hardwood, for cooking (121). Together, MWCNT and wood smoke constitute potential pulmonary health hazards to many people of all ages and demographics. Furthermore,

studying these exposures offered opportunities to better characterize their adverse health effects and to improve our understanding of how those impact inflammatory mechanisms.

Our studies demonstrated that introduction of nickel-containing MWCNT to the lung after HDM allergen sensitization results in significantly exacerbated inflammatory responses. Specifically, sensitized mice instilled with FA21 displayed augmented eosinophilia and elevated biosynthesis of cysteinyl leukotrienes. This is suggestive of a causative role for cys-LT in the recruitment of eosinophils to the airways; dissecting the full mechanism of this action will require further study. Interestingly, our investigation into the interaction of allergic inflammation and wood smoke exposure may offer another avenue of exploration into that process. Adult female mice exposed to ambient wood smoke for a period of three weeks prior to HDM sensitization displayed significantly increased eosinophilia and cys-LT production after allergen challenge. Furthermore, the offspring of those mice, having been exposed to wood smoke only *in utero*, experienced the same effect: augmented eosinophil recruitment and biosynthesis of cys-LT. These observations indicate the possibility of a single mechanism of lung eosinophil recruitment, mediated by cys-LT, which is activated by inhalation of carbonaceous particulate regardless of source.

Collectively, the studies detailed in this manuscript have supplied novel insights into the connections between inhaled pollutants and pulmonary allergic inflammation. These data help to advance our understanding not only of the harmful impacts of MWCNT and wood smoke on the body, but also of the cellular processes underlying inflammation in allergic asthma.

## 4.2 Future Directions

Several aspects that contribute to pulmonary inflammatory responses to inhaled MWCNT, wood smoke, and the delineation of similarities between these remain unresolved. Additional experiments are needed to further dissect molecular events contributing to the effects of particulate exposures.

### *4.2.1 Unresolved Issues Pertaining to MWCNT-Induced Airway Inflammation*

A major hope when investigating the inflammatory properties of MWCNT is that specific parameters may be defined which prove to be predictive of the host response to the particle. The most notable factors influencing the host response to these particles were the presence of nickel in the particle and the production of cys-LT in the airway.

*(i)* *The presence of nickel clearly correlated with the particles' inflammatory potential.* To further evaluate the inflammatory response elicited by different MWCNT particles containing different levels of residual nickel catalyst, additional experiments could be performed either by removing the metal from $Ni^{high}$ particles like FA21 or by supplementing it in preparations of $Ni^{low}$ particles like FA04. The nickel present in FA21 is likely covalently bound into the particle, making removal of the metal difficult. Whether acid washes remove the metal (to be assayed by ICP mass spectrometry) and thereby the resultant effect on inflammatory potential would be interesting. Alternatively, the effect of adding the metal back to FA04 could be addressed by simply adding it to the dispersion media prior to sonication.

(ii) *Inhibitors of 5-LO reduced the lung inflammatory response elicited by FA21.* The principal aspects that remain unresolved pertain to the cellular source of the observed leukotrienes and the relative contributions of $LTB_4$ and cys-LT (both products of arachidonic acid by action of 5-LO) to the inflammatory process. Mice lacking $LTB_4$ receptors (BLTR1 and 2) would subsequently serve as an approach to resolve their relative importance. This would be addressed by evaluating the respective levels of $LTB_4$ and cys-LT present in the BALF after instillation of MWCNT. More definitive involvement of these mediators could also be ascertained by administering MWCNT to 5-LO -/- mice (obtained from Jackson Labs).

(iii) *Prenatal exposure to wood smoke has profound effects on the pulmonary inflammation elicited by HDM allergen in later life.* This observation raises the possibility that prenatal exposure to MWCNT might mediate a similar effect. In these experiments, dams would be exposed to MWCNT and their pups would be evaluated for signs of exacerbated inflammatory responses.

### *4.2.2 Unresolved Issues Pertaining to Wood Smoke-Induced Airway Inflammation*

The maternal exposure to wood smoke clearly had profound consequences for allergen responsiveness in the offspring. However, several issues remain unresolved, largely as a consequence of the inherent complexity of this model. They include:

(i) *Develop a more detailed characterization of the cellular events that underpin the elevated HDM-responsive inflammation resulting from* in utero *exposure to wood smoke.* The relative roles of ILC2 and CD4+ Th2 cells and their

associated cytokines (IL-4, IL-5, IL-9, and IL-13) need to be resolved. The relative contributions of ILC2 and CD4+ T cells could be initially investigated by exposing pregnant IL-13 and IL-5 reporter mice to wood smoke and monitoring the cellular sources of these cytokines in the offspring, using both control (steady-state) and HDM-sensitized mice.

*(ii)* *Examine the BALF collected from mice exposed to wood smoke* in utero *for cys-LT and $LTB_4$ levels.* The effect of treating dams with 5-LO inhibitors during wood smoke exposure should provide information as to the value of these mediators in conferring the observed exacerbated responsiveness to HDM allergen to the offspring. Antibodies exist to permit flow cytometry analysis of CysLT1. It may also be possible to stain receptors and leukotrienes for colocalization via confocal microscopy, though the short half-lives of most cys-LT would likely limit this method to the study of $LTE_4$.

*(iii)* *Isolate the component of wood smoke most critical to exacerbating HDM responsiveness to the offspring.* This would rely on a dose-response study of varying concentrations of $PM_{2.5}$ to experimentally determine the most hazardous concentrations and thereby improve the model's relevance to real-world exposures. It would also be necessary to resolve the relative roles of the gaseous and particulate phases of wood smoke by improving their characterization. Namely, it may be possible to fractionate the smoke and examine independently the effects of $PM_{10}$ and the gaseous components in addition to those of $PM_{2.5}$.

*(iv)* *Assess the effects of wood smoke exposure on fecundity.* While conducting our wood smoke study, we observed what appeared to be unusually large litters produced by smoke-exposed dams. This bears further investigation to determine whether wood smoke inhalation somehow impacts litter size and if so, how.

*(v)* *Investigate the role of maternal milk in conveying altered immunity from wood smoke-exposed dams to pups.* The event responsible for the observed skewing of immunity toward Th2 responses following wood smoke exposure remains unclear. It is possible that potentiation of allergic inflammatory responses in the progeny may occur in neonates via immunologically active factors present in maternal milk. The possibility that maternal immune modulatory factors are transferred in the colostrum to offspring during lactation has been demonstrated previously and asthma susceptibility in humans can be conferred by breast milk. To test the potential contribution of milk-borne mediators, we will examine the effect of adoptive nursing in our model of wood smoke exposure and allergen sensitization. Litters of C57BL/6 mice will be swapped between smoke-exposed and air-exposed dams within 24 hours of birth.

***(vi)*** *Determine whether maternal exposure to wood smoke retards development of the lung and associated innate immunity in the offspring.* This may manifest itself in elevated numbers of lung ILC2s or dendritic cells. To resolve this possibility, one possible strategy would involve harvesting pups immediately after birth and comparing cellular indicators of development in the BALF and the lung epithelium.

### *4.2.3 Inflammatory Mechanisms Common to MWCNT and Wood Smoke Exposures*

Additional experiments will seek to examine whether common inflammatory events contribute to both MWCNT and wood smoke. Given the important role that nickel played in our nanotube studies, further investigation is warranted into its function in wood smoke-induced pulmonary inflammation. This would involve characterizing the metal content of wood smoke via ICP-mass spectrometry and empirically evaluating the differences between nickel-bearing MWCNT and purified MWCNT. To gain insight into the processes by which residual nickel catalyst becomes chemically available *in vivo*, powdered nickel could be added to a preparation of nickel-free MWCNT prior to sonication. A second area worthy of investigation is whether the action of cys-LT detailed in the MWCNT study also plays a central role in smoke-induced exacerbations of inflammation. If this assertion were found to be correct, then the next logical step would be to resolve whether cys-LT are operative in the dams or in their offspring. Lastly, investigations by our laboratory have revealed that NK cells are involved in suppressing the development of HDM-induced lung inflammation. Conceivably, the elevated responsiveness arising from either exposure could be a result of diminished numbers or functionality of pulmonary NK cells. It would be interesting to detail the behavior of NK cells in both models and to determine the extent of NK cells' role following direct and indirect exposure to MWCNT and wood smoke.

## Appendix A

## A Role for $PGI_2$ in the Maturation of Hepatic NK Cells

**Abstract:**

During the characterization of mice lacking the $PGI_2$ receptor, IP, it was observed that the most overt characteristics of the IP-/- mice were changes in the NK cells present in these animals. These changes included a marked increase in the level of NKp46 expression by NK cells in the spleen and lungs. During the course of this study we examined the level of cytokine expression by NK cells in IP-/- mice compared to WT controls. We reported that the level of IFNγ produced in response to NK1.1 cross-linking by splenic NK cells was elevated in IP-/- mice. The liver has been reported to contain large numbers of NK cells, including one population which is resident in the tissue and another indistinguishable from circulating NK cells. Given that the number and characteristics of lung tissue NK cells were altered in IP-/- mice, we examined whether such differences were also evident in the liver. Comparison of surface phenotypes and cytokines produced by NK cells in IP-/- and WT mice revealed that hepatic NK cells were more numerous in IP-/- mice. Activation of hepatic mononuclear cells using immobilized NK1.1 in the presence of exogenous IL-2 failed to induce significant levels of IFNγ secretion in either IP-/- or WT cells. These findings suggest that the livers of the IP-/- mice contain elevated of NK cells, but these cells were atypical since they did not produce IFNγ. Whether this was an artifact of collagenase dispersion or an inherent property of these NK cells remains unclear. In addition, the hepatic T cells in C57BL/6 mice produced over five-fold more

IFNγ than was produced by IP-/- T cells. These findings reveal major differences in both NK cells and T cells in the livers of IP-/- mice.

**Introduction:**

*Natural Killer Cells*

Natural killer (NK) cells are a unique innate lymphocyte population that acts as part of the first line of host defense. These cells are employed to kill virus-infected or malignant cells and accordingly play an important role in tumor defense (167). Approximately 10% of resident lymphocytes in the lung are NK (168). These are characterized as $CD11b^{high}CD27^{low}$ and express higher levels of DX5, CD122, Ly49s, and CD43 than do splenic NK (169). The rapid response time of lung NK and their ability to bridge the gap between innate and adaptive immunity make them ideally suited to combatting respiratory infections. NK depletion has been demonstrated to reduce the efficiency of pathogen clearance, worsen lung pathology, and decrease the Th1 population (168). This suggests that NK cells are central to the induction of protective immunity in the lung, which can also be influenced by prostacyclin-IP signaling.

Conventional NK cells (cNK) arise from a common lymphoid progenitor in the bone marrow. Immature NK express NK1.1 at later stages of development; this is followed by the expression of NKp46 and subsequently DX5, which appears immediately prior to NK maturation and departure from the bone marrow. Mature NK go through an additional four developmental stages, which can be distinguished according to varying levels of CD11b and CD27 expression.

Though it has long been believed that NK cells are limited to nonspecific immune functions (170), more recent studies have presented evidence that they possess a degree of immunological memory. NK cells can modulate elements of adaptive immunity independent of T and B cells, as observed in a murine model of contact hypersensitivity:

*Rag2-/-* mice were capable of mounting an adaptive immune response to hapten challenge despite the absence of T and B lymphocytes, but were rendered incapable of this following depletion of NK cells. These studies identified the surface expression of Thy-1, Ly49C, and CXCR6 as markers for so-called memory NK cells in mice, along with the absence of DX5. In humans, sensitized memory NK cells have been shown to produce increased amounts of IFNγ when activated by IL-12, IL-15, and IL-18 (171). NK cells also interact closely with dendritic cells (DC). Activation of NK induces maturation of the DC through mechanisms dependent on TNFα, IFNγ, and cell-to-cell contact. Mature DC, in turn, support the polarization of T cells to favor the Th1 response and decrease inflammation (168).

NK cells are a major source of IFNγ throughout the body. IFNγ is an important effector molecule for anti-viral responses and also plays a role in regulating other immune functions. It is therefore considered likely that IFNγ is closely involved with the immunological tolerance observed in the liver (172). IFNγ is also known to be a central component of the inflammation involved in delayed-type hypersensitivity (173). Traditionally thought to be made exclusively by NK and T cells, IFNγ has more recently been identified as a product of innate immune cells, including macrophages, DC, and neutrophils, as in the case of hypersensitivity pneumonitis. Synthesis of IFNγ by innate cells is key to the establishment of Th1 responses before T cells begin production as part of the adaptive immune response (174).

*Hepatic Natural Killer Cells*

NK cells make up approximately 40% of all lymphocytes present in the liver. Liver-resident NK are functionally and phenotypically distinct from those circulating in the blood; hepatic NK are involved in viral defense, tumor suppression, and fibrosis prevention. The main site of NK cell development is the bone marrow, in both mice and humans (171). But the source of liver-resident NK cells is still unclear; they may originate in the liver itself or mature from precursors arising in the bone marrow. Two phenotypically different populations of NK cells have been described in the livers of mice. One population is CD3-CD19-NK1.1+DX5+ and indistinguishable from circulating NK cells, while the other is CD3-CD19-NK1.1+DX5- and believed to reside in the liver without entering circulation.

While conventional NK cells are present in the liver, nonconventional trNK cells also appear. trNK cells are CD49a+DX5-, whereas cNK are CD49a-DX5+. Unlike cNK, trNK develop in the absence of the transcription factors NFIL3 and Eomes. Instead, trNK rely on T-bet, which is expressed at an earlier point in development in the liver than in the bone marrow; this yields mature NK deficient in DX5. This suggests that trNK may arise from a developmental pathway distinct from that of bone marrow derived cNK. Both cNK and trNK in the liver produce IFNγ and are capable of releasing perforin and granzymes; however, trNK also produce TNFα, which stimulates the recruitment of neutrophils to the liver (169).

The liver is integral to both innate and adaptive immunity. It is a site of extrathymic T cell proliferation, development of immunological tolerance, and deletion of activated T cells (175). The hepatic environment favors the induction of tolerance over immunity when exposed to foreign antigens. This sets the liver apart as an immune-privileged site,

wherein allografts, certain pathogens, and exogenous proteins are for the most part immunologically ignored. This local tolerance often begets systemic tolerance of those antigens which are persistently expressed in the liver. The tolerogenic mechanism has therapeutic implications in that hepatic expression of autoantigens can significantly reduce the incidence of autoimmune diseases (172).

Even though the liver cedes much of its hematopoietic ability to the bone marrow as the animal ages, the adult human liver does retain the capacity to resume hematopoiesis. This may make it possible for resident NK to originate directly from the liver. However, there is also evidence that tissue-resident NK develop from precursors originating in the bone marrow. Hepatic NK precursors are swiftly replaced by precursors recruited from the bloodstream if depleted, as in the case of liver transplants. Contrarily, mature NK do not leave the hepatic tissue to reenter circulation. This suggests that while NK cells may originate in the bone marrow, they remain stationary after migrating to the liver and maturing there (176).

*$PGI_2$ Receptor, IP, and NK Cells*

The IP receptor and its ligand, prostacyclin ($PGI_2$), have been shown to be involved in the development of hypersensitivity reactions such as contact dermatitis and allergic asthma in both humans and mice (96). Like all prostanoids, prostacyclin is a bioactive lipid derived from arachidonic acid through the enzymatic activity of cyclooxygenases. Prostacyclin in particular has been examined due to its vasodilatory function and its capacity to prevent aggregation of platelets. These studies have led to the creation of a number of synthetic analogs (177). Those analogs have in turn been found to possess the

ability to suppress Th2 responses, including bronchoconstriction and the recruitment of Th2 cells to airways, thereby significantly decreasing Th2-type inflammation. Furthermore, IP deficiency can exacerbate Th1 responses, leading to increased viral inflammation and elevated production of IFNγ by T cells (178).

As the Th2 response is central to the progression of allergic asthma, the potential of prostacyclin-IP signaling to modulate related symptoms bears further investigation. To that end, we have previously examined this receptor and described a disparity in the innate immune responses of C57BL/6 vs. IP-/- mice (69). Most notable was the difference in populations of NK cells in the lungs and spleen, with the lung of mice deficient in the prostacyclin receptor containing roughly twice as many NKs as those of wildtype mice. As the liver is home to large numbers of NK cells, we examined the relative populations of wildtype and IP-/- hepatic NK cells.

**Methods:**

*Mice*

C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) (10-16 weeks old) were used throughout this study. Mice were bred under pathogen-free conditions in a barrier facility and experimental animals maintained in micro-isolator cages and treated in accordance with National Institutes of Health guidelines and the American Association of Laboratory Animal Care regulations. Animal experiments were approved by the University of Montana, Institutional Animal Care and Use Committee, IACUC according to National Institute of Health guidelines.

*Media*

Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (CM-RPMI), (Hyclone Fetal Calf Serum, Thermo Scientific), L-glutamine, penicillin & streptomycin, HEPES, Sodium Pyruvate (Gibco Life Technologies) and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO).

*Isolation of Hepatic Immune Cells*

Cells were collected for analysis by collagenase digestion of whole liver tissue. Following mechanical deconstruction, liver samples were incubated for a minimum of 1 hour in a 37°C water bath with shaking. Supernatant was then strained and diluted to a per-sample volume of 40 mL with culture media. Lymphocytes were isolated by centrifugation over Percoll density gradients.

*Flow Cytometry*

Hepatic mononuclear cells were FcγR blocked using 2.4G2 antibody (ATCC) and stained with combinations of the following mouse conjugated mAb (all purchased from BioLegend): allophycocyanin (APC) or FITC anti-CD3, APC/Cy7 anti-CD4, PE anti-CD8a, APC or PE anti-CD11c, PE or APC/Cy7 anti-I-A/I-E, APC/Cy7 anti-Ly6G, APC or APC/Cy7 anti-Ly6C, APC/Cy7 anti-Ly-6G/Ly6C (Gr-1), PE, FITC or Brilliant Violet 421 anti-CD11b, APC or PE anti-F4/80. In addition, PE anti-Siglec-F (BD Biosciences) was used to stain eosinophils. Flow cytometric acquisition was performed on a FACSAria II (BD Biosciences) by 4-color analysis using FACSDiVa software and FlowJo, with a minimum of 50,000 live, single-cell events per sample collected.

*Statistical Analysis*

Data were analyzed using GraphPad Prism 5.0 (GraphPad, La Jolla, CA). Results involving two variables were analyzed by two-way ANOVA with a Bonferroni post-hoc test. Data comparing two groups were analyzed using an unpaired t test. Figures show combined data from multiple studies or independent repeats (two or more). Data shown are mean ± SEM. A p value $< 0.05$ was considered statistically significant. Significance denoted by *, **, or *** is defined as $p<0.05$, $p<0.01$, or $p<0.005$, respectively.

**Results:**

Our laboratory has previously reported that the number and properties of NK cells are altered in IP-/- mice. Given that large numbers of memory NK cells are typically found in the liver, it was logical to characterize hepatic NK cells in IP-/- mice.

*Analysis of IP-/- hepatic NK cells by flow cytometry*

To detail the phenotype of hepatic NK cells, livers from wildtype C57BL/6 mice and IP-/- mice (C57BL/6 background) were dispersed using collagenase. The resulting cell suspension was fractionated using Percoll density gradient centrifugation, and mononuclear cells collected for analysis. Cytometry revealed an abundance of hepatic lymphocytes in IP-/- mice compared to WT mice, particularly NK cells. IP-/- mice had seven times more mononuclear cells than WT C57BL/6 mice. Of the lymphocytes collected, 5.8% were DX5+NK1.1+ NK cells in IP-/- mice, whereas only 1.2% were identified as DX5+NK1.1+ in WT mice (Fig. A1). This change in the proportion of NK phenotypes may indicate a defect in nonspecific immune reactivity in the absence of functional IP-prostacyclin signaling. Deficiency in the IP receptor also resulted in higher levels of NKp46 expression by NK cells evidenced by IP-/- hepatic MNC being 7.6% NK1.1+NKp46+ compared to 0.3% in C57BL/6 (Fig. A1)., i.e., IP-/- mice possessed more than 20 times as many hepatic NKp46+NK1.1+ NK cells as WT mice.

*Analysis of IFNγ production by splenic and hepatic NK cells in IP-/- mice*

IFNγ is the predominant cytokine produce produced by NK cells. In order to investigate the functional properties of hepatic NK cells in the IP-/- and C57BL/6 mice

hepatic MNC were cultured overnight on plates pre-coated with either anti-NK1.1 (20 μg/mL) or PBS in the presence of exogenous IL-2 (10 pg/mL) and the level of IFNγ present in culture supernatants assayed by ELISA. Positive controls consisted of hepatic MNC that were cultured in the presence of immobilized anti-CD3 (2 μg/mL) in order to activate resident T cells. Controls comprised cells cultured in media of different concentrations of α-galactosylceramide (Gal-cer, a ligand for NK-T cells which are known to produce IFNγ). Curiously, although NK cells were present in hepatic MNC preparation, activation using immobilized NK1.1 did not induce IFNγ production above what was observed in media alone, even with the addition of Gal-cer. Interestingly, stimulation of hepatic MNC with immobilized anti-CD3 did induce the production of large amounts of IFNγ; however, C57BL/6 cells produced markedly more cytokine than did cells from IP-/- mice (Fig. A2) with levels reaching only one fifth of the IFNγ produced by WT cells. These findings were in marked contrast to the production of IFNγ by NK cells in the spleens, since application of NK1.1 induced higher levels of IFNγ production in C57BL/6 spleens compared to IP-/- (Fig. A3).

**Discussion:**

NK1.1 and DX5 (CD49b) are prototypic NK cell surface markers. The former is an allotypic marker expressed by NK cells in C57BL/6 mice, while DX5 is a NK cell marker of particular utility as it is expressed by all mouse strains. We observed increased expression of NKp46 by CD3-CD19- cells that expressed NK1.1, signifying an expanded population of NK cells in the livers of IP-/- mice compared to wildtype mice. NKp46 is one of the natural cytotoxicity receptors (NCR) commonly expressed by both activated and non-activated NK cells. Recognition by this receptor typically results in immediate degranulation of the NK cell and lysis of the activating target cell (179). While this might be expected to be associated with increased NK cell cytotoxicity, it has recently been discovered that the immunomodulatory capacity of NKp46+ NK cells is critical to defense against certain inflammatory disorders in the liver. The progression of nonalcoholic fatty liver disease is determined in large part by the activation of Kupffer cells, liver-resident macrophages which respond to the release of damage-associated molecular patterns from damaged hepatocytes by recruiting inflammatory monocytes to the liver tissue. NKp46+ NK cells are able to hamper this inflammatory response by polarizing liver macrophages toward a wound-healing and anti-inflammatory M1 phenotype by secreting IFNγ. Hepatic inflammation is significantly exacerbated and prolonged in the absence of NKp46+ NK cells and ultimately results in fibrosis (180).

A major problem encountered in this study was that the production of IFNγ in response to NK1.1 cross-linking by both IP-/- and WT NK cells was minimal. The reason for this lack of response remains unclear and conceivably could be an artifact associated with the use of collagenase to release MNC from the liver tissues. In contrast, stimulation

of cultured hepatic MNC with anti-CD3 antibody did induce cytokine production; hepatic T cells from IP-deficient mice still produced substantially less IFNγ than did NK derived from wildtype mice. The reason for this marked reduction in the IFNγ response likely reflects a reduction in the number of T cells producing this cytokine. These findings indicate that prostacyclin-IP signaling plays a pivotal role in the maturation of hepatic T cells, specifically in the selection of cells producing IFNγ.

**Figure Legends:**

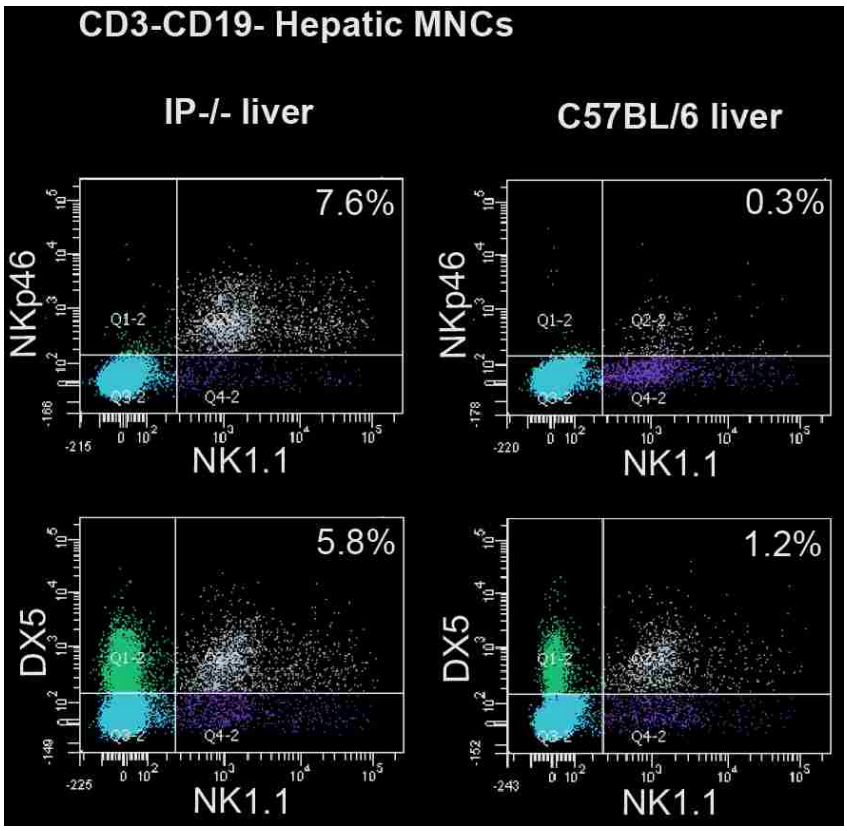



**Figure A1: Hepatic DX5+NKp46+NK1.1+ NK cells are more abundant in IP-/- mice.**

Livers from IP-/- and C57BL/6 mice were dispersed using collagenase and the number and characteristics of the NK cells present evaluated by flow cytometry.

Flow cytometry revealed that hepatic DX5+NK1.1+ NK cells make up 5.8% of cells present in IP-/- mice compared to only 1.2% present in wildtype mice. In addition, the level of NKp46 expression by NK cells was elevated in IP-/- mice.

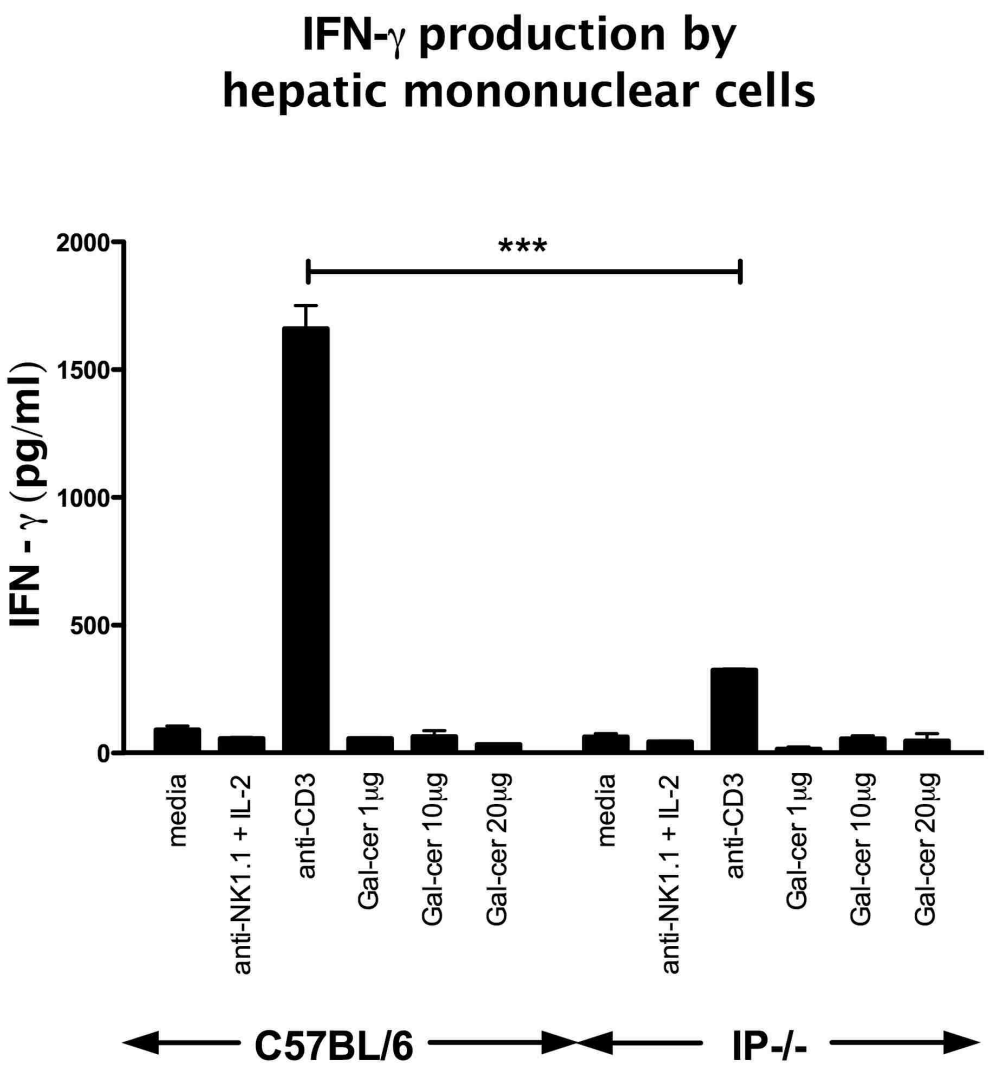



**Figure A2: IFNγ production in response to anti-CD3 is reduced in hepatic MNC from IP-/- mice.**

Hepatic MNC from C57BL/6 and IP-/- mice were cultured in the presence of plate-bound anti-NK1.1 with 10 ng/mL IL-2 (20 μg/mL) or anti-CD3 (2 μg/mL) for 24h. Supernatants were assayed for IFNγ revealed by ELISA. Hepatic MNC from C57BL/6 produced significantly higher levels of IFNγ than IP-/- cells following anti-CD3 activation (300 pg compared to 1500 pg).

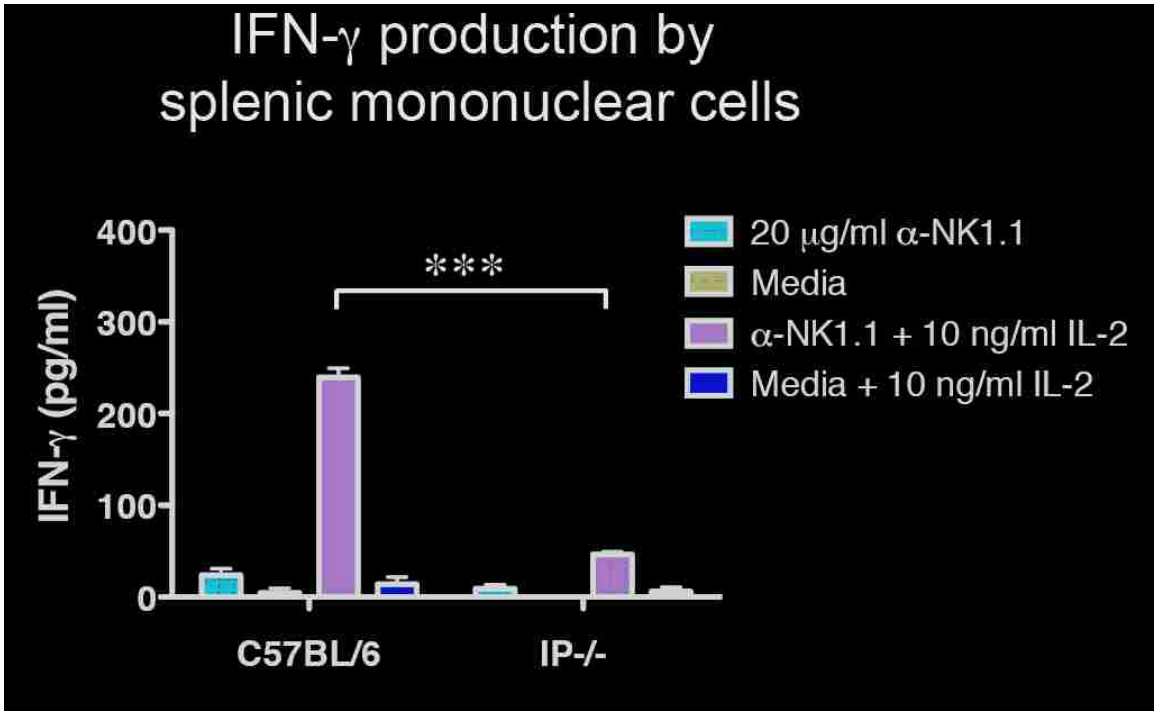



**Figure A3: IFNγ production by splenic MNC in response of anti-NK1.1.**

Spleens cells were stimulated on plates pre-coated with anti-NK1.1 and exogenous IL-2 and the level of IFNγ produced after 24h assayed by ELISA.

## Appendix B

# Prenatal tobacco smoke exposure predisposes offspring mice to exacerbated house dust mite-elicited allergic airway inflammation associated with altered innate effector function

Maria Ferrini[1], Britt Postma[1], Sophia Carvalho[1], Lucas Miranda Marques[1], Kent Pinkerton[2], Kevan Roberts[†1*] and Zeina Jaffar[†1*]

[1]Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, College of Health Professions and Biomedical Sciences, University of Montana, Missoula, Montana, USA.

[2]Department of Anatomy, Physiology and Cell Biology, Center for Health and the Environment, University of California, Davis, CA, USA.

[†]Zeina Jaffar and Kevan Roberts contributed equally to this work.

[*]Corresponding authors: Zeina Jaffar or Kevan Roberts, Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, University of Montana, Missoula, MT 59812. E-mail: zeina.jaffar@umontana.edu or kevan.roberts@umontana.edu

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**Abstract:**

**Background:** Epidemiological studies suggests that prenatal and early life environmental exposures have adverse effects on pulmonary function and are important contributors in the development of childhood asthma and allergic disease. The mechanism by which environmental tobacco smoke (ETS) exposure in utero promotes the development of allergic asthma remains unclear. In this study, we investigated the immunological consequences of prenatal exposure to ETS in order to understand events responsible for the development or exacerbation of allergic asthma.

**Methods:** Pregnant C57BL/6 mice were exposed to ETS or filtered air throughout gestation and the effects on pulmonary inflammation in the offspring were examined and compared. Specifically, the effects on eosinophilic inflammation, airway hyperreactivity, goblet cell hyperplasia, properties of pulmonary natural killer (NK) cells and type 2 cytokines elicited in response to inhaled house dust mite (HDM) allergen were investigated in the progeny.

**Results:** Exposure to ETS prenatally significantly exacerbated HDM-induced airway eosinophilic inflammation, hyperreactivity, mucus secretion, cysteinyl leukotriene biosynthesis and type 2 cytokine production in the offspring. Consistently, lung mononuclear cells from ETS-exposed offspring secreted higher levels of IL-13 when stimulated *in vitro* with anti-αβ TCR antibody or HDM allergen. Moreover, offspring from ETS-exposed dams exhibited a higher frequency of $CD11b^+$ dendritic cells and $CD3^+CD4^+$ T lymphocytes in the lungs following allergen inhalation compared to air-exposed mice. Unexpectedly, the exacerbated allergic inflammation in the ETS-exposed offspring was

associated with a reduction in $CD3^{-}CD19^{-}NK1.1^{+}CD94^{+}$ NK cell numbers and their IFN-γ production, highlighting a role for innate immunity in the enhanced allergic response.

**Conclusion:** Our results reveal that prenatal exposure to ETS predisposes offspring to an exacerbated allergic airway inflammation that is associated with a reduction in pulmonary NK cell function, suggesting that NK cells play a key role in controlling asthma severity.

**Keywords:** Prenatal exposure, Environmental tobacco smoke, Allergic asthma, Innate immunity

## Appendix C

## Publications

(i) Prenatal wood smoke predisposes mice to exacerbated allergic lung inflammation. Sophia Carvalho, Maria Ferrini, Britten Postma, Kevan Roberts, Zeina Jaffar. *In preparation.*

(ii) A role for cysteinyl leukotrienes in promoting airway inflammation elicited by multi-walled carbon nanotubes. Sophia Carvalho, Maria Ferrini, Zeina Jaffar, Kevan Roberts. *In preparation.*

(iii) Prenatal tobacco smoke exposure predisposes offspring mice to exacerbated house dust mite-elicited allergic airway inflammation associated with altered innate effector function. Maria Ferrini, Sophia Carvalho, Britten Postma, Lucas Miranda Marques, Kent Pinkerton, Yoon Hee Cho, Kevan Roberts, Zeina Jaffar. 2017. *Accepted: Particle and Fibre Toxicology.*

(iv) $PGI_2$ controls pulmonary NK cells that prevent airway sensitization to house dust mite allergen. Bryan Simons, Maria Ferrini, Sophia Carvalho, David Bassett, Zeina Jaffar, Kevan Roberts. 2017. *Journal of immunology* 198: 461-471.

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