I, Vanessa C Saunders, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Immunobiology

It is entitled:

Mechanisms of Particulate Matter-Induced Experimental Asthma

Student Signature: Vanessa C Saunders

This work and its defense approved by:

Committee Chair: Marsha Wills-Karp, PhD

Fred Finkelman, MD

George Deepe, MD

Chris Karp, MD

Kristen Page, PhD
Mechanisms Of Particulate Matter-Induced Experimental Asthma

A dissertation submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for the degree of

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by

Vanessa Christine Saunders

B.A. FISK University

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Committee Chair: Marsha Wills-Karp, Ph.D.
Abstract

Asthma is the result of a failure in respiratory tolerance to innocuous inhaled substances, leading to maladaptive Th2 immune responses. Over the last few decades, the prevalence of asthma has increased at a rate far greater than that which could be attributed to the contribution of genetic susceptibility factors, thus, indicating that the environment plays a critical role in the rapid increase in disease. Epidemiological studies have shown a strong association between exposure to airborne particulate matter (PM) and increased incidences of asthma. However to date, these studies are only correlative and have not firmly established a relationship between PM exposure and the increases in asthma prevalence. We have previously shown that the direct exposure of mice to ambient PM collected in urban Baltimore (AUB) can induce the cardinal features of asthma, yet the exact mechanism(s) of this induction remain unknown. To begin to determine the mechanisms by which PM exposure may contribute to the rise in asthma, we tested the hypothesis that AUB can directly induce the development of airway hyperresponsiveness (AHR), airway inflammation, cytokine production and oxidative stress, through the initiation of both innate and adaptive immune responses in the airway. In this body of work, we firmly establish that AUB activates the airway epithelium to produce a number of factors (CCL20, GM-CSF) that recruit and activate immunogenic myeloid dendritic cells to the airway wall and drive the development of pathogenic Th2/Th17 cell responses that subsequently induce the pathophysiological manifestations of asthma in mice. Collectively, these studies support the hypothesis that PM exposure may, in part, contribute to the recent increases in asthma prevalence and morbidity.
Preface

This thesis consists of an introduction (Chapter 1), an overview of the general methods (Chapter 2), four chapters (Chapters 3 – 6) which are comprised of individual manuscripts, a summary and a discussion (Chapter 7). The work in Chapter 4 was submitted to Environmental Health Perspectives. The work herein was performed by the author under the guidance of Dr. Marsha Wills-Karp in the Division of Immunobiology, Cincinnati Children’s Hospital, and Department of Pediatrics at the University of Cincinnati College of Medicine.
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To my advisor, Dr. Marsha Wills-Karp, thank you for believing in me. Your gift for mentoring is an inspiration. You are an excellent scientist, but more importantly and simply put, you are an excellent person. I am sincerely grateful for your enthusiasm and the time you invested in advising me, motivating me and challenging me as a scientist.

I would like to thank my thesis committee, Dr. George Deepe, Dr. Fred Finkelman, Dr. Christopher Karp and Dr. Kristen Page for their interest in my growth and development as a scientist. Each of you has opened your doors to me and provided me with research tools, scientific advice and career advice.

Additionally, I would like to thank my friends. Despite never studying immunology, you listened to me talk about experimental protocols and scientific manuscripts and supported me throughout my journey as a scientist. This work is truly the result of a combined effort that included everyone mentioned above plus many others that have come into my life along the way. Without you this thesis would not exist.
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<tr>
<td>PM</td>
<td>Particulate matter</td>
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<td>AUB</td>
<td>Ambient Urban Baltimore Particulate Matter</td>
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<td>GINA</td>
<td>Global Initiative for Asthma</td>
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<td>ISAAC</td>
<td>International Study of Asthma and Allergies in Childhood</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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Chapter 1

Introduction
I. Asthma: Definition and Symptoms

‘Spasmodic asthma’, was defined in the 1860’s by Henry Salter, as “paroxysmal dyspnea of a peculiar character, generally periodic, with intervals of healthy respiration between attacks”, that leads to “the sense of impending suffocation” and “the agonizing struggle for the breath of life”\(^1\). It was believed that asthma was a disease of complex origins that could be characterized by muscular spasms of the bronchial tubes\(^1\). Today, the Global Initiative for Asthma (GINA) provides the following operational description of asthma, “Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness (AHR) that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment”\(^2\). The complex nature of asthma and the overlap of pathological features such as airway inflammation, airway obstruction and mucus production with disorders such as acute respiratory distress syndrome and chronic obstructive pulmonary disease often make it difficult to accurately diagnose asthma. Indeed the Global Initiative for Asthma addressed the complexity of diagnosis in children under the age of five stating that “…neither airflow limitation nor airway inflammation, the main pathologic hallmarks of the condition, can be assessed routinely in this age group”\(^3\). Thus the standardization of care is difficult and treatment must be carefully tailored to the nature of the symptoms presented.
II. Asthma: Prevalence, Risk Factors and Morbidity

Within the past several decades an alarming increase in the prevalence of asthma has been documented. Approximately 300 million individuals worldwide suffer from asthma and it is estimated that an additional 100 million people will suffer from asthma by 2025. To date, asthma is the most prevalent chronic childhood disease. Results from phase three of the International Study of Asthma and Allergies in Childhood (ISAAC), which surveyed approximately 1.19 million participants from 233 centers in 97 countries, indicate that the mean prevalence of wheezing was 11.5% among 6-7 year old children and 14.1%, among 13 – 14 year old adolescents. Globally 4.9% of children and 6.9% of adolescents exhibit symptoms of severe asthma. These unsettling prevalence rates are grossly underestimated and it is predicted that globally 20% of adolescents and 16.5% of children experiencing wheezing and symptoms of severe asthma were never diagnosed. The predominance of such undiagnosed cases exist in developing countries within the African, Eastern Mediterranean and Indian subcontinents, in which limited access to healthcare can result in those of lower socioeconomic status remaining undiagnosed.

Data from ISAAC showed that a higher prevalence of wheezing was associated with higher gross national income. Indeed, the highest mean asthma prevalence rates are reported to occur in westernized societies such as the United Kingdom/Republic of Ireland (16.1%), North America (11.9%) and the Caribbean (10.4%) in comparison to less westernized societies such as Southeast Asia (3.3%), Southern Asia (3.5%), and China/Taiwan/Mongolia (2.1%). In the Unites States alone, data from the U.S. National
Center for Health Statistics estimates that approximately 34 million Americans have received a diagnosis of asthma in their lifetime with 16.2 million adults and 6.7 million children currently living with asthma.

The negative impact of airway obstruction resulting from asthma episodes includes sleep disturbance, missed days at school and work, limited ability to participate in social activities, the need for costly treatments, hospitalizations and death. In addition to its negative social impact asthma creates a substantial economic burden. In 2007, it was estimated that 14.7 billion dollars went to direct asthma care in the U.S. Furthermore, indirect costs due to loss of productivity are estimated to be as high as 5 billion dollars, resulting in a total U.S. economic cost of 19.7 billion dollars per year.

III. Pathogenesis of Asthma

The characteristic features of allergic asthma, which include bronchial hyperresponsiveness, mucus production, smooth muscle hyperplasia, eosinophilia, and IgE production result from specific immune responses driven by both infectious (viruses, fungi) and non-infectious (pollens, animal dander, pollutant, mite and cockroach excrement) agents. However, the induction and magnitude of such responses can also depend on genetic susceptibility. Because T cells orchestrate the key characteristics associated with asthma (e.g., IgE and eosinophilia), numerous studies have sought to define a role for the T cell in allergic asthma.
i. **CD4⁺ T cells**

Numerous studies demonstrated that the induction and regulation of allergic airway disease relied heavily on lymphocyte recruitment, activation and expansion. The increased numbers of pulmonary T cells in asthmatics suggested that these cells were involved in the development of asthma symptoms. Several lines of evidence in mouse models of experimental asthma demonstrate that CD4⁺ T cells are indeed required for the development of asthma. Specifically, airway hyperreactivity induced in mice that received a passive transfer of sensitized lymphoid cells was abrogated with T cell depletion of lymphoid populations. In addition, the antibody depletion of CD4⁺ T cells abrogated AHR in animal models of allergen and ozone-induced AHR. Studies performed in athymic (nude) mice and those lacking mature lymphocytes (Rag1/−) showed that a lymphocyte deficiency abrogated the development of AHR and reduced allergen-induced eosinophilia. In addition, the reconstitution of Rag1/− mice with CD4⁺ T cells restored the development of allergen induced AHR and eosinophilia. Collectively, these studies confirm that CD4⁺ T cells are necessary for the development of AHR.

In 1986 the understanding of T cells in various diseases improved greatly with the identification of antigen-specific Th1 and Th2 subsets. Th1 cells are generally characterized by the secretion of the cytokine IFNγ and mediate protection against microbial pathogens and cell-mediated immunity. Th2 cells are generally characterized by secretion of the cytokines IL-4, IL-5, IL-9 and IL-13 and mediate humoral responses and those to helminths and extracellular parasites. A role for Th2 cytokines in allergic airway diseases has been suggested by their ability to induce
several features of allergic diseases such as IgE production and the regulation of eosinophil activation. Specifically, the Th2 cytokine IL-4, has several functions that are relevant to the development of allergic inflammation such as: 1) it is an important growth factor for Th2 cells; 2) it promotes B lymphocyte isotype switching to IgE; and 3) it enhances mast cell growth and induces chemokines that recruit Th2 cells. The Th2 cytokine IL-5 is necessary for the differentiation, survival and activation of eosinophilic inflammation in the asthmatic airway.

Robinson and colleagues originally described an association between Th2 cytokine production and asthma when they reported that BAL cells from asthmatics exhibited a notably higher Th2 cytokine profile (IL-4, IL-5) when compared to normal controls. This association was later confirmed in numerous studies of asthmatic airways. In support of a role for Th2 cytokines in asthma, genetic studies have linked asthma to a region of human chromosome 5q, which encodes the genes for Th2 cytokines. A more definite role for Th2 cytokines in asthma was provided by experimental animal studies in which the adoptive transfer of a clonal population of Th2 cells into the mouse lung induced allergic airway symptoms. Although a role for Th2 cells is clear, the contribution of individual Th2 cytokines in allergic inflammation was more difficult to delineate. IL-4 was shown to be important in the initiation of allergic inflammation. However, studies revealed that IL-4 played a limited role in the induction of AHR, but was instead necessary for the eosinophilia that is commonly associated with asthma. In contrast, IL-13 was shown to play a limited role in initiation of disease, however it can mediate the development of the downstream sequelae of allergic asthma. Although IL-5 clearly is important in pulmonary eosinophilia and has been shown to be important
in allergic asthma in some animal models, its role in human asthma remains controversial. Current reports indicate that the administration of mepolizumab (anti-IL-5) to asthmatics significantly attenuates pulmonary eosinophilia. In addition, despite reports of no significant effects on pulmonary function (FEV₁, PEFR, PC₂₀), asthmatics receiving mepolizumab experience a reduced number of exacerbations. Although questions still remain, these studies support the contention that Th2 cells play an important role in the pathogenesis of asthma. Taken together, these reports confirm that the Th2 cell is a central mediator of asthma, as depicted in Figure 1.
Figure 1. The Th2 cell is a central mediator of asthma. The production of Th2 cytokines leads to the pathophysiological features of asthma including IL-4-induced IgE synthesis, IL-5-induced eosinophil recruitment and degranulation, and IL-13-driven mucus production and AHR.
While the pathological features of asthma are mediated through Th2 cytokines, Th1 derived cytokines can negatively regulate allergic asthma. We 46 and others 47-49 have previously shown that cytokines driving Th1 responses such as IL-12 and IFNγ reduce allergen-induced AHR, eosinophilia, lymphocyte recruitment and Th2 cytokine production in mouse models of experimental asthma.

In addition to the established role of Th1 subsets in the regulation of asthma, data from our lab 50 and others 51 indicate that the regulatory T cell (Treg) confers resistance to the development of allergen-induced AHR through its ability to promote respiratory tolerance and limit chronic airway inflammation to non-pathogenic stimuli 51. Mechanisms of Treg-induced regulation of adaptive immune responses include the production of the cytokines IL-10 and TGF-β 52. The increased presence of the regulatory T cell associated cytokine IL-10 in cell culture supernatants correlates with reduced peripheral blood mononuclear cell (PBMC) Th2 cytokine production and decreased PBMC antigen specific proliferation 53 and is necessary for peptide-induced amelioration of allergic asthma 54. Recently in an elegant study, Nguyen et al. 55 compared the capacity of natural regulatory T cells from allergic asthmatics, non-allergic asthmatics and healthy individuals to suppress dendritic cell (DC) activation. The ability of Tregs to suppress DC activation was reduced in Tregs from asthmatics. Lastly, in a mouse model of allergic asthma, the adoptive transfer of Tregs attenuated ovalbumin (OVA) allergen-induced AHR, pulmonary inflammation and Th2 cytokine production in an IL-10 dependent manner 56, 57. Taken together these reports indicate that the balance of Tregs in the lungs is essential for protection against the development of asthma.
Over the last decade, a role for the newly described Th17 family of cytokines in asthma has emerged. The first family member (IL-17A) was originally identified in 1995. Subsequently between the years 2000 – 2002, five additional IL-17 family members were identified. Numerous reports establish a role for Th17 cells in the recruitment and activation of macrophages and neutrophils. The increased presence of IL-17A and IL-17F in asthmatic sputum and airways, as well as in human BAL fluid and BAL cells indicated that these cells may significantly contribute to the development of airway disease. Studies in animal models confirmed the role of IL-17 in the development of AHR in allergen-induced, and ozone-induced airway disease. Moreover, IL-17 can synergize with Th2 cytokines to exacerbate allergic asthma. However, other studies show a protective role, or no contribution of IL-17 in allergic inflammation. Thus the contribution of IL-17 to asthma pathogenesis is complex and requires further study.

ii. DCs in Experimental Asthma

DCs were identified in the early 1970’s and were subsequently found to influence naïve T cell differentiation. Immature DCs have increased antigen uptake capacity and continuously sample the environment. Upon maturation DCs exhibit a reduced phagocytic capacity, increased capacity for antigen presentation, and they migrate to draining lymph nodes where they prime naïve T cells to differentiate into various subsets. Specifically, DCs provide three types of signals to T cells. Firstly, MHCII dependent antigen presentation; secondly signals provided via cell-mediated interactions (CD80/CD86, OX40/OX40L) and thirdly cytokines that direct the development of
Th2 (IL-6, IL-9) \(^{91-93}\), Th1 (IL-12) \(^{94}\), Treg (TGFβ/IL-10) \(^{57,95,96}\) and Th17 cells (IL-6/TGFβ/IL-23) \(^{92,97,98}\). Thus factors provided by DCs to T cells are likely important drivers of the aberrant T cell responses observed in asthmatic individuals.

In the airways, DCs form networks surrounding the airway epithelial cell layer \(^{99}\) from which they can project their dendrites through epithelial cell tight junctions to sample environmental allergens without compromising barrier function \(^{100-104}\). Within hours of exposure, antigen or particles deposited in the airways are taken up by pulmonary DCs and transported to the lung draining lymph nodes for presentation to T cells \(^{105,106}\).

Interestingly, the numbers of pulmonary DCs in human infants (< 1 year) are low or undetectable and increase following respiratory tract infections \(^{107}\) and other environmental exposures. This is consistent with studies in rodents \(^{108}\) that show that environmental stimulation is necessary for the recruitment/expansion of pulmonary DCs. Both human and animal model observations suggest a role for DCs in asthma. The numbers of DCs are increased in the bronchial mucosa of asthmatics as compared to healthy individuals \(^{109,110}\). In previous studies, van Rijt et al. \(^{111}\) show that the depletion of CD11c positive cells attenuated allergen-driven Th2 cytokine production, eosinophilic inflammation and AHR in response to methacholine (MCh). Although it is important to note that pulmonary macrophages also express CD11c \(^{112}\) and thus were potentially depleted, these studies begin to indicate that pulmonary DCs are necessary for the development of allergic asthma \(^{83-90}\).

Recently numerous pulmonary DC subsets were identified to be mediators of airway immune responses. Of particular interest are the myeloid and the plasmacytoid
DCs. Myeloid DCs (mDCs) are recognized for their high costimulatory molecule expression, high expression of MHCII, surface expression of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs 1-6, 8) and the production of pro-inflammatory and T-cell skewing cytokines. These cells are important activators of anti-microbial and anti-fungal T-cell responses. Alternatively, plasmacytoid DCs (pDCs) are recognized for their high expression of TLR 7 and 9 and the production of antiviral cytokines such as type-1 interferons. Pulmonary DC subsets play a dual role in asthma regulation. In the lung, mDCs can be identified by high expression of the transmembrane protein CD11c and the lack of the granulocyte marker Gr-1 (CD11c<sup>hi</sup>, Gr-1<sup>-</sup>), while pDCs can be identified by the intermediate expression of CD11c and the expression of Gr-1 (CD11c<sup>int</sup>, Gr-1<sup>+</sup>). The in vivo depletion of myeloid lineages of DCs reduced eosinophil inflammation and Th2 cytokine production in a model of OVA-induced asthma, while the administration of mDCs drove AHR. As mDCs regulate the Th2 response that occurs in allergic asthma, they are considered to be pro-asthmatic or immunogenic DCs.

Alternatively, in an experimental model of allergic asthma, antibody depletion of pDCs abrogated OVA-induced tolerance, resulting in eosinophilic inflammation and Th2 cytokine secretion during subsequent challenge in OVA-tolerized mice. PDCs induce the differentiation of suppressive regulatory T cell subsets and the adoptive transfer of allergen pulsed pDCs, prior to OVA sensitization and challenge, was found to inhibit eosinophilic inflammation and Th2 cytokine production. Together these reports indicate that pulmonary pDC and mDC subsets promote tolerance or drive the
development of immune responses to allergens respectively and that the balance of these subsets in the lung critically regulates the development of airway disease.

iii. Epithelial Cells as Mediators of Asthma

The average adult inhales approximately 10,000 L of air daily. Because the airway epithelium lines the respiratory tract it serves as the initial interface of the host with inhaled environmental substances. Epithelial cell shedding and airway remodeling are characteristic of both allergic and obstructive airway disease. Cells in the airway epithelium create a protective barrier against toxic substances and invasion by foreign organisms through the formation of tight junctions that prevent the diffusion of inhaled products larger than 10 µM and limit microbe invasion. The disruption of tight junctions thus leads to increased pulmonary particle exposure and microbial invasion.

Although it was initially thought that the epithelium acted solely as a physical barrier, previous studies indicated that epithelial cell derived relaxation factors are necessary for the maintenance of smooth muscle tone. Studies supporting this hypothesis showed that removal of the respiratory epithelial cell layer increased airway contractions to bronchoconstrictors such as acetylcholine and histamine. In addition to the maintenance of airway tone, recent evidence suggests that the pulmonary epithelium confers protection through the removal of particles through the mucociliary escalator and the production of a variety of proteins (e.g. mucus, antimicrobials, complement, cytokines, chemokines) that directly protect against foreign microbes or recruit and activate immune cells necessary for airway defense. The
expression of PRRs such as TLRs on airway epithelial cells also makes them important sensors of danger signals and initiators of airway inflammation. Previous reports indicate TLR4 expression is upregulated in epithelial cell lines after antigen exposure. Furthermore, TLR pathways have been implicated in epithelial cell derived-proallergic cytokine production. *In vitro* studies suggest that airway epithelial cells are capable of phagocytosing foreign particles and releasing mediators that support airway inflammation, although this is controversial.

Epithelial cells can indirectly orchestrate Th2 responses by secreting cytokines that activate and condition DCs in a manner that promotes the skewing of naïve T cells. Multiple reports indicate that airway epithelial cells produce DC differentiating cytokines (GM-CSF, TSLP) after exposure to allergen and particulate matter (PM). Granulocyte macrophage colony-stimulating factor (GM-CSF) directly stimulates the differentiation of progenitor cells into mDCs, while suppressing the development of pDC populations. Studies by Bleck et al. show that human bronchial epithelial cells (HBEC) treated with diesel exhaust particles (DEPs) induce *in vitro* immature monocyte derived DC maturation and T-cell stimulating capacity in a GM-CSF dependent manner. Studies indicate that GM-CSF overexpression can overcome inhalation tolerance and the necessity for adjuvant in the development of eosinophilia, and Th2-differentiation to purified allergen (recombinant Der p1, OVA), which is associated with an increase in lung DCs. Furthermore, antibody neutralization of GM-CSF reduces cigarette smoke-, allergen- and diesel exhaust-mediated eosinophilia and Th2 cytokine production. GM-CSF neutralization abrogated airway resistance, inflammation and the appearance of mucus-containing cells.
Taken together, these data indicate that GM-CSF promotes the pathological features of allergic asthma.

Another epithelial derived mediator that has been shown to direct the development of Th2 skewing DCs is thymic stromal lymphopoietin (TSLP) \textsuperscript{83,149-152}. In the appropriate settings, TSLP upregulates DC costimulatory molecule expression \textsuperscript{83,152} leading to increased Th2 cytokine production. Moreover, TSLPR-/- mice exhibit decreased antigen-induced Th2 responses \textsuperscript{153}.

Activated epithelial cells can directly recruit T cells through chemokine production. Epithelial cell-derived chemokines that directly regulate pulmonary DC and T cell recruitment/migration include: CCL3/MIP-1\textalpha \textsuperscript{154,155}, CCL4/MIP-1\textbeta \textsuperscript{154,156}, CCL20/MIP-3\textalpha \textsuperscript{136,157-160}, MCP-1 \textsuperscript{161-164} and thymus activating and regulatory chemokine (TARC) \textsuperscript{165}.

Previous reports indicate that TARC \textsuperscript{166,167} and MCP-1 \textsuperscript{168} directly modulate pro-allergic responses. Kawasaki and colleagues \textsuperscript{166} showed that the bronchial epithelium stained positive for TARC. TARC binds the CCR4 and CCR8 receptors, which are upregulated on Th2 cells. The administration of antibodies against TARC resulted in a decrease in OVA-induced BAL eosinophilia, lymphocyte recruitment, Th2 cytokine production and bronchoconstriction to intravenous MCh \textsuperscript{166}. Thus, epithelial cell production of TARC may play a role in allergic disease. Mice deficient in MCP-1 have greatly reduced Th2 cytokine gene expression and protein production as compared to wildtype controls \textsuperscript{169}, and antibody depletion of MCP-1 reduced allergen induced AHR and BAL histamine levels \textsuperscript{168}. Furthermore, the direct administration of recombinant MCP-1 was sufficient to induce airway hyperreactivity in normal mice \textsuperscript{168}.
Lastly, the chemokine CCL20 binds the receptor CCR6, which is preferentially expressed on immature DCs and Th17 cells, suggesting that it plays a dual role in allergic airway disease through the recruitment of DCs as well as the preferential recruitment of pro-inflammatory Th17 cells. Taken together, these studies suggest that airway epithelial cell activation may contribute to the initiation and maintenance of allergic inflammation.

iv. **Oxidative Stress and Asthma**

It has been postulated that the disruption of the pulmonary oxidant/antioxidant balance may play a significant role in the development of allergic airway disease. Reactive oxygen species (ROSs) are produced during normal cellular homeostasis and regulate processes such as aerobic respiration and enzyme reactions. However, when antioxidant defenses can no longer control those levels, ROSs such as superoxide anions, $\text{H}_2\text{O}_2$ and hydroxyl radicals, quickly react with cellular components leading to cellular damage and cell death. Evidence that oxidative stress may be important in asthma comes from the observation that genetic deficiencies in antioxidant genes are significant risk factors for the development of asthma. Numerous studies have indicated that polymorphic variants in pro-oxidant (NADPH oxidase) genes are associated with protection against allergic bronchial asthma, whereas polymorphic variants in antioxidant genes (glutathione S-transferase) are associated with increased susceptibility to persistent wheezing and asthma. From such studies one can conclude that genetic variations that lead to a decrease in oxidation protect against asthma.
Rossi et al.\textsuperscript{176} initially proposed in 1964 that the NADPH oxidase complex consists of multi-component transmembrane proteins that transport electrons to oxygen molecules, which results in the production of the oxygen radical superoxide (\(O_2^-\)). The major subunits of the prototypic NADPH oxidase complex (NOX2) include two transmembrane subunits (gp91\textsuperscript{phox} and gp22\textsuperscript{phox}), three cytosolic subunits (p40\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox}) and the GTPase, Rac. Epidemiological studies show that polymorphisms in the p22\textsuperscript{phox} subunit, which results in decreased NADPH oxidase enzymatic activity, are associated with a decreased risk of developing allergic bronchial asthma\textsuperscript{173}. Further support for an association between altered NADPH oxidase activity and asthma is provided by the observation that inhalation of a pharmacological inhibitor of NADPH oxidase activity, apocynin, reduced ozone-induced bronchial hyperreactivity (decreased FEV\textsubscript{1} response after MCh) and airway narrowing\textsuperscript{177}. Furthermore, superoxide is dismutated to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in the airways and numerous studies associate increased H\textsubscript{2}O\textsubscript{2} with airway disease. For example, multiple reports have shown elevated concentrations of H\textsubscript{2}O\textsubscript{2} in asthmatic exhaled breath condensate\textsuperscript{178, 179} and epithelial lining fluid\textsuperscript{180}, as compared to healthy controls. In one such study, H\textsubscript{2}O\textsubscript{2} levels are negatively correlated with FEV\textsubscript{1} and the provocative concentration of inhaled histamine that caused a 20\% fall in FEV\textsubscript{1} (PC\textsubscript{20})\textsuperscript{178}. Studies by Muijers et al.\textsuperscript{181} have shown that pharmacological inhibition of NADPH oxidase activity with apocynin abrogates allergen-induced AHR in mice. Interestingly, however, Savina et al.\textsuperscript{182} demonstrated that murine DCs deficient in the gp91\textsuperscript{phox} subunit have a reduced phagosomal pH, leading to the reduced capability to process and present antigen and to defective T-cell proliferation.
Cumulatively these studies indicate that the balance of pro-oxidant pathways may significantly contribute to the development of allergic airway disease.

Antioxidant control of the Th1/Th2 balance and pulmonary oxidative stress is a significant factor in the regulation of airway disease. Although multiple antioxidants play a role in the maintenance of the pulmonary oxidant/antioxidant balance, the most thoroughly studied is the thiol antioxidant glutathione (GSH) and glutathione S-transferase (GST). The GST superfamily consists of cytosolic and microsomal enzymes that catalyze the conjugation of reduced GSH with electrophiles. Epidemiological reports across multiple ethnicities indicate that polymorphisms in the GST Pi 1 (GSTP1) and the GST Mu 1 (GSTM1) genes are significantly associated with the development of asthma. Additional studies in pediatric populations show that asthmatic children possessed significantly lower levels of reduced GSH in exhaled breath condensate and epithelial lining fluid, as compared to healthy controls. In addition increased levels of oxidized glutathione (GSSG), a significant indicator of oxidative stress, are seen in the serum and epithelial lining fluid of asthmatics.

Antioxidants are significant regulators of antigen presentation and T-cell IL-2 responses. In 1995, Jeannin et al. introduced a new role for thiol antioxidants [N-acetyl cysteine (NAC), and GSH], as modulators of Th1 and Th2 responses, showing that antioxidant treatment decreased in vitro IL-4 and IgE production. In 1998 Peterson et al. showed that systemic GSH depletion decreases in vitro antigen specific-IFNγ production from primed lymphocytes, with no effect on IL-4. In vitro coculture assays revealed that unlike untreated splenocytes, GSH-depleted splenocytes had reduced IL-12 production that limited their ability to support T-cell derived IFNγ production.
Subsequently, multiple studies have indicated that GSH is critical in determining Th1/Th2 immunity through its effects on the production of APC-derived IL-12 \(^{196-200}\). In one such study, Kim et al. \(^{200}\) showed that systemic GSH depletion protects against Th1-mediated delayed type hypersensitivity (DTH), leading to a reduction in IFN\(\gamma\) mRNA expression at the site of antigen challenge, with no effect on IL-4 mRNA expression. The authors suggested that the mechanism behind this protection was a decrease in the ability of GSH-depleted DCs to produce the Th1-skewing cytokine IL-12. Allergen challenge significantly reduced basal IL-12 \(^{201}\) and IFN\(\gamma\) production, which was restored with GSH administration \(^{201,202}\). However, studies in allergen models have also shown that administration of exogenous GSH during the time of allergen challenge reduced OVA-driven BAL Th2 cytokine production (IL-4, IL-5) BAL eosinophils \(^{201,202}\), serum IgE levels \(^{202}\) and allergen-induced AHR \(^{201}\). Taken together these studies indicate that cellular GSH levels can regulate allergen driven AHR and Th2 cytokine production and promotes the induction of Th1 responses that are protective against the development of AHR.

IV. Etiology of Asthma

The etiology of asthma is generally unknown, however both genetic and environmental factors are thought to contribute. Studies supporting the role of one’s genetic makeup in the development of asthma show a 50% probability that an individual will develop asthma if both parents are asthmatics, which reduces to 25% if only a single parent is affected \(^{203}\). Furthermore, the probability that identical twins will both present with asthma is significantly higher than that for fraternal twins \(^{204,205}\). However the rate at
which asthma is increasing far surpasses that which can be attributed to genetic factors. Thus, numerous studies have addressed the question as to what environmental changes have led to the increase in asthma prevalence.

A number of environmental exposures have been associated with asthma including: infectious agents, allergen and PM. Indeed, the environmental contribution to the induction of asthma is well documented and according to the American Lung Association, more than 50% of affected individuals in the U.S. possess specific allergies to airborne substances. However recent reports suggest that respiratory viral infection, as opposed to allergen exposure, is the major cause of acute wheeze in asthmatic children. Yet infection rates fail to track with the increase in the prevalence of asthma. Alternatively, increased modernization is associated with a decrease in the rate of infections, as addressed in the ‘hygiene hypothesis’. As it is indisputable that increased urbanization has brought with it increased airborne PM from sources such as automobile traffic, this thesis will focus on the role of ambient PM in the induction of asthma.

V. Contributions of Air Pollutants to Asthma

The Global Alliance against Chronic Respiratory Disease (GARD) lists exposure to outdoor air pollutants as one of the major risk factors for the increased morbidity and mortality resulting from cardiovascular and respiratory illness, which includes the increased frequency of symptomatic asthma attacks, increased need for pulmonary medication and decreased pulmonary function.
The U. S. Environmental Protection Agency defines the six major pollutants in the U.S. as: ground-level ozone (O\textsubscript{3}), particle pollution (PM\textsubscript{2.5} and PM\textsubscript{10}), lead (Pb), nitrogen dioxide (NO\textsubscript{2}), carbon monoxide (CO), and sulfur dioxide (SO\textsubscript{2}). Among these, the effects of PM on asthma incidence appear to be larger and more consistent when compared to gaseous pollutants\textsuperscript{210}. The current EPA definition of PM is: “a complex mixture of extremely small particles and liquid droplets. Particle pollution is made up of a number of components, including acids (such as nitrates and sulfates), organic chemicals, metals, and soil or dust particles”. Particles greater that 10 µm in diameter have a high probability of being cleared in the nasal passages, however those ≤10 µm (PM\textsubscript{10}) have a greater probability of reaching the airways and can be further characterized by size fractions. Coarse PM (PM\textsubscript{10}, 2.5 µm -10 µm in diameter) is generally composed of biological material such as dust, whereas fine PM (PM\textsubscript{2.5}, diameters ≤ 2.5 µm) is generally composed of combustion products and ultrafine particles (≤0.1 µm in diameter)\textsuperscript{211, 212, 213}.

Multiple mechanisms of particle deposition may occur based on the size of inhaled PM, however the primary modes of deposition include: diffusion (affected by diameter but not particle density), impaction (particle size or density prevent movement with airflow) and sedimentation (settling due to gravity). Diffusion and impaction can occur during both inspiration and expiration. Diffusion and sedimentation are the main modes of deposition in the lungs for particles 0.1 – 1 µm, whereas larger particles are deposited through impaction and sedimentation\textsuperscript{214}. Particles ranging from 5 – 10 µM are generally deposited in the lower airways, while those <5 µM are more readily deposited in the and alveoli\textsuperscript{210}. 

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VI. Epidemiological Association Between PM Exposure and Asthma

Studies spanning multiple cities in the USA report positive correlations between increased respiratory illness and increased levels of airborne particles. In one such study, Schwartz et al. (1993) collected questionnaires on respiratory illness in the first National Health and Nutrition Examination Survey, which encompassed 53 different urban areas in which the Environmental Protection Agency recorded total suspended particles (TSP) levels for the preceding year. The results of these studies indicated that elevated levels of TSP were associated with the risk of chronic bronchitis and respiratory illness in ‘never’ smokers \(^{215}\). In addition, numerous epidemiological studies have reported positive correlations between PM exposure and increased emergency room (ER) visits for asthma \(^{216-220}\). Epidemiological studies of air pollution among genetically similar populations highlight the importance of environmental pollutants as risk factors for the development of asthma. For example, a study done in Singapore from 1990-1994 reported positive correlations between increases in pediatric ER visits for asthma with every 20 µg/m\(^3\) increase in daily atmospheric TSP \(^{221}\).

Further evidence for an association between particles and respiratory health was derived from an occurrence in the Utah Valley in the years preceding, during and following a labor dispute that resulted in the closure of a local steel mill that contributed to a reduction of > 80% in the local levels of PM \(^{222,223}\). Closure of the mill was associated with decreased bronchitis, asthma \(^{224}\) and total respiratory hospital admissions \(^{225}\). One of the important aspects of this study was that the decrease in PM levels in this single geographical area occurred over the relatively short timeframe of 13 months, thus
limiting additional environmental variables. Furthermore, the reopening of the Mill provides an appropriate control for the decreases in morbidity associated with closure of the mill, as there was an increase in pediatric hospital admissions for bronchitis and asthma upon reopening of the mill.

In other examples, the Study of Particles and Health in Atlanta (SOPHIA) analyzed approximately 4.4 million ER visits across 13 hospitals over a seven-year period in Atlanta. From these studies, Peel at al. reported that increased levels of PM\textsubscript{10} can be individually associated with upper respiratory infections and increases in asthma visits in comparison to weak or null associations with the control (finger wound) ER visits\textsuperscript{219}. In a time series analysis of air pollutants and cardiopulmonary ER visits in seven Canadian cities, Stieb and colleagues\textsuperscript{216} found that PM was associated with a 14% increase in ER visits per 20.6 µg/m\textsuperscript{3} PM\textsubscript{10} and a 7.6% increase in visits per 8.2 µg/m\textsuperscript{3} PM\textsubscript{2.5}. In studies conducted in northern California during two winter drought seasons (1988-99, 1991-1992) in which residential wood combustion (RWC) was the largest source of PM\textsubscript{10} in Santa Clara County, Lipsett et al.\textsuperscript{218} found that PM\textsubscript{10} levels were significantly correlated with ER visits. In these studies, meteorological data suggested that the combination of low temperature and high particle levels was an important predictor of ER visits. However, temperature was not strongly associated with ER visits when the particle of interest was PM\textsubscript{10}, but was when associated with ozone and NO\textsubscript{2}.

In addition to the strong association between particles and asthma morbidity, numerous studies show an association between PM levels and increased mortality from cardiopulmonary disease. Follow up studies from the original Utah Valley study indicate that decreases in mortality were associated with decreases in PM\textsubscript{10} concentrations (15
µg/m³) during the steel mill closure 226. Another example supporting the role of PM in mortality can be seen in the Harvard Six City study, in which outdoor air pollution was monitored at a central site across multiple U.S. cities. In these studies, 811 subjects (25 – 74 years old) completed standard questionnaires and underwent spirometric testing from 1974 – 1977. Data from these studies indicate that a stronger association occurred between fine or sulfate particles and mortality in comparison to the level of association with sulfur dioxide, nitrogen dioxide or acidity. Along with smoking, air pollution was positively associated with mortality due to lung cancer and cardiopulmonary disease. Furthermore, when subjects experiencing high blood pressure or diabetes were excluded from the analyses, associations between pollution and mortality remained unchanged 227. Follow up studies from the Six City study reported an increase in mortality with each 10 µg/m³ increase in daily PM$_{2.5}$ and the risk for mortality was significantly decreased in cities that exhibited the greatest reduction in PM$_{2.5}$ 228. In a study conducted locally in Cincinnati, death records showed that TSP and not sulfur dioxide was a significant risk factor for mortality 229.

As a side effect of modernization, industrial manufacturing plants and modern conveniences such as the automobile release combustion products such as diesel exhaust and particle pollution into the atmosphere 230, 231 which typically correlates with the increases in asthma prevalence. The majority of epidemiological studies strongly associate both residence in an industrial area 224, 225, 232, 233 or close proximity to heavily utilized highways and other areas containing high automobile traffic 234-239 with an increase in the prevalence of asthma. However a few reports fail to correlate the incidence of allergic disease with proximity to a major road 240, 241 or traffic-related air
pollution\textsuperscript{242,243}, which may be the result of variations in study design, traffic patterns, seasonal changes and varying environmental factors, such as viruses and local airborne allergens such as pollen. In an effort to preserve U.S. air quality the Clean Air Act enacted by Congress led to the development of the National Ambient Air Quality Standards for the six major pollutants (Table 1). According to the 2009 American Lung Association State of the Air Report, one in six Americans (47 million) are exposed to unhealthy chronic levels of particle pollution as determined by the EPA standards, thus increasing their risk for asthma and lung disease.
Table 1. National Ambient Air Quality Primary Standards

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Level</th>
<th>Average Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Monoxide</td>
<td>9 ppm (10 mg/m$^3$)</td>
<td>8-hour</td>
</tr>
<tr>
<td></td>
<td>35 ppm (40 mg/m$^3$)</td>
<td>1-hour</td>
</tr>
<tr>
<td>Lead</td>
<td>0.15 µg/m$^3$</td>
<td>Rolling 3-Month Average</td>
</tr>
<tr>
<td></td>
<td>1.5 µg/m$^3$</td>
<td>Quarterly Average</td>
</tr>
<tr>
<td>Nitrogen Dioxide</td>
<td>0.053 ppm (100 µg/m$^3$)</td>
<td>Annual (Arithmetic Mean)</td>
</tr>
<tr>
<td>Particulate Matter (PM$_{10}$)</td>
<td>150 µg/m$^3$</td>
<td>24-hour</td>
</tr>
<tr>
<td>Particulate Matter (PM$_{2.5}$)</td>
<td>15.0 µg/m$^3$</td>
<td>Annual (Arithmetic Mean)</td>
</tr>
<tr>
<td></td>
<td>35 µg/m$^3$</td>
<td>24-hour</td>
</tr>
<tr>
<td>Ozone</td>
<td>0.075 ppm (2008 std)</td>
<td>8-hour</td>
</tr>
<tr>
<td></td>
<td>0.08 ppm (1997 std)</td>
<td>8-hour</td>
</tr>
<tr>
<td>Sulfur</td>
<td>0.12 ppm</td>
<td>1-hour (Applies only in limited areas)</td>
</tr>
<tr>
<td></td>
<td>0.03 ppm</td>
<td>Annual (Arithmetic Mean)</td>
</tr>
</tbody>
</table>

Available from [http://www.epa.gov/air/criteria.html](http://www.epa.gov/air/criteria.html)
VII. PM-Induced Airway Inflammation and Disease

As a follow up to epidemiological studies associating increased levels of PM with physician diagnoses for asthma, human exposure studies have shown that significant increases in airway inflammation occurred after PM exposure. Specifically, studies of segmental lung challenges of healthy volunteers with PM$_{2.5}$ collected in Hettstedt, a county in Germany with high levels of industrialized pollutants, reveal that even short term deposition of PM in the lungs resulted in increases in total BAL monocytes and the production of the pro-inflammatory cytokines IL-6 and TNF-$\alpha$. In addition, healthy volunteers receiving instillations of PM$_{10}$ collected in the Utah Valley experienced significant increases in pulmonary neutrophils, several indices of lung injury (protein, albumin, fibronectin, $\alpha$1-antityrpsin) in the BAL and an increase in the BAL levels of the proinflammatory cytokines IL-8, TNF-$\alpha$ and IL-1$\beta$. From these studies, we see that segmental challenge with both fine and coarse fractions of PM can induce an inflammatory milieu in the lung. In both studies, biologically active fractions exhibited increased capacity to induce oxidative stress, which correlated with the increased presence of transition metals. It is important to note, however, that bronchial responses observed after PM challenge may be innate responses to environmental insults as both the influx of monocytes and neutrophils and the production of pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-$\alpha$, are observed in studies using a single bronchial segmental challenge of healthy volunteers with bacterial endotoxin. However, the endotoxin levels were low to undetectable in the PM samples collected in Germany and in the dust taken from the Utah Valley, suggesting that endotoxin contamination is not a major determinant of PM-induced airway inflammation in these studies.
In a chamber exposure study, Nightingale et al.\textsuperscript{248} found that DEP exposure (2 hour) increased the levels of exhaled carbon monoxide, neutrophil numbers, and myeloperoxidase in the sputum of healthy nonsmoking volunteers four hours after exposure. DEP exposure, however, did not induce changes in FEV1 or FVC, sputum numbers of lymphocytes or eosinophils, levels of IL-6 or IL-8 in the sputum, or plasma levels of the inflammatory cytokines IL-6, TNF-\(\alpha\) or p-selectin. Although the amount of PM inhaled is less controlled in chamber studies, they eliminate the potential that physical damage to the airways occurs when PM is instilled via a bronchoscope.

Asthma symptoms increase in severity during physical exercise\textsuperscript{4}. Furthermore, physical activity increases the resting tidal volume, thereby increasing an individual’s exposure to aerosolized particles. Thus, exposure protocols that incorporate exercise can further reproduce natural instances in which one would observe exacerbations. In an assessment of ultrafine particle exposure among both healthy and asthmatic volunteers, with intermittent exercise, Gong et al.\textsuperscript{249} showed that exposure to PM (2 hour) collected in an area of Los Angeles with substantial vehicular pollution resulted in a significant, time dependent fall in FEV\(_1\). In these studies, a minimal decrease in FEV\(_1\) was seen solely among asthmatics 4 hours post exposure and an approximately 2.4% decrease in FEV\(_1\) was observed approximately 22 hours post challenge. No significant changes were observed in FVC. Although physiological changes between healthy and asthmatic individuals were not statistically significant, trends in asthmatics suggest that environmental PM can alter pulmonary function at an accelerated rate in asthmatics. Ultrafine PM exposure also resulted in a 0.55% decrease in the percentage of oxygenated hemoglobin in the arterial blood (SpO\(_2\)) of both healthy and asthmatic individuals.
immediately after exposure. Although the percent changes in lung function were not significantly different between healthy and asthmatic individuals, the baseline oxygenation was 99.3% in healthy individuals versus 98.2% in asthmatics. Thus, PM exposure exacerbates an already compromised parameter in asthmatics thereby decreasing the threshold leading to the presentation of clinical symptoms that are not seen in healthy individuals. In a study comparing ultrafine, fine and coarse fractions of concentrated PM, Samet et al. were unable to detect changes in FEV₁ or FVC among healthy, non-smoking individuals 18 hours post exposure. However, increases were seen in the number of BAL neutrophils after exposure to both fine and coarse PM. Thus PM exposure may create a lung milieu that enhances immune responses to environmental insults.

To understand the role of individual components of PM in the development of airway inflammation, many studies have focused on DEPs, which are fossil fuel combustion products typically found in ambient air. In healthy individuals, short-term exposure (1 hour) to DEP (300 µg/m³) induces increases in the BAL levels of neutrophils, mast cells, CD4 and CD8 cells, and increased mRNA expression of the neutrophilic chemotactic factors IL-8 and GRO-α in the airways. However, in studies by Behndig in 2006, using a lower dose of DEP (100 µg/m³), neutrophils were the only cells present after DEP exposure despite a longer exposure time (2 hours). Conversely, during a comparison of non-allergic healthy individuals and mild asthmatics exposed to an average of 108 µg/m³ DEP (2 hours), Stenfors and colleagues showed that decreases in FEV₁ and FVC were below clinical significance or were unchanged after DEP exposure. Because these studies only assessed single short-term (1-2 hour)
exposures that may be insufficient to induce substantial changes in lung function \(^{211, 249, 250, 253}\), it can be hypothesized that multiple challenge studies may prove more useful to determine whether PM exposure alters pulmonary function. However, due to the ethical issues associated with exposing humans on multiple occasions to PM, animal studies have been utilized to test the biological effects of multiple exposures to PM on airway function.

VIII. Animal Models of PM Exposure

i. PM-Induced Exacerbation of Experimental Allergic Asthma

Studies on \textit{in vivo} PM exposure have provided insight into the effects of PM exposure on both the induction and exacerbation of asthma. Multiple studies have been conducted to begin to determine whether PM exposure alone can drive asthma symptoms or whether it can enhance responses during co-exposure with allergens. In one such study, a single PM instillation was given to OVA-peptide specific T cell receptor (DO11.10) transgenic mice, prior to exposure to OVA. Sensitization of these animals with PM collected in an urban environment resulted in the enhancement of OVA-induced AHR and inflammation, in a dose-dependent manner\(^{75, 254}\). These studies suggest that PM-induced exacerbation of allergic disease can occur despite prior allergen sensitization. In addition, the simultaneous immunization with traffic PM and birch pollen results in a synergistic increase in pollen specific IgE and bronchial hyper-reactivity in mice \(^{255}\). Also, in studies of murine models of OVA-induced asthma, exposure to oil combustion components such as residual oil fly ash (ROFA) after an aerosolized OVA challenge caused synergistic increases in Th2 cytokine production,
increased BAL albumin and protein content, increased BAL eosinophilia and increased airway responsiveness to MCh. The administration of ambient PM post OVA sensitization and challenge resulted in an additive increase in OVA-induced AHR and BAL eosinophilia. Thus, PM exposure can significantly exacerbate allergic asthma when administered throughout the course of disease.

ii. Direct Induction of Airway Inflammation and AHR by PM

In addition to the exacerbation of disease, previous reports indicate that exposure to either PM alone and its individual components can induce features of experimental asthma in naïve mice. The repeated intranasal exposure of mice to DEPs results in an increase in AHR, BAL cellularity and mucus cell proliferation. This induction was accompanied with increased production of GM-CSF, indicating that DEP exposure creates an environment that promotes the recruitment and expansion of DC populations. Interestingly, other studies using individual PM components, such as carbon black particles (CBP) and DEP, fail to show an induction of AHR and eosinophilia after in vivo exposure. This lack of responsiveness may be attributed to variations in the time, and duration of exposures as well as the exact composition of PM. In a real-world setting ambient PM is highly complex, suggesting that the combination of various components may be necessary for the development of airway disease. Studies from our lab and others, using real-world PM collected in urban Baltimore (AUB) have shown that exposure of naïve A/J mice to AUB directly induced AHR to cholinergic agonists, T cell cytokine production, and eosinophilic inflammation.
IX. Potential Mechanism(s) of PM Induced Airway Disease

Although the composition of PM varies with source, location, and time of collection, several characteristics of PM have been associated with their biological effects. These features include particle size, the association with microbial products (bacterial endotoxin, fungal antigens), metals and polycyclic aromatic hydrocarbons (PAHs).

i. Size

Coarse PM ($PM_{10}$, 2.5 µm -10 µm in diameter) is generally composed of biological material such as dust, whereas fine PM ($PM_{2.5}$, diameters $\leq$ 2.5 µm) is generally composed of combustion products and ultrafine particles ($\leq$0.1 µm in diameter). As smaller particles are more widely distributed in the airways, studies have examined particle size as a factor in PM-induced immune responses. In a comparison of particles of varying size ranges, Samet and colleagues found that coarse and fine PM fractions, but not ultrafine particles were able to induce pulmonary neutrophilia in normal individuals. Conversely, in a mouse model, Samuelsen et al. found that polystyrene particles within the coarse size range induced higher levels of proinflammatory cytokines and increased neutrophil numbers in the BAL, when compared to cytokine production and inflammation induced by particles in smaller size ranges. However, Oberdörster, and colleagues show that ultrafine titanium dioxide ($TiO_{2}$) particles induced larger inflammatory reactions in rats versus fine particles of the same composition. These studies employ particles obtained from identical sources and times and thus control for additional environmental agents that may alter PM composition and subsequent
responses. From the current data, the importance of size as a contributing factor to particle toxicity is debatable. Therefore, it is necessary to focus on other components of PM fractions as a source of immune activation.

ii. **Associations with allergen**

Allergens, which are typically proteins or glycoproteins ranging from 10 – 50 kD in size, generally require particle carriers to be inhaled. The concentration of allergen present on the particle surface may be determined by particle size and shape. For example, mite and cockroach antigens are more commonly carried on particles with mass median diameters of 10 – 50 µM, whereas cat, dog, and mouse allergens are generally carried by particles with mass median diameters of 5 µM.\(^{210}\)

Common particulate allergen carriers include organic products such as feces, dander, pollen grains and fungal spores.\(^{268}\) However, evidence exists that combustion products such as DEPs act as allergen carriers, which may be a potential mechanism of fine-PM induced exacerbation of allergic disease. Allergen charge plays a potential role in the binding of PM. Knox and colleagues\(^{269}\) showed that the binding of the outdoor grass pollen antigen Lol p 1, but not Lol p 5 to diesel particles was possibly due to the increased negative charge on the surface of Lol p 1. In a study of indoor allergens, Ormstad and colleagues\(^{270}\) showed the binding of the dog allergen (Can f 1), cat allergen (Fel d 1) and birch pollen allergen (Bet v 1) on soot particles in homes. House dust mite allergens (Der p 1) were not detected on soot particles, however, all allergens were able to bind DEPs, thus the nature of both the particle and the antigen are necessary for
allergen-PM interactions. In this study binding to DEP was associated with increased negative charges present on all tested allergens.

iii. Endotoxin

TLRs and 1→3 β-D-glucan receptors may recognize bacterial endotoxin and carbohydrate moieties present in PM fractions. PRR recognition of biological constituents may play an important role in activating immune responses through the induction of pro-inflammatory signals and oxidative stress pathways. Previous reports indicate that low levels of bacterial lipopolysaccharide (LPS) can stimulate TLR4 to induce the development of Th2 responses that are dependent on signaling through the myeloid differentiation primary response gene-88 (MyD88) pathway. In an assessment of PM-induced TLR4 activation, Cho et al. show that ROFA-induced lung injury (increased BAL protein, macrophages and polymorphonuclear leukocytes) and cytokine gene expression (IL-β, IL-6, TNF-α) were significantly reduced in C3H/HeJ mice, which have a nonfunctional TLR4, when compared to normal (C3H/OuJ) controls. Furthermore, Soukup et al. show that treatment with polymyxin B, an antibiotic that binds bacterial endotoxin, reduced PM-induced alveolar macrophage cytokine production. Thus, residual endotoxin present in PM fractions can exacerbate the development of asthmatic responses. In addition non-biological fractions of PM may also induce airway disease.
iv. Transition Metals

Numerous epidemiological studies indicate that the association between PM and the increase in physician diagnoses\textsuperscript{244}, ER visits and hospitalizations for asthma\textsuperscript{224, 275} occur in areas in which PM sources contain increased levels of biologically active transition metals. For example, Hirshon et al\textsuperscript{275} showed an association between an increase in the 24-hour concentration of zinc in the ambient air and the increased risk for emergency department visits and hospitalizations for asthma in Baltimore. Additionally, studies conducted in Germany compared the effects of PM exposure on allergic disease in individuals residing in two counties strongly impacted by industrial pollution (Bitterfeld and Hettstedt) as compared to those in a neighboring county without any sources of industrial pollution (Zerbst). Interesting data from these studies indicate that children residing in Hettstedt exhibited an approximate 50% increase in the prevalence of physician-diagnosed allergies, eczema, and bronchitis when compared to children from Zerbst. Furthermore, children in Hettstedt presented with approximately twice the number of respiratory symptoms (wheeze, shortness of breath, and cough without cold) and increased sensitization to common aeroallergens when compared to children in Zerbst\textsuperscript{275}. In a study in Utah, Pope et al.\textsuperscript{224} compared the effects of PM levels during the fall and winter periods when a steel mill was open, closed, and reopened, on hospital admissions. In the winter of 1985/86, when the mill was open the 24-hour PM\textsubscript{10} levels exceeded 150 µg/m\textsuperscript{3} on 13 occasions. When the mill was shut down during the winter of 1986/87 the 24-hour PM\textsubscript{10} levels never exceeded 150 µg/m\textsuperscript{3} and subsequently during the winter of 1987/88, following the reopening of the mill, the 24-hour PM\textsubscript{10} levels exceeded 150 µg/m\textsuperscript{3} on 10 occasions. Data on hospital admissions revealed that pediatric hospital
admissions for bronchitis and asthma were approximately 2-3 times higher when the steel mill was open, as compared to when it was closed.

As a follow up to these studies, numerous reports have implicated PM associated metals as mediators of asthmatic responses. In studies using PM collected in urban Baltimore, Walters et al.\textsuperscript{260} show that, when compared to a reference PM sample that failed to induced AHR in mice, ambient PM composed of higher concentrations of copper, manganese, and zinc, lead and nickel induced significant increases in AHR. Gavett et al.\textsuperscript{276} studied the direct effects of PM\textsubscript{2.5} collected in 1999 from Hettstedt and Zerbst on the induction of airway inflammation and the exacerbation of allergic asthma, in a mouse model. Data from these studies show that the direct aspiration of PM\textsubscript{2.5} collected in Hettstedt, but not Zerbst significantly increased several indicators of pulmonary injury and inflammation (BAL protein levels, N-acetyl-β-D-glucosaminidase, IL-1β, IL-6, MIP-2) when compared to exposures with control filter extracts. In addition, PM\textsubscript{2.5} from both cities, when administered before OVA challenge in allergic mice, increased PenH, however, increases induced by Hettstedt PM (190%) were higher than those induced by Zerbst PM (120%). In these studies Hettstedt PM\textsubscript{2.5} contained higher levels of magnesium, lead, copper, cadmium, tin and arsenic than Zerbst PM\textsubscript{2.5}\textsuperscript{276}. In subsequent studies, segmental lung challenges of healthy individuals to PM\textsubscript{2.5} collected in 2002 from Hettstedt and Zerbst show that PM\textsubscript{2.5} from Hettstedt, but not from Zerbst, significantly increased the percentage of monocytes, and the concentration of IL-6 and TNF-α in the alveolar space, when compared to saline challenge. Furthermore, BAL cells from lung segments exposed to Hettstedt PM, but not Zerbst PM, had increased oxidant radical generation as compared with cells after saline challenge. PM from Hettstedt
containing higher levels of nickel, copper, zinc and cadmium in comparison to PM collected in Zerbst\textsuperscript{247}. Studies by Ghio et al.\textsuperscript{222} showed significant increases in BAL inflammation (neutrophils IL-8, TNF, IL-1β) and indices of lung injury (protein, albumin, fibronectin, α-1-antitrypsin) in individuals exposed to PM extracts collected while a steel mill was functioning (1986 and 1988), but not when the mill was closed (1987), when compared to saline controls. Iron, copper, zinc and lead were the most abundant metals in PM extracts taken when the steel mill was open\textsuperscript{222}. Taken together, these reports support the concept that PM-associated transition metals are important players in PM-induced or PM-exacerbated airway disease.

X. Hypothesis

Based on the evidence described above, it has been hypothesized that AUB exposure alone plays an important role in the increase in the incidence and severity of asthma over the last few decades. In this thesis, we propose to further evaluate this hypothesis and begin to determine the mechanisms by which exposure to inhaled AUB alone might induce the features of asthma. Specifically, we will test the hypothesis that components of the complex mixture of real world PM activate the airway epithelium, which leads to the recruitment and activation of DCs and subsequent activation of CD4+ T cells in the lung. Once activated, CD4+ T cells elaborate cytokines, which in turn, induce the features of allergic disease.
XI. Specific Aims

To test our hypothesis, we propose the following specific aims:

**Specific Aim 1**: To characterize the innate and adaptive immune responses in mice after single and multiple PM exposures.

**Specific Aim 2**: To determine whether PM-induced changes are dependent on lymphocytes.

**Specific Aim 3**: To determine if PM induces changes in DC recruitment, activation, phenotype and function and to define the mechanism of these changes.

**Specific Aim 4**: To define the early epithelial cell responses to PM that may drive subsequent innate and adaptive immune responses.
Figure 2. Potential mechanisms of PM-induced AHR. DCs and airway epithelial cells act as sensors of PM and collaborate to induce pro-inflammatory responses against PM. Once DC subsets are conditioned by either PM-exposed epithelial cells or through direct PM activation these cells can induce pro-asthmatic or tolerogenic T-cell responses.
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Chapter 2

General Methods
I. Animals

Male and female A/J, Cybb^{tm1Din}/J and C57BL/6, Balb/c, Rag1 deficient (Rag1 -/-) mice (4 to 10 weeks old, Jackson Laboratories, Bar Harbor, ME), and MyD88 deficient (MyD88 -/-) mice were housed in an environmentally controlled specific pathogen-free facility at Cincinnati Children’s Hospital Medical Center with access to food and water ad libitum. Mice were handled in accordance with the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee.

II. Collection of PM in Urban Baltimore

Ambient PM was collected in Baltimore as previously described. Briefly, collection of PM was conducted in urban Baltimore using a high volume cyclone that was continuously operated for periods of three months. The cyclone was operated at a flow rate of 0.6 m$^3$/minute, which allowed for a theoretical size cutoff of 0.85 µm in aerodynamic diameter. PM size was determined using light microscopy and the median particle size was determined to be 1.78 µm with a standard deviation of 2.21 µm. Endotoxin levels in PM were determined using the Limulus amebocyte lysate (LAL) assay, and averaged 10 EU/ml endotoxin. Microscopic examination revealed the presence of fungal hyphae.

III. In Vivo PM Exposure

Mice were anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (45 mg/kg) and xylazine (8 mg/kg) and exposed to either PBS or AUB (0.5 mg in a 50 µl volume of PBS) on days 0, 3 and 6 via aspiration challenge. For exposures
mice were placed on a 60° incline, the tongue was extended to prevent swallowing and 50 µl aliquots of AUB (0.5 mg) or sterile PBS were instilled in the oral cavity. The nose was pinched gently after instillation to improve aspiration efficiency. In a comparison of multiple routes of particle exposure Foster et al. ⁴ found that approximately 80% of radiolabeled particles aspirated in a 50 µl volume were present in the lungs after direct instillation.

IV. Airway Responsiveness Measurements

Airway responsiveness to intravenous acetylcholine (ACh) was assessed as previously described ². Twenty-four hours after the final AUB exposure (D7). Mice were anaesthetized, paralyzed with decamethonium bromide (25 mg/kg), intubated and ventilated at a rate of 120 breaths per minute with a constant tidal volume (0.2 ml). ACh (50 mg/kg) was injected into the inferior vena cava and the dynamic change in airway pressure (cm H₂O x sec), referred to as the airway pressure time index (APTI), was assessed for 5 minutes.

V. Measurements Of Serum IgE

Immediately after AHR measurements terminal blood collection was performed from the posterior vena cava. Blood was collected in serum separator tubes (BD Microtainer®, Franklin Lakes, NJ) and centrifuged for separation. Serum was removed from the top layer and immediately stored at -80°C. ELISA assays were performed using matched antibody pairs (BD Pharmigen™, Franklin, Lakes NJ) to determine the total levels of serum IgE.
VI. Bronchoalveolar Lavage (BAL)

Immediately after airway responsiveness measurements a pulmonary lavage was performed with a 1.0 ml aliquot of cold Hanks’ balanced salt solution (HBSS) without calcium or magnesium (Mediatech Inc., Herndon, VA). Using a 1 ml syringe fitted with an I.V. catheter tip attached (Medex Jelco), 1ml of HBSS was slowly instilled into the lungs and the pulmonary lavage was performed for a total of three instillations and aspirations using the same HBSS. The recovered lavage fluid (70 to 80%) was centrifuged (300 x g for 8 min). Supernatants were removed and stored at -80°C for the determination of cytokine, chemokine and growth factor levels. The cell pellet was resuspended in 1.0 ml of 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) for the determination of the total cell count.

VII. Quantification Of Total Cells And Differential Cell Counts From The BAL Fluid

Total cells were counted under a light microscope with the use of a hemocytometer. For BAL cell differential counts, slides were prepared by cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA), and stained with Diff-Quik (Dade Behring, Du¨dingen, Switzerland). Morphologic criteria were determined with the evaluation of ≥ 500 cells/slide.

VIII. Determination Of Immune Cell Phenotype In The Lungs
The lungs were excised, minced and incubated in 6 ml of digestion media containing RPMI 1640, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 mg/ml liberase CL (Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/ml) (Sigma, St. Louis, MO) for a period of 45 – 60 min at 37°C. Digested tissue was processed through a 70-micron cell strainer, following which erythrocytes were lysed with ACK lysis buffer (Invitrogen, Carlsbad, CA) for 5 minutes. Lysis was stopped using 10 ml RPMI supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Immune cell populations were determined using flow cytometry. Lung cell suspensions were analyzed on the LSRII flow cytometer (BD Biosciences, San Jose, CA). Populations were gated on forward scatter, side scatter and 7AAD fluorescence to exclude debris and dead cells. T cell were identified using APC conjugated anti-mouse CD4 (clone: L3T4) and FITC conjugated anti-mouse TCRβ. Myeloid DCs (CD11c<sup>hi</sup>, CD11b<sup>+</sup>, Gr1<sup>-</sup>) and pDCs (CD11c<sup>low</sup>, CD317<sup>+</sup>, Gr1<sup>+</sup>) were identified using APC, Pacific Blue or Alexa Fluor 647 conjugated anti-CD11c (clone: N418), anti-CD11b-PECy7 (clone: M1/70), anti-Gr1-Alexa Fluor 750 (clone: RB6-8C5) and anti-CD317-Alexa Fluor 488 (clone: 120g8). All pre-conjugated antibodies were purchased from eBioscience, (San Diego, CA) with the exception of anti-CD317, a kind gift from Giorgio Trinchieri (National Cancer Institute, Fredericksburg, MD), which was conjugated to AF488 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Compensations of spectral overlaps were performed using the BD FACSDiva (BD Bioscience, San Jose, CA) and FlowJo (Treestar, Inc., Ashland, OR) analysis software.
For T-cell cytokine determination, cells were cultured in 96 well plates at a concentration of 250,000 cells in a 300 µl volume of media or with concanavalin A (5 µg/ml) for 72 hours. Th1, Th2 and Th17 associated cytokine levels were determined with ELISAs using matched antibody pairs as per manufacturer’s recommendation.

IX. Histological Examination Of Lung Sections

To assess airway inflammation and mucus cell content in the airway wall the lungs were excised and fixed in a 10% formalin solution for a minimum of 24 hours. After fixation the individual lobes were washed in methanol, dehydrated, embedded in paraffin and cut into 5 µm sections. Sections were mounted on slides and received hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) staining. To assess inflammation H&E stained slides were read in a blinded fashion and given an arbitrary histological score. The following scale was used 0: no inflammation, 1 – 1.99: 1-25% inflammation of section, 2 – 2.99: 26% - 50 % inflammation of section, 3 – 3.99: 51% - 75% inflammation of section, 4 – 4.99: 76%-100% inflammation of section.

The number of PAS positive cells per section was counted and averaged over four sections per mouse lung.

X. Isolation And Culture Of Tracheal Epithelial Cells

Tracheas were removed between the larynx and the main bronchial branches and enzymatically digested in 1mg/ml pronase (Roche-Applied Science, Indianapolis, IN) for 16 – 18 hours then centrifuged at 350 x g for 10 minutes. The cells were then resuspended and cultured on Primaria™ (Falcon, Becton Dickinson, Franklin Lakes, NJ) plates for a
period of four hours to remove contaminating adherent cells. Non-adherent cells were centrifuged and resuspended in Dulbecco’s modification of Eagle’s Medium and Ham’s F-12 in a 50/50 mix (Mediatech, Herdon, VA), supplemented with defined antibiotics and growth factors. Cells were cultured on rat-tail collagen (BD Biosciences, Bedford, MA) coated on Transwell® membranes (Costar®, Corning® Life Sciences, Lowell, MA). Cells were cultured until confluent as determined by the observation of Trans Epithelial Electric Resistance measurements greater than 1000 ohms using the EVOM™ Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL). Confluent epithelial cell monolayers were treated on the apical side in a volume of 0.5 ml for 24 hours.

XI. Isolation And Culture Of Bone Marrow Derived DCs

Bone marrow cells (1.5x10^7) were harvested from 6 – 8 week old mice. Following harvest, red blood cells were lysed for 5min in ACK lysis buffer. The remaining cells were cultured in 75 cm² flasks (Falcon, Becton Dickinson, Franklin Lakes, NJ) containing 5 ng/ml mouse GM-CSF (PeproTech, Rocky Hill NJ). Fresh GM-CSF was added on day 3 and 6. By day 6 DC cultures are 95 – 98% myeloid in nature, expressing high levels of CD11c and CD11b. On day 7, cells were harvested and plated in 96 well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a density of 1x10^6 cells/well for in vitro treatments.
XII. ELISA

Cytokine levels from the BAL and *in vitro* cell supernatants were determined via ELISA using matched antibody pairs according to the specific manufacturer’s instructions.

XIII. RNA Extraction And Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using the TRIzol® (Invitrogen, Carlsbad, CA) reagent according to the manufacturer’s recommendations. To assess gene expression in epithelial cell and whole lung samples, purified RNA was treated with 1 unit/µl DNase I (Invitrogen, Carlsbad, CA) at 65°C for 5 minutes. Reverse transcription was carried out on 1 µg of RNA in a 20 µl reaction containing 1X reverse transcriptase buffer, 10 mM dNTP, 0.1M DTT, 40 units/µl of RNase out (Invitrogen, Carlsbad, CA), and 200 units of Superscript II Reverse transcriptase. RT-PCR reactions were prepared using the iQ™ SYBR® green Supermix (Bio-RAD, Hercules, CA) and were analyzed on the iCycler (Bio-RAD). RT-PCR primers were identified using the online PrimerBank ⁴, ⁵ and the qPrimerDepot, quantitative PCR primer database (http://mouseprimerdepot.nci.nih.gov/).

XIV. Western Blot

BMDCs (3×10⁶) were plated in 12 well plates and serum starved for twenty-four hours prior to treatment with AUB (50 µg/ml) for 0, 15, 30, 45, 60 and 120 minutes. Supernatants were removed and cells harvested in a cell lysis buffer containing 50 mmol/L Tris, pH 7.5; 40 mmol/L β-glycerophosphate, 100 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 200 µmol/L Na3VO4, 200 µmol/L phenylmethylsulfonyl
fluoride, and 1% Triton X-100. Protein concentrations were determined using the Coomassie Plus Kit (Pierce, Rockford, IL) according to manufacturer’s recommendations. Cell extracts (30 µg) were resolved on NuPage® Novex Bis-Tris gels (Invitrogen, Carlsbad CA), and transferred to a nitrocellulose membrane, according to manufacturer’s recommendations. Protein levels were visualized using Ponceau red staining to ensure equivalent amounts of protein were loaded. Membranes were rinsed in TBS-T to remove the Ponceau red and blocked in 5% milk/TBST-T overnight, followed by incubation with anti-IκB-α (C-21) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody (1:500 dilution) overnight in 5% milk/TBST-T. Membranes were incubated with goat anti-rabbit (EMD Biosciences, Gibbstown, NJ) secondary antibody in 5% milk/TBST-T 1 hour, rinsed repeatedly in TBST-T and signals were amplified and visualized using Amersham™ ECL Western Blotting Detection Reagents (RPN2209) (Amersham Biosciences, Pittsburgh, PA).

XV. Statistical Analysis

Data are expressed as mean ± SEM. Differences between multiple groups were determined using one-way ANOVA followed by a Tukey’s multiple comparison test, for comparison of 2 groups a student’s t test was performed (GraphPad Prism; GraphPad Software Inc., La Jolla, CA. Significance was assumed at P ≤ 0.05.
References


Chapter 3

Characterization of the Immune Response After Repeated Exposure to Ambient Particulate Matter
Abstract

**Background.** Epidemiological studies indicate that elevations in airborne PM significantly increase the risk for developing disease, however a causal relationship has not been firmly established as the vast majority of controlled acute PM exposure studies in healthy individuals have failed to show the induction of the hallmark features of asthma (i.e. AHR, Th2-mediated inflammation) after PM exposure. **Objective.** To address these disparities, we examined the effects of the direct delivery of AUB on the induction of the pathophysiological features of asthma in mice over time. **Methods.** To this end, we assessed the effects of single and multiple short-term intratracheal AUB (0.5 mg) exposures on pulmonary inflammation, T-cell cytokine production, and oxidant/antioxidant gene expression. **Results.** Twenty-four hours after a single AUB aspiration challenge, we observed a mixed pulmonary Th1/Th17 cytokine profile that shifts towards a Th2/Th17 profile after three challenges. GM-CSF and IL-1β are increased in the BAL after a single AUB exposure, but are no longer detectable after multiple challenges, suggesting that they play role in the initiation of pulmonary inflammation. In contrast, the BAL levels of pulmonary cytokines (IL-6, TNF-α) and chemokines (KC, CCL20, MCP-1, MIP-1α, MIP-1β) were elevated after each AUB challenge. In addition, AUB increased expression of pro-oxidant (NADPH oxidase) genes, while reducing antioxidant (GST) gene expression. **Conclusion.** Our results indicate that AUB exposure induced several features of asthma, including AHR and eosinophilic inflammation. The development of these pathophysiological features of asthma was associated with a time dependent shift from an ‘anti-asthmatic’ Th1/Th17 phenotype toward a mixed Th2/Th17 phenotype. These results suggest that indeed AUB
is able to induce the major features of asthma, but that multiple challenges are required for these features to develop. These studies provide an explanation for the disparity in epidemiological and acute human exposure experiments and provide initial insights into the mechanisms driving these effects.
Introduction

Asthma is a heterogeneous disease of unknown etiology characterized by the development of maladaptive Th2 type immune responses to otherwise innocuous triggers. Although the cause of asthma is unknown, epidemiological studies have shown a strong association between the levels of ambient PM and increased ER visits and hospital admissions for asthma\(^1\)-\(^5\). However epidemiological studies fail to differentiate between a causative role for PM in asthma versus a role for PM as an aggravator of pre-existing respiratory stress induced by other pollutants (e.g. ozone), viruses, animal dander, and pollens. Acute controlled exposure studies in healthy individuals have implicated both PM-induced airway inflammation and oxidative stress in the development of the allergic diathesis\(^6\)-\(^13\), yet ethical limitations on PM concentration, exposure time and the number of exposures results in a general failure of such studies to show PM-induced effects on lung function. To this end, we sought to determine whether PM exposure induces the allergic phenotype in mice, which allowed us to control variables that would be virtually impossible to standardize in human studies (host genetics, prior respiratory infection, diet). We examined the \textit{in vivo} effects of PM exposure on indices of asthma in mice after single or multiple PM inoculations.

Although Th2 responses are necessary and sufficient for the development of experimental asthma\(^14\),\(^15\), recent reports also suggest a role for IL-17 in the development of experimental asthma\(^16\)-\(^23\). Indeed one such study shows that the depletion of IL-17 abrogates ozone induced AHR\(^19\). Yet, studies using ambient PM only weakly associate the induction of AHR and the development of Th2 immunity. This may be, in part, due to technical limitations because the majority of human exposure studies utilize single PM
challenges. Furthermore, these studies have not addressed the effects of PM on the induction of Th17 responses, nor have they characterized the levels of lung pro-inflammatory chemokines and cytokines that may contribute to the development of experimental asthma.

Ambient PM is a complex mixture containing microbial products, transition metals and PAHs. Multiple reports have implicated metal-induced oxidative stress as a potential mechanism of PM-induced airway inflammation in healthy individuals. Moreover, epidemiological evidence associates a null polymorphic variant in antioxidant gene expression (GST) with susceptibility to PM-induced asthma. A recent report from the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) shows that children who are carriers of a GSTP1 polymorphism (Ile→Val), which reduces enzymatic function of the antioxidant enzyme GSTP1, have an increased risk for wheezing when exposed to high levels of DEP, whereas infants with normal GST function are protected against such wheezing. Consistent with the association of asthma susceptibility with impaired antioxidant pathways, DCs lacking the NF-E2-related factor-2 (Nrf2), a major transcriptional activator of phase 2 antioxidant gene expression, have increased PM-driven production of cytokines. Likewise, the exogenous administration of the thiol antioxidants N-acetyl-cysteine (NAC) or GSH has been shown to reduce allergen-driven airway hyperresponsiveness, eosinophilia and Th2 cytokine production in mice. Pharmacological inhibition of the pro-oxidant NADPH oxidase pathway abrogated the development of allergen-induced AHR. Taken together, these studies suggest that PM effects on the pulmonary oxidative balance may be critical in the
development of asthma symptoms. Yet, to date, studies have not simultaneously assessed the effect of PM exposure on both the pro- and antioxidant pathways.

The objective of this study was to directly compare the development of AUB-induced AHR, airway inflammation, T-cell cytokine production and the oxidant balance after single and multiple AUB inoculations. We show that AUB induces a non-significant increase in AHR after a single challenge that reaches statistically significant levels after multiple inoculations. These pathophysiological changes were temporally associated with a shift from a mixed pulmonary Th1/Th17 response after a single challenge to a mixed Th2/Th17 response after multiple challenges. The presence of Th17 cytokines following AUB exposure correlates with the predominantly neutrophilic inflammatory response seen after AUB exposure. Interestingly, AUB-exposure suppresses the development of antioxidant gene expression, while supporting pro-oxidant NADPH oxidase gene expression. These results suggest that a PM-driven shift away from an ‘anti-allergic’ Th1/Th17 phenotype toward a Th2/Th17 phenotype is associated with the gradual development of AHR following multiple exposures.

Materials and Methods

AUB Exposure

Ambient PM was collected in Baltimore as previously described\textsuperscript{24}. Briefly, collection of PM was conducted in urban Baltimore using a high volume cyclone collector with a theoretical cut-point of 0.85 µm aerodynamic diameter when operated at a flow rate of 0.6 m\textsuperscript{3}/min. Mice were anesthetized with an i.p, injection of a mixture of
ketamine (45 mg/kg) and xylazine (8 mg/kg) and exposed to either PBS or AUB (0.5 mg in a 50 µl volume of PBS) on days 0, 3 and 6 by i.t. instillation. Mice were sacrificed 24 hours after the initial or final AUB inoculation for the assessment of airway function and BAL cellularity.

**Airway Responsiveness Measurements**

Airway responsiveness to intravenous ACh was evaluated 24 hours after the final AUB exposure. Mice were anaesthetized, paralyzed with decamethonium bromide (25 mg/kg), intubated and ventilated at a rate of 120 breaths per minute with a constant tidal volume (0.2 ml). ACh (50 mg/kg) was injected into the inferior vena cava and the change in airway pressure was assessed for 5 minutes and referred to as the airway pressure time index (APTI).

**Determination of BAL Cellularity and Chemokine Levels**

Immediately after airway responsiveness measurements the lungs were lavaged three times with 1ml of cold Hanks’ balanced salt solution without calcium or magnesium (Invitrogen, Carlsbad, CA). The recovered lavage fluid (70 to 80%) was centrifuged (300 x g for 8 minutes) and the cell pellet resuspended in 1 ml of 10% FBS in PBS. Total cells were counted with a hemocytometer. To determine the cell types present in the BAL, slides were prepared by cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA) and stained with Diff-Quik (Dade Behring, Du¨dingen, Switzerland). Morphologic criteria were determined in ≥ 500 cells/slide with a light microscope. BAL chemokine levels were determined with ELISAs using matched
antibody pairs that were purchased from R&D Systems (Minneapolis, MN). ELISAs were performed according to the manufacturer's instructions.

**Lung T-Cell Cytokine Measurements**

The lungs were excised, minced and incubated in 6 ml of digestion media containing RPMI 1640, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 mg/ml liberase CL (Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/ml) (Sigma, St. Louis, MO) for a period of 45 – 60 minutes at 37°C. Digested tissue was processed through a 70-micron cell strainer, and erythrocytes were lysed by incubation with ACK lysis buffer (Invitrogen, Carlsbad CA) for 5 minutes. Lysis was stopped using 10 ml RPMI supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

For T cell cytokine determination 250,000 pulmonary cells were cultured in 96 well plates containing a 300 µl volume of media or with concanavalin A (5 µg/ml) for a period of 72 hours. Cytokine levels were determined via ELISAs using matched antibody pairs purchased from R&D Systems (Minneapolis, MN).

**RT-PCR Analysis**

Three hours after a single challenge (3hr), or twenty-four hours after a single (D1) or three (D7) aspiration challenges, the lungs were excised post lavage to determine changes in mRNA levels of antioxidant genes previously associated with asthma. RT-PCR primer sequences for *Ncf4* were obtained from the qPrimerDepot, quantitative PCR
primer database (http://mouseprimerdepot.nci.nih.gov/) and all other sequences were
determined using the online PrimerBank $^{34,35}$. Primer sequences are listed in Table 2.
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Results

**AUB Induces AHR and BAL Cellularity**

To assess the kinetics of AUB-induced AHR and inflammation, we analyzed the airway responses of A/J mice to the cholinergic agonist ACh. These mice exhibit increased sensitivity to antigen induced AHR $^{36-38}$. Twenty-four hours after a single exposure, AUB induced a non-significant increase in AHR (Figure 1A) and significant increases in BAL eosinophils, lymphocytes, macrophages and neutrophils (Figure 1B) when compared to their respective PBS controls. This response was predominantly neutrophilic in nature. In contrast, after three exposures to AUB a significant increase in AHR was induced (Figure 1C, p <0.0001) when compared to PBS controls. As with a single exposure, AUB significantly increased BAL eosinophils, lymphocytes, macrophages and neutrophils after multiple exposures (Figure 1D). Although neutrophil numbers remained the highest there was a substantial increase in the numbers of eosinophils, lymphocytes and macrophages, with no change in neutrophil numbers as compared to the levels seen after a single challenge.
Figure 1. AUB exposure induces significant AHR and inflammation.

AHR to ACh (50 mg/kg) 24 hours after one (Figure 1A) or three (Figure 1 C) i.t. exposures to PBS (open bars) or AUB (0.5 mg/exposure, filled bars). (B, D) BAL populations of eosinophils (Eos), lymphocytes (Lys), macrophages (Mac), and neutrophils (Neu). Values are mean ± SEM, N = 7-10 mice/group. *p < 0.0001, †p < 0.05 vs. PBS.
AUB exposure increases pro-inflammatory chemokines and cytokines in the BAL

To begin to understand the mechanism(s) of AUB-induced increases in lung cellularity, we determined the effects of AUB exposure on the BAL levels of the granulocyte/macrophage growth factor (GM-CSF) and chemoattractants (MCP-1, MIP-1α, MIP-1β, CCL20) that are known to contribute to cell mediated inflammation. We observed that 24 hours after one (Figure 2A) or three (Figure 2B) exposures AUB induced a significant increase in the levels of each of the chemokines in the BAL (Figure 2A-B). Interestingly, GM-CSF only appeared to be present after the initial challenge.

To determine whether AUB induced significant elevations in proinflammatory mediators, we determined the effects of exposure on the BAL levels of the proinflammatory cytokines (IL-1β, IL-6, TNF-α) and the chemokine KC after a single (Figure 3A) or multiple challenges (Figure 3B). We show that at both times, AUB induced a significant increase in the levels of proinflammatory cytokines (IL-6, TNF-α) and the chemokine KC in the BAL (Figure 3A-B). Within our study, the chemokine KC was the most abundantly produced factor in the BAL, which may contribute to the predominantly neutrophilic response observed (Figure 1 B, D). Interestingly, IL-1β only appeared to be present after the initial challenge.
Figure 2. AUB exposure increases BAL growth factor and chemokine levels.

BAL levels of GM-CSF, MCP-1, MIP1α, MIP1β and CCL20 were measured 24 hours after one (A) or three (B) AUB challenges. Values represent mean ± SEM, N = 7-10 mice/group. *p < 0.0001, †p < 0.05 vs. PBS.
Figure 3. AUB exposure increases BAL pro-inflammatory cytokine production.

BAL pro-inflammatory cytokine (IL-1β, IL-1, TNF-α) and chemokine (KC) levels 24 hours after one (A) or three (B) AUB challenges. Values represent mean ± SEM, N = 7-10 mice/group. *p < 0.0001, †p < 0.05 vs. PBS.
**AUB induces a mixed T-cell cytokine profile**

To assess the nature of the lymphocyte cytokine response to AUB, we measured the pulmonary levels of cytokines associated with Th2, Th17 and Th1 effectors from lung cell suspensions restimulated *in vitro* with concanavalin A. We show that cells from mice that received a single AUB exposure produced a higher level of the Th17 (IL-17A) and Th1 (IFNγ) cytokines in comparison to their respective PBS exposed controls (Figure 4A). While there was no significant induction of the Th2 associated cytokines IL-4 and IL-13, there was a trend toward increased production of the Th2 cytokine IL-5 (Figure 4A). Interestingly, after three challenges restimulated lung cells from mice exposed to AUB have significantly upregulated Th17 (IL-17A) and Th2 (IL-4, IL-5, IL-13) cytokine production in comparison to their respective PBS exposed controls, while there was no significant induction of the Th1 associated cytokine IFNγ (Figure 4B).
Figure 4. AUB skews lung T-cells towards a mixed T cell cytokine profile.

T cell cytokine protein levels were assessed in the supernatants of concanavalin A stimulated lung cells harvested from mice exposed to AUB once (D1) or 3 times (D7). Values represent mean ± SEM, N = 7-10 mice/group. *p < 0.0001, †p < 0.05 vs. PBS.
**AUB shifts the pulmonary oxidative balance**

To gain additional insight into the mechanisms by which AUB induces pulmonary inflammation and T-cell activation we assessed the pulmonary mRNA expression of antioxidant genes after AUB exposure. Previous reports from our lab associate the biological activity of AUB with higher levels of transition metals\(^\text{24}\), which is in support of *in vitro* studies that suggest that transition metal-induced oxidative stress contributes to PM-induced airway reactivity\(^\text{12, 25, 27}\). Indeed, AUB-induced DC production of T-cell skewing cytokines is exacerbated in mice that are deficient in transcription factors that induce antioxidant and detoxification genes\(^\text{32}\).

We found that AUB exposure significantly reduced the expression of the GST family members Gsta1 (Figure 5A), Gstm1 (Figure 5B) and Gstp1 (Figure 5C). We did not observe significant changes in the expression of the transcription factor Nrf2 (Figure 5D), nor the adaptor Keap1 (Figure 5E), which together play a critical role in the transcription of antioxidant genes. To determine whether AUB altered antioxidant defenses as well, we assessed the pulmonary expression of pro-oxidant genes in the NADPH oxidase family including: (A) Cybb, (B) Ncf4, (C) Ncf2, (D) Noxo1, and (E) Noxa1 after AUB exposure (Figure 6A-E). Counter to the apparent trends in antioxidant gene expression, we observed an increase in expression of genes in the NADPH oxidase gene family (Figure 6A-E).
Figure 5. AUB reduces pulmonary antioxidant gene expression.

RT-PCR analysis showing PBS (circle) or AUB (square)-induced pulmonary expression of the antioxidant genes (A) Gsta1, (B) Gstm1, (C) Gstp1, (D) Nrf2, and (E) Keap1 three hours (3hr) and twenty-four hours after a single challenge (D1) or 3 AUB aspiration challenges (D7). Values represent mean ± SEM, N = 4-8 mice/group. *p < 0.0001, †p < 0.05 vs. PBS.
Figure 6. AUB increases pulmonary NADPH oxidase gene expression.

RT-PCR analysis showing PBS (circle) or AUB (square)-induced pulmonary expression of the pro-oxidant NADPH oxidase genes (A) Cybb, (B) Ncf4, (C) Ncf2, (D) Noxo1, and (E) Noxal three (3hr) or twenty-four hours after a single challenge (D1), and twenty-four hours after 3 challenges (D7). Values represent mean ± SEM, N = 4-8 mice/group. *p < 0.0001, †p < 0.05 vs. PBS.
Figure 6.
Discussion

Although acute controlled PM exposure experiments in healthy individuals have consistently shown elevations in BAL pro-inflammatory cytokine production and neutrophil content \(^6,9-13,39\), they have generally failed to show changes in lung function. Thus, we examined the kinetics of inflammatory cell influx and cytokine production \textit{in vivo} in mice exposed to AUB. Our studies showed that a single challenge with AUB increased BAL PMN levels, and BAL levels of IL-1\(\beta\), IL-6, TNF-\(\alpha\) and KC (the mouse ortholog of IL-8) \(^{12}\). We observed that the cytokine IL-1\(\beta\) is increased in the BAL after a single AUB exposure but is no longer detectable after multiple challenges, suggesting that IL-1\(\beta\) may play a role in the initiation of pulmonary inflammation. This finding is consistent with previous reports suggesting a role for IL-1\(\beta\) in the initiation of chemical \(^{40}\) and PM-induced \(^{41}\) pulmonary inflammation. In one such study, Ishii et al. show that supernatants from PM-activated alveolar macrophages can induce the expression of proinflammatory cytokines (RANTES, TNF-\(\alpha\), ICAM-1, MCP-1, IL-8, VEGF) in airway epithelial cells, which is attenuated when supernatants were depleted of IL-1\(\beta\) \(^{41}\). Moreover, the administration of recombinant IL-1\(\beta\) increased allergen-induced neutrophilia \(^{42}\) and \textit{in vivo} antibody depletion of IL-1\(\beta\) attenuated increases in BAL inflammatory cells (eosinophils, lymphocytes, alveolar macrophages) in models of chemical \(^{40}\) and cigarette smoke \(^{43}\) induced airway disease. The levels of the neutrophil chemoattractant KC are also elevated at all the time points measured, as are the levels of PMNs in the BAL suggesting, but not proving, that the induction of KC by AUB is responsible for the sustained elevations in lung neutrophils.
While a single exposure to AUB did not induce significant increases in AHR, multiple AUB challenges induced significant elevations in AHR. These results may explain the lack of induction of lung function changes in controlled human exposure regimes, which for ethical reasons generally only include a single exposure to PM. Furthermore, these results provide support for the strong correlation between impaired lung function, hospitalizations, and enhanced exacerbations of asthma following repeated, environmental exposures that have been reported in numerous epidemiological studies\textsuperscript{1-5, 31, 44-49}.

The kinetics of AUB-induced AHR correlated well with the kinetic pattern of T cell cytokine production. Specifically, 24 hours after a single exposure, when no increases in AHR were observed, the lung cells were primed to produce Th17 and Th1 cytokines. In contrast, 24 hours after repeated exposure, the increase in AHR was associated with cells primed to produce a mixed Th2/Th17 cytokine response.

As chemokines are linked to the recruitment of inflammatory cells into the airways, we assessed the kinetic pattern of lung chemokine expression following AUB exposure. Twenty-four hours after both challenges, we observed an increase in the chemokines MIP-1\(\alpha\) and MCP-1. However, after a single exposure the levels of MIP-1\(\alpha\) were higher than those of MCP-1, a trend that is reversed after multiple challenges. Previous reports indicate that the chemokine MIP-1\(\alpha\) and its receptor CCR5 are predominantly expressed in association with Th1 differentiation\textsuperscript{50} and mice deficient in CCR5 have Th2 skewed cytokine production\textsuperscript{51}. Alternatively the chemokine MCP-1 is implicated in Th2 differentiation and previous reports indicate that the levels of MCP-1 are increased in the airway of asthmatics and are associated with the development of Th2
responses. In addition, the removal of MCP-1 attenuated antigen induced AHR, eosinophilia and Th2 cytokine production in mice. Taken together our findings suggest that the kinetic pattern of chemokine induction by AUB- after single and multiple challenges correlates well with the observed transition from a pulmonary Th1/Th17 phenotype toward a Th2/Th17 response.

Consistent with the induction of T cell responses, we observed that AUB induced significant increases in the levels of the chemokine CCL20, which binds the receptor CCR6 on immature DCs and Th17 cells. Interestingly, we observed an increase in the myeloid growth factor GM-CSF after a single AUB challenge that dissipates by the third exposure. Taken together these data suggest that AUB may induce the initial recruitment, differentiation, and survival of immunogenic mDCs in the lung through its ability to upregulate CCL20 and GM-CSF.

Although the mechanisms underlying the induction of inflammation in response to AUB exposure are not well understood, oxidative stress is thought to be a significant contributor to the development of both pollutant- and allergen-induced airway responses. Consistent with this hypothesis, we found that expression of the individual subunits of the pro-oxidant NADPH-oxidase complex, were significantly elevated following AUB exposure. Because the activation of the NADPH oxidase complex leads to the production of superoxide anions, our results indicate that AUB creates a pro-oxidant milieu in the lung. In addition to its ability to directly induce oxidative stress, we see that AUB shifts the oxidant/antioxidant balance even further as it also significantly decreased the expression of several antioxidant genes (Gsta1, Gstm1, Gstp1). These findings are consistent with recent reports from the Cincinnati Childhood Allergy and Air Pollution
Study (CCAAPS) that shows that children who are carriers of a GSTP1 polymorphism (Ile$^{105}$→Val$^{105}$), which reduces GSTP1 enzymatic function, have an increased risk for wheezing when exposed to high levels of DEP, whereas infants with normal GST function are protected against such wheezing $^{31}$. Thus, there is an additive risk for the development of PM-induced airway disease in infants with reduced anti-oxidative capacity. Reports in animal models indicate that the administration of the antioxidant glutathione decreased pro-asthmatic IL-4 production, OVA-specific serum IgE, and pulmonary eosinophilia in OVA-immunized mice when compared to those receiving PBS after OVA challenge $^{58}$. The antioxidant enzymes GSTM1 and GSTP1, which are protective against oxidants in the lung, have been shown to directly inhibit IgE and IL-4 production $^{58,59}$.

Taken together, our results demonstrate that repeated exposures to AUB alone can induce the phenotypic features of asthma (AHR, airway inflammation), concomitant with gradual shifts in the lung cytokine profile from a mixed Th1/Th17 to a mixed Th2/Th17 cytokine pattern. These changes are temporally associated with increases in oxidant gene expression and a loss in antioxidant defenses. Our finding that Th2/Th17 cytokine responses and AHR developed after multiple exposures to AUB may explain the lack of detection of Th2 cytokine production and lung function changes after single PM challenges in humans and provide further support for the hypothesis that environmental PM exposures contribute to the recent rise in asthma prevalence.
References

Chapter 4

Particulate Matter Induced Airway Hyperresponsiveness is Lymphocyte Dependent
Abstract

**Background.** Exposure to airborne PM, which is a major component of air pollution, has been associated with increases in both exacerbations and hospitalizations for asthma. We have previously shown that exposure to AUB induces AHR, eosinophilic and neutrophilic inflammation, and the recruitment of T cells. However, the mechanism(s) by which it induces these features of asthma remain unknown. **Objective.** To determine whether T lymphocytes play a role in AUB-induced AHR. **Methods.** We compared the effects of AUB exposure on the allergic phenotype in wildtype Balb/c and Rag1-/- mice, which lack mature lymphocytes. **Results.** We found that exposure of wildtype mice to AUB induced AHR concomitant with increases in the numbers of BAL lymphocytes, eosinophils and neutrophils, and mucus containing cells in the lungs of wildtype mice. Interestingly, we show for the first time that these effects were associated with significant elevations in lung cell IL-17A, IL-17F and Th2 (IL-13, IL-5) cytokine levels and reductions in the levels of the suppressive cytokine, IL-10. Rag1-/- mice failed to develop AUB-induced AHR, however, AUB induced BAL cellularity and mucus cell changes were only partially inhibited in Rag1-/- mice. **Conclusions.** Taken together our results suggest that AUB exposure increases the pathophysiological features of asthma via activation of lymphocyte-dependent pathways. These results provide a plausible biological mechanism for the strong association between PM exposure and the increased severity of asthma.
Introduction

Asthma is a chronic inflammatory disease of the lung characterized by airway inflammation, AHR, and mucus hypersecretion. The current disease burden has reached epidemic proportions and there is now an estimated 300 million people worldwide suffering with asthma. Although the etiology of asthma is unknown, there is evidence that both genetic and environmental triggers contribute to disease. The recent rise in disease prevalence is unlikely to be explained by changes in the genetic make-up of the society as a whole, as it does not change that dramatically in such a short time frame. Thus, changes in the environment are likely to be the culprit driving the marked increase in prevalence of this disease.

Environmental triggers of asthma include: allergens, viruses, environmental tobacco smoke, and PM. Of these environmental triggers, several lines of evidence suggest that exposure to ambient PM may be associated with the increase in asthma morbidity. For example, numerous epidemiological studies have reported positive correlations between PM exposure and increased medication use, physician visits and ER visits for asthma. Acute controlled exposures of healthy humans to PM have shown a wide variety of responses from no significant effects on airway function or inflammation to significant increases in cellular inflammation. Despite wide variations in the study designs, sources and composition of PM used in these studies, the most consistent findings have been that PM exposure increases BAL neutrophils and inflammatory cytokines such as IL-8 and IL-6, with some studies demonstrating increases in T lymphocytes (CD4+) in bronchial biopsies of healthy human volunteers. Studies in animals, have shown that direct instillation of biologically relevant sources of PM into the lungs of naïve mice...
induces many of the pathophysiological features of asthma. Although many hypotheses have been put forth to explain the ability of PM to directly induce or exacerbate asthma-like symptoms, to date the exact mechanisms underlying the adverse pulmonary effects of PM are not well understood.

As numerous studies in animal models have shown that exposure to other environmental triggers such as allergens and ozone induce AHR through a T cell dependent process, and PM has been shown to drive T cell cytokine production in vivo and in culture systems, we hypothesized that PM-induced AHR and airway inflammation occurs through a lymphocyte dependent process. Thus, the objective of the current study was to directly explore the role of lymphocytes in the development of PM-induced AHR and airway inflammation. To this end, we compared the effects of AUB on airway reactivity and allergic inflammation in wildtype Balb/c and Rag1−/− mice, which lack mature lymphocytes. We show that exposure to AUB induces significant increases in airway reactivity, eosinophilic and neutrophilic inflammation, and mucus metaplasia, concomitant with increases in both Th2 and Th17 cytokine production. In marked contrast, Rag1−/− mice do not develop AHR or Th2/Th17 cytokine production after AUB exposure. Furthermore, AUB induced increases in BAL and tissue inflammation as well as mucus production were only partially lymphocyte dependent. Collectively, our results demonstrate that pulmonary exposure to a real-world source of PM induces the recruitment and activation of T cells leading to the induction of the pathophysiological features of asthma. These results provide a plausible biological mechanism for the strong association between PM exposure and increases in asthma prevalence observed in epidemiological studies.
Materials And Methods

Mice

Male and female C.129S7(B6)-Rag1tm1Mom/J (Rag1-/-) and Balb/c (wildtype) control mice (9 to 10 weeks old, Jackson Laboratories, Bar Harbor, ME) were housed in an environmentally controlled specific pathogen-free facility at Cincinnati Children’s Hospital Medical Center. The mice received access to food and water ad libitum. Mice were treated humanely and with regard for alleviation of suffering in accordance with the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee.

PM Exposure

Ambient PM was collected from a sixth floor window in urban Baltimore during the months of March to May in 2005 using a high volume cyclone collector with a theoretical cut-point of 0.85 μm aerodynamic diameter when operated at a flow rate of 0.6 m³/min. Mice were anesthetized with an i.p. injection of a mixture of ketamine (45 mg/kg) and xylazine (8 mg/kg) and exposed to either PBS or AUB (0.5 mg in a 50 μl volume of PBS) on days 0, 3 and 6 by i.t. instillation.

Airway Responsiveness Measurements

Airway responsiveness to intravenous ACh was evaluated 24 hours after the final AUB exposure as previously described. Briefly, mice were anaesthetized, intubated, and respiration at a rate of 120 breathes per minute with a constant tidal volume (0.2 ml)
and paralyzed with 25 mg/kg decamethonium bromide 72 hours after final allergen challenge. After a stable baseline was achieved, 50 mg/kg ACh was injected into the inferior vena cava, and dynamic airway pressure (cm H2O x sec) was followed for 5 minutes.

**Determination of BAL Cellularity and Chemokine Levels**

Lungs were lavaged three times with a 1.0-ml aliquot of cold Hanks’ balanced salt solution. Recovered lavage fluid (70–80%) was centrifuged at 300 x g for 8 minutes, and the cell pellet was resuspended in 1.0 ml of 10% FBS in PBS. Slides were prepared by cytocentrifugation and stained with Diff-Quik (Dade Behring). BAL total and cell differential counts were determined using morphologic criteria under a light microscope with the evaluation of ≥ 500 cells/slide. BAL chemokine levels were determined with ELISAs (R&D Systems, Minneapolis, MN).

**Lung T-Cell Identification and Cytokine Measurements**

Whole lungs were perfused with ice-cold PBS, removed, minced and placed in 6 ml RPMI 1640 containing 0.5 mg/ml Liberase CI (Roche Diagnostics) and 0.5 mg/ml DNase I (Sigma-Aldrich) and incubated at 37°C for 45 minutes. Single cell suspensions were pelleted, and stained with APC conjugated anti-mouse CD4 (L3T4) and FITC conjugated anti-mouse TCRβ for flow cytometric analysis (eBioscience, San Diego, CA) using a FACSVantage SE flow cytometer. Analysis was done using FlowJo software (Tree Star, Inc.). For T cell cytokine determination, lung cells (250,000) were cultured in
media or concanavalin A (5µg/ml) for a period of 72 hours. Cytokine levels were measured with ELISAs.

**Histological Examination of Lung Sections**

To assess the effects of AUB on airway inflammation and mucus cell content in the airway wall, lungs were excised and fixed in 10% formalin, washed in methanol, dehydrated, embedded in paraffin and cut into 5µm sections. Sections were mounted on slides and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Slides were read in a blinded fashion and scored according to the following scale: 0: no inflammation, 1 – 1.99: 1-25% inflammation of section, 2 – 2.99: 26% - 50% inflammation of section, 3 – 3.99: 51% - 75% inflammation of section, 4 – 4.99: 76% - 100% inflammation of section. The number of PAS+ cells per section was counted and averaged over 4 sections per mouse lung.

**Measurement of Serum IgE Levels**

Immediately after AHR measurements terminal blood collection was performed from the posterior vena cava. Blood was collected in serum separator tubes (BD Microtainer®, Franklin Lakes, NJ) and centrifuged for separation. Isolated serum was immediately stored at -80°C until total IgE levels were measured by ELISA using matched antibody pairs (BD Pharmingen™, Franklin Lakes, NJ).
Statistical Analysis

Differences between multiple groups were determined using one-way ANOVA, with Tukey multiple comparison post-test comparisons, a Student’s t-test was performed for the comparison of 2 groups (GraphPad Prism; GraphPad Software, Inc, La Jolla, CA). Significance was assumed at p <0.05.

Results

AUB-Induced AHR Is Lymphocyte Dependent.

To assess the contribution of lymphocytes to the development AUB-induced AHR, we compared the airway responses of wildtype and Rag1−/− deficient mice to the cholinergic agonist acetylcholine. Exposure to AUB induced a significant increase in AHR in wildtype mice when compared to PBS controls (Figure 1, p <0.0001). In contrast, AUB did not induce significant increases in AHR in Rag1−/− as compared to their wildtype controls. Interestingly, lymphocytes also contributed to the baseline airway response to acetylcholine as the response was lower in PBS-challenged Rag1−/− mice as compared to their respective wildtype controls, although the observed difference did not achieve statistical significance.
Figure 1. AUB-induced AHR is lymphocyte dependent.

AHR to ACh (50 mg/kg), as defined by the time-integrated change in airway pressure (APTI, cmH2O•s), was determined in wildtype (Balb/c) mice and Rag1/-/- mice after three i.t. exposures to AUB (0.5 mg/exposure). Values are mean ± SEM, N = 7-10 mice/group. *p < 0.0001 vs. PBS wt, ##p < 0.01 vs. AUB wt.
AUB Induction of Mucus is Partially Lymphocyte Dependent

As mucus metaplasia is a consistent feature of allergic asthma, we determined the effects of AUB exposure on mucus production as assessed by PAS staining. As shown in Figure 2A-E, AUB exposure significantly increased the numbers of airways containing PAS+ mucus cells in the lungs of both wildtype (Figure 2B) and Rag1-/- (Figure 2D) mice in comparison to their respective controls (Figure 2A, C). Quantification of the numbers of PAS+ cells in lung sections from groups of mice showed that the numbers of PAS+ cells in AUB-exposed Rag1-/- mice were significantly elevated over their PBS-controls, but significantly lower than that observed in the wildtype AUB-exposed mice (Figure 2E, p <0.0001).
Figure 2. AUB-induced a partially T-cell dependent increase in mucus cells. PAS staining in lung sections from PBS (A and C) and AUB (B and D) exposed wildtype (A and B) and Rag1−/− mice (C and D). The number of PAS+ cells (E) in airways was counted by light microscopy. Arrows indicate areas showing PAS+ staining. Values are mean ± SEM. The number of PAS+ cells in four sections per mouse lung were averaged.
(N = 4 mice/group). *p < 0.0001 versus PBS wt, #p < 0.0001 versus AUB wt. Bar = 50 µm.

**AUB-Induced BAL Inflammation Is Partially Lymphocyte Dependent.**

To determine whether AUB induces allergic airway inflammation in a lymphocyte dependent manner, we compared the cellularity of the BAL fluids from Rag1-/- and wildtype mice following PBS or AUB exposure. In PBS controls, the majority of the cells in the lavage of both strains of mice consisted primarily of alveolar macrophages (Figure 3). Following AUB exposure of wildtype mice, significant elevations were observed in the numbers of BAL macrophages, neutrophils, lymphocytes and eosinophils in wildtype mice. Increases in each cell type were also observed in Rag1-/- mice upon AUB exposure. However, the levels of neutrophils were significantly lower in the BAL collected from Rag1-/- mice exposed to AUB as compared to PBS controls, while no significant differences were found in the numbers of BAL macrophages, or eosinophils between Rag1-/- and wildtype controls. Of note, although Rag1-/- mice are devoid of T and B lymphocytes, we observed a small number of cells in the BAL with morphological characteristics of lymphocytes. These are most likely NK cells, as it has been previously shown that NK cell numbers are increased in naïve Rag1-/- mice. Consistent with the inflammatory patterns seen in the BAL following AUB exposure, we observed widespread perivascular and peribronchial inflammation in the lungs of both wildtype and Rag1-/- mice as compared to their PBS-exposed controls (Figure 4). Consistent with the partial effect of Rag1 deficiency on AUB-induced increases in BAL cellularity, the degree of AUB-induced inflammation in the lung sections from Rag1-/- mice was significantly reduced in comparison to that seen in wildtype mice (Figure 4E, p
<0.05), but still significantly higher than in their PBS-controls. Based on cell morphology, the inflammatory foci consisted primarily of neutrophils and macrophages. Of note, we detected AUB particles in the sections presumably engulfed by macrophages (Figure 4B, D). Taken together these results suggest that AUB exposure induces a marked cellular infiltration of the mouse lung, which is only partially lymphocyte dependent.
Figure 3. AUB-induced increases in BAL cellularity are partially lymphocyte dependent.

BAL populations of macrophages and neutrophils (A) and lymphocytes and eosinophils (B) were assessed in both WT and Rag1-/- mice after AUB exposure. Values are mean ± SEM, N = 7-10 mice/group. *p < 0.0001, **p < 0.01, versus PBS wt; #p < 0.0001 versus AUB wt, ###p < 0.05 versus AUB wt, ††p < 0.01 versus PBS Rag1-/-.
Figure 4. AUB-induced lung inflammation is partially lymphocyte dependent.

Hematoxylin and eosin staining of wildtype (A and B) and Rag1-/- (C and D) mice exposed to either PBS (A and C) or AUB (B and D). Arrows indicate AUB particles. The degree of inflammation was scored according to an arbitrary scale defined in the Materials and Methods section. Values are mean ± SEM, N = 8 independent sections/group. *p < 0.0001, versus PBS wt, ##p < 0.01 versus AUB wt; †p < 0.0001 versus PBS Rag1-/-.. Bar = 50 μm.
Figure 4.

A.  

B.  

C.  

D.  

E.  

Inflammatory Score  

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<th></th>
<th>PBS - WT</th>
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<th>PBS - Rag1 −/−</th>
<th>AUB - Rag1 −/−</th>
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* P-value < 0.05
## P-value < 0.01
† P-value < 0.001
AUB Induces the Recruitment of CD4+ T-Cells.

To gain additional insight into the type of T lymphocytes recruited by AUB exposure, we assessed the effects of AUB exposure on the numbers of CD4+ TCRβ+ cells by flow cytometric analysis of whole lung cell digests. Our results revealed that AUB exposure induced significant increases in the percentage of conventional CD4+ TCRβ+ T cells in the lungs of wildtype mice (Figure 5A-B). As expected there were no detectable CD4+ T cells in Rag1-/- animals (data not shown).

To begin to understand the mechanism(s) of AUB-induced CD4+ T-cell subset recruitment, we assessed the effects of AUB exposure on the levels of several chemoattractants (TARC, C3, MCP-1, MIP-1α) that are known to contribute to T-cell mediated inflammation. We show that AUB induced a significant increase in the levels of each of these chemokines in the BAL (Figure 5D-G). However, only TARC production appeared to be dependent upon the presence of lymphocytes as it was significantly lower in Rag1-/- mice exposed to AUB as compared to the levels in AUB-exposed wildtype mice (Figure 5D). These results demonstrate that AUB exposure likely initially induces the recruitment of inflammatory cells into the lungs through the production of chemoattractants such as C3a, MCP-1, MIP-1α and TARC which are likely produced by either airway epithelial cells or monocyte/macrophage populations.
Figure 5. AUB increases CD4+ T-cell recruitment in the lung.

Representative flow cytometry plots of CD4+ and TCRβ+ cells in the BAL fluid of wildtype mice exposed to PBS (A) or AUB (B). Quantification of the percentage of CD4 positive T-cells (C) was performed in the lungs of wildtype BALB/c and Rag1-/- mice before and after AUB. TARC (D), C3a (E), MCP-1 (F), and MIP-1α (G) levels are measured in the BAL of AUB or PBS exposed mice. Values are mean ± SEM, N = 7-10 mice/group. *p < 0.0001, **p < 0.01 versus PBS wt, #p < 0.0001 versus AUB wt, †p < 0.0001, ††p < 0.01 versus PBS Rag1-/-.
Figure 5. Saunders et al.

A. 

B. 

C. 

D. 

E. 

F. 

G.
AUB Induces T-cell Cytokine Production

To assess the nature of the lymphocyte cytokine response to AUB, we measured the levels of cytokines associated with Th2, Th17, Th1 and Treg effector cell function from lung cell suspensions restimulated in vitro with concanavalin A. Restimulated lung cells from wildtype mice exposed to AUB produced a higher level of the Th2 cytokines IL-4, IL-5 and IL-13 than their PBS exposed controls (Figure 6A), while there was no significant induction of the Th1 associated cytokine IFNγ (Figure 6B). Consistent with the increase in inflammation in the lungs and the BAL following AUB exposure there was a significant reduction in the immunosuppressive cytokine IL-10 in cells from AUB-exposed wildtype mice (Figure 6C). AUB exposure also resulted in the production of significantly higher levels of the Th17 associated cytokines IL-17A and IL-17F as compared to their PBS-exposed controls (Figure 6D). As expected, lung cells from Rag1-/- mice did not respond to concanavalin A stimulation (data not shown).

To determine whether AUB exposure induces allergic sensitization, we measured total serum IgE levels from wildtype and Rag1-/- mice exposed to AUB. AUB exposure did not increase the levels of total serum IgE levels when compared to those seen in PBS-exposed controls (Figure 6E). IgE levels were not detected in Rag1-/- mice, which is expected as these mice lack mature B cells. These results suggest that acute exposures to AUB do not promote atopy and that the AHR and inflammatory responses observed in AUB-exposed mice are not dependent upon IgE-driven processes.
Figure 6. AUB skews lung T-cells towards a mixed Th2/Th17 profile.

Cytokine production from concanavalin A restimulated lung cells showing; Th2 cytokines IL-4, IL-5, and IL-13 (A), the Th1 cytokine IFNγ (B), the Treg cytokine IL-10 (C) and the Th17 cytokines IL-17A and IL-17F (D). Total serum IgE levels in AUB-exposed and wildtype Balb/c mice (E). Values represent mean ± SEM. N = 7-10 mice/group. *p < 0.0001, **p < 0.01, versus PBS wt, #p < 0.0001 versus AUB wt.
Discussion

In the present study, we demonstrate that exposure to AUB induced marked increases in airway responsiveness to cholinergic stimuli, concomitant with an increase in eosinophilic and neutrophilic inflammation, CD4+ T-cell recruitment, and mucus cell metaplasia. These findings are consistent with epidemiological studies linking PM exposure and recent increases in asthma prevalence and morbidity. Moreover, our results support previous human PM exposure studies and studies in mouse models from our group and others showing that different sources of ambient PM (AUB, fly ash, DEPs) can directly induce the pathophysiological features of asthma as well as enhance immune responses to other allergens.

Our results demonstrate that AUB-induced AHR is dependent upon lymphocytes as AHR is significantly attenuated in mice lacking mature lymphocyte populations (Rag1-/- mice). These results are consistent with other reports showing that the development of AHR to other environmental triggers of asthma such as allergens, oxidants and irritants is dependent upon T lymphocytes. Although the development of AHR in response to AUB was dependent upon lymphocytes, the AUB-induced influx of inflammatory cells into the lungs as assessed by both BAL and histological examination of lung sections, was only partially dependent upon lymphocytes. This apparent disassociation between inflammation and AHR is in agreement with previous reports from our lab indicating that airway inflammation does not correlate with the development of antigen- or PM-induced AHR. Likewise, AUB-induced mucus cell metaplasia was only partially abrogated in Rag1-/- mice. As numerous other studies have shown the importance of CD4+ T cells and the Th2
cytokine, IL-13, in mucus production following antigen, these studies are somewhat surprising. These results may suggest that either AUB directly induces mucus cell changes in the airway epithelium or that other innate immune cells such as neutrophils contribute to the induction of this response in Rag1−/− mice. Indeed, several studies have implicated neutrophil-derived mediators (neutrophil elastase) in mucus production.

As Th2 cytokines have been closely associated with the development of antigen-induced AHR, we examined the cytokine profile in the lungs of mice exposed to AUB. We find that AUB induced a marked influx of CD4+ T cells into the lungs and elevations in the levels of both Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A, IL-17F) cytokines, concomitant with a reduction in the Treg cytokine, IL-10. No significant changes were seen, as compared to baseline levels, in the Th1 cytokine IFNγ. Taken together these results suggest that AUB induces allergic inflammation by both suppressing tolerogenic immune responses (IL-10) and by inducing a Th2/Th17 mixed immune response in the airways.

We show for the first time that AUB-induced AHR is associated with the induction of a mixed Th17/Th2 cytokine response in the lung. Our observation is consistent with recent studies in human asthmatics and in animal models of ozone and allergen-induced AHR implicating IL-17 in the development and progression of allergic asthma. Specifically, recent studies have shown that IL-17A levels in asthmatics correlate with the incidence of AHR and severity of disease. Likewise aerosolized pollutants (organic dust and ozone) have been shown to induce IL-17A in human BAL cells and in the mouse lung. Indeed, recent studies have demonstrated a primary role for IL-17 in ozone-induced AHR as both antibody blockade of IL-17 and genetic deficiency in the IL-17A...
17R protects against ozone-induced AHR. Interestingly, studies of IL-17 in allergen-induced models of AHR suggest a more complex picture with some studies showing that IL-17 plays an important role in allergen-driven AHR, while others show that either IL-17 does not play a role at all, or that it can either stimulate or inhibit the development of allergic inflammation depending on the timing of IL-17 blockade.

Despite the evidence implicating IL-17 in AHR, it alone does not appear to be sufficient to induce AHR, as neither transfer of Th17 cells nor administration of rIL-17 induce AHR. Interestingly, co-transfer of Th17 and Th2 cells enhances AHR over that seen with Th2 cells alone. Although the exact mechanisms of the synergism between IL-17 and Th2 cytokines are not currently well understood, IL-17 is a potent stimulator of neutrophil recruitment and activation and IL-17-dependent AHR has recently been shown to be neutrophil dependent. A role for neutrophils in the induction of AHR in our model is suggested by the fact that AUB induced a significant influx of neutrophils into the mouse lung and that the numbers of neutrophils in the lavage are reduced in Rag1-/- mice concomitant with the suppression of AHR. Taken together, these results suggest that the development of AHR in response to airway delivery of antigens/pollutants is dependent on the synergistic actions of Th2 and IL-17.

The source of Th2 and Th17 cytokines appears to be lymphocytes as both baseline and AUB-stimulated cytokine levels are absent in Rag1-/- mice. Specifically, although not proven, we propose that the cells producing these cytokines are CD4+ T cells as we observed a marked influx of CD4+ T cells following AUB exposure, which was absent in Rag1-/- mice. However, a contribution by other lymphocyte populations cannot be ruled out. Indeed, recent studies suggest that NKT cells may contribute to the
development of AHR induced by ozone through their ability to recognize lipid antigens and produce cytokines early after exposure. NKT cells likely do not play a role in our studies, as Rag1/-/- mice do not have mature NKT cells. However, NK cells, which also recognize lipids antigens, may play a role as it has been shown that the development of these cells is upregulated in Rag1/-/- mice. These cells may account for the lymphocyte-like populations we identified morphologically in the BAL. They may also be the source of the lymphocyte independent production of IL-5 and recruitment of eosinophils and other inflammatory cells we observed in the lungs of Rag1/-/- mice.

The mechanisms by which PM activates lymphocytes are currently unknown. We have previously reported that PM collected in urban Baltimore contains a variety of potentially biologically active components such as endotoxin, metals, and PAHs. Although we have found that AUB does not contain classic antigens such as house dust mite, cat, and cockroach, we cannot rule out the possibility that it may contain unrecognized protein antigens. Recent studies suggest that non-proteinous pollutants such as ozone activate lymphocytes in the mouse lung. Although it had been previously thought that substances such as ozone damage the airways through free radial production, leading to the presentation of altered self-proteins, recent studies suggest that ozone may also induce airway inflammation through TLR4-mediated processes. As we have reported that Baltimore PM contains low levels of endotoxin, the activation of immune responses in our model may be at least partially TLR4 dependent. Alternatively, the oxidative potential of transition metals (copper, manganese, zinc) and PAHs contained in our PM source may also drive AUB-induced T cell activation. Substantial evidence suggests that metals and oxidative stress play a significant role in
the strong epidemiological association between indices of allergic airway disease and PM exposure in epidemiological studies conducted both in the Utah Valley and in Germany \(^7,^{10,11,49}\). Specifically, the PM collected in the Utah valley during the time a steel mill was open induced significantly greater indices of lung injury than did PM collected during the mill closure period \(^10,11\). PAHs are also thought to be strong inducers of oxidative stress as the ability of DEP containing high levels of PAHs to enhance OVA sensitization in mice is inhibited by pre-treatment of mice with thiol antioxidants \(^51,52\). As we have previously shown that the water-soluble fraction of AUB does not contribute to its ability to induce AHR, the organic fraction of AUB containing numerous PAHs, may play a significant role in T cell activation in our model. Each of these components is likely to activate T cell mediated immune responses through effects on DC function, rather than direct effects on T cells, as it has been shown that DEP collected in urban Baltimore does not directly activate T cells \(^23\). In contrast, AUB has been shown to directly activate DCs in culture with elevations in CD80, CD86, CD40, and MHCII expression \(^23,24,53\). Moreover, the induction of these costimulatory molecules and cytokine production (IL-12p40, IL-6, IL-10, TNF-\(\alpha\)) was oxidant dependent as these indices were elevated in mice lacking the antioxidant gene Nrf2 or partially reversed by antioxidant treatment \(^53\). Taken together these results suggest that AUB activates DC-T cell activation through multiple additive or synergistic effects driven by the individual components of real world ambient air PM. Further studies are clearly required to tease out the exact mechanisms by which AUB drives T-cell mediated AHR.

In summary, our studies demonstrate that exposure of the mouse lung to real world ambient PM directly induces several features of asthma (i.e. AHR, granulocytic
inflammation, mucus hypersecretion) concomitant with the activation of an adaptive immune response characterized by the recruitment and activation of CD4+ T lymphocytes. The induction of a Th2/Th17 skewed cytokine environment in the lung may directly drive asthmatic symptoms as shown herein, as well as lead to the sensitization to or enhancement of ongoing immune responses to heterologous antigens in susceptible individuals. These studies provide a plausible biological mechanism for the strong association between PM exposure and the increase in asthma prevalence and morbidity.
References


Chapter 5

*In Vivo* Ambient Particulate Matter Exposure Alters Dendritic Cell Recruitment and Phenotype
Abstract

Background. We have previously shown that ambient PM collected in urban Baltimore directly induces airway inflammation and AHR through the activation of T-cells. However, the mechanisms by which AUB activates T cells are not well understood.

Objective. As T cell differentiation is controlled through multiple signals derived from DCs, we sought to determine the effects of PM on pulmonary DC recruitment, activation, and phenotype. Methods. To this end, we exposed mice in vivo to AUB (50 µg, days 0, 3, 6) and assessed the lung DC phenotype (mDC, pDC) and activation status by flow cytometry as well as DC cytokine producing capability. Lastly, we evaluated the contribution of GM-CSF to AUB-induced changes in DC phenotype and T-cell cytokine profiles. Results: We found that in vivo AUB exposure induced a preferential increase in the numbers of immunogenic mDCs recruited to the lung, with an apparent reduction in tolerogenic pDC numbers, concomitant with the development of a mixed Th2/Th17 cytokine response. Direct exposure of DCs to AUB induced a mixed Th2 (IL-6)/Th17 (IL-1β, IL-6, IL-23) skewing cytokine profile that was NF-κB dependent and partially MyD88-dependent. Moreover, we show that these changes in DC phenotype were at least partially dependent on GM-CSF as anti-GM-CSF antibody treatment reversed AUB-induced increases in mDC recruitment, activation, and Th17 cytokine production.

Conclusion: Taken together, these results indicate that AUB-induced GM-CSF production drives the selective expansion of lung mDC populations, which prime naïve T cells to produce a mixed Th2/Th17 pulmonary cytokine profile. These results provide a plausible explanation for the ability of AUB to directly induce allergic airway inflammation.
Introduction

Over the past several decades an alarming increase in the prevalence of asthma has been documented. Approximately 300 million individuals worldwide suffer from asthma and it is estimated that an additional 100 million people will suffer from asthma by 2025\textsuperscript{1}. Aberrant Th2 cytokine production (IL-4, IL-5, IL-13) in response to environmental allergens is necessary for many of the pathological features of allergic asthma including IgE production, eosinophilic inflammation, and AHR\textsuperscript{2-8}. Recent studies also suggest that other subsets contribute to increased susceptibility (Th17)\textsuperscript{9-14} or tolerance (Th1, Treg)\textsuperscript{15-20} to the development of asthma. Although susceptibility to the development of allergic asthma is known to be dependent upon genetic factors\textsuperscript{21}, the recent rise in asthma prevalence, morbidity and mortality are not likely due to changes in genetic factors alone. Thus, changes in the environment in westernized countries have been implicated in the recent rise in asthma prevalence.

One environmental exposure that has been shown to be tightly associated with asthma morbidity and mortality is ambient PM generated as a result of the combustion of fossil fuels by automobiles\textsuperscript{22-26}. Specifically, PM exposure in combination with allergen has been shown to enhance experimental allergic-asthma in mice\textsuperscript{27-29}. In addition, we\textsuperscript{30, 31} and others\textsuperscript{27, 28, 32} have previously reported that exposure to particulate air pollution can directly induce the cardinal symptoms of asthma including increased AHR, pulmonary eosinophilia and T cell cytokine production\textsuperscript{27, 28, 30-32}. Despite evidence that PM induces adaptive immune responses, the mechanisms by which PM stimulates adaptive immunity are not well understood.
Multiple mechanisms exist for the priming of the naïve T-cell response. For example, cytokines such as TSLP\textsuperscript{33-35}, GM-CSF\textsuperscript{36-39} and IL-33\textsuperscript{39-41} when produced can indirectly drive naïve T-cell differentiation toward a Th2 phenotype by conditioning DCs, which then have the capacity to stimulate such a transition. DCs drive naïve T-cell differentiation and activation through MHCII dependent antigen presentation, co-stimulatory molecule (CD80/CD86) engagement, and the production of cytokines that skew naïve T-cells into Th2 (IL-6, IL-9)\textsuperscript{42,43,44}, Th1 (IL-12)\textsuperscript{45}, Treg (TGFβ/ IL-10)\textsuperscript{46-48} or Th17 cells (IL-1β, IL-6/TGFβ/IL-23)\textsuperscript{43,49-52}. It has recently become appreciated that DC cytokine production is dependent upon “danger signals” conveyed through the activation of PRRs such as the TLRs. TLRs have been designated to function as viral (TLR 3, 7, 9) or fungal and bacterial (TLR 1, 2, 4, 5, 6) sensors\textsuperscript{53}. With the exception of TLR 3, all TLRs were previously shown to signal at least in part through the MyD88 adaptor protein. Upon TLR activation MyD88 initiates several signaling pathways, which leads to the activation of MAP kinase pathways and the NF-κB pathway\textsuperscript{53,54}.

DCs in the airways are situated beneath the airway epithelium\textsuperscript{55}, a position from which they can extend their dendrites into the airway lumen and sample antigens without disturbing the integrity of the airway epithelial cell barrier\textsuperscript{56-58}. Previous reports indicated that there were few DCs in the airways of newborn humans\textsuperscript{59} and of animals kept in clean animal facilities\textsuperscript{60}. Following exposures to environmental factors such as allergens, infectious agents, and toxins in early life, the numbers of airway DCs increase\textsuperscript{61-63}. The numbers of DCs in the lungs of asthmatic individuals are elevated above and beyond those observed in healthy controls\textsuperscript{59}. Likewise, increases in pulmonary DCs are observed in animal models of allergic disease\textsuperscript{19,64}. DCs are the primary antigen
presenting cells necessary for both the initiation and the perpetuation of allergic inflammation \(^{19,64}\). Moreover, it has been shown that specific DC subsets are associated with the development of allergic inflammation. Specifically, immunogenic mDCs (CD11c\(^{hi}\), CD11b\(^{+}\)) \(^{65}\), have been shown to be necessary for the development of allergen induced AHR \(^{66}\), eosinophilic inflammation and Th2 cytokine production \(^{19,67}\). Conversely, pDCs (CD11c\(^{low}\), Gr1\(^{+}\), CD317\(^{+}\)) can inhibit allergen induced eosinophilic inflammation and Th2 cytokine production \(^{65,68}\) and it is thought that their actions occur through regulatory T cells.

Evidence is accumulating that AUB may activate DCs, however, the majority of studies to date have been performed in vitro in cells derived from human peripheral blood \(^{69,70}\) or mouse bone marrow derived DCs \(^{71}\). These studies have been performed under conditions that drive the DCs towards a myeloid phenotype, precluding the determination of the effects of PM on DC subset recruitment. In such studies, PM has been shown to activate human peripheral blood derived DC \(^{69,70}\) and mouse bone marrow derived DC \(^{71}\) cytokine production, MHCII expression and costimulatory molecule expression. However in one study, which was designed to evaluate the adjuvant effects of carbon black particles on allergen-driven responses, the numbers of mDCs was shown to increase following combined allergen-PM exposure, as compared to allergen exposure alone. However, the effects of PM alone on in vivo DC numbers were not determined \(^{72}\). Thus, determination of the impact of PM alone on pulmonary DC subset recruitment and cytokine phenotype requires further investigation.

The mechanisms driving the expansion of immunogenic DCs in the allergic airway are not well understood, but may include the production of the cytokine GM-CSF,
which directly stimulates the differentiation of progenitor cells into mDCs, while suppressing the development of pDC populations. Previous reports indicate that the overexpression of GM-CSF overcomes the necessity for adjuvant for the development of eosinophilia and Th2-differentiation in response to purified allergen (recombinant Der p1, OVA) exposure. These effects are associated with an increase in lung DCs. Furthermore, antibody neutralization of GM-CSF reduces allergen, cigarette smoke, and diesel exhaust-mediated eosinophilia, Th2 cytokine production and AHR. However, to date, studies have not addressed the role of GM-CSF on pulmonary mDC/pDC ratios and activation after PM exposure and its relationship to PM-driven T-cell activation.

Because we have previously reported that real-world PM collected in an urban environment directly induces airway inflammation, we sought to determine the effects of in vivo exposure to ambient PM collected in urban Baltimore on the phenotype of pulmonary DCs. Moreover, we examined the role of GM-CSF in the regulation of pulmonary DC phenotype. Our studies show that exposure to AUB results in a GM-CSF dependent shift towards the preferential recruitment and activation of immunogenic mDCs producing a Th2/Th17 (IL-6, IL-23) skewing cytokine profile, with a concomitant suppression in tolerogenic pDC recruitment. We found that AUB-induced DC activation and cytokine production is NF-κB dependent, while only partially MyD88 dependent. These results provide a plausible explanation for the ability of AUB to directly induce allergic airway inflammation.
Materials and Methods

Mice

Male and female A/J, C57BL/6 mice (6 to 10 weeks old, Jackson Laboratories, Bar Harbor, ME) and MyD88 deficient (MyD88−/−) 78 mice were housed in an environmentally controlled, specific pathogen-free facility at Cincinnati Children’s Hospital Medical Center. The mice received access to food and water ad libitum. Mice were handled in accordance with the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee.

Reagents

As previously reported 31, AUB was collected using a high volume cyclone that was continuously operated at a flow rate of 0.6 m³/minute for periods of three months. We have previously reported that AUB contains highly reactive metals, aromatic hydrocarbons, bacterial endotoxin and protein 31. For in vitro stimulation, cells were treated with AUB (50 μg/ml), ragweed (50 μg/ml, RGW, Greer Laboratories, Lenior, NC), Aspergillus fumigatus (50 μg/ml, ASP, Greer Laboratories, Lenior, NC), the NF-κB inhibitor isohelenin (10 μM, EMD Biosciences, Gibbstown, NJ), or DMSO (1 μl, Fisher Bioreagents, Fair Lawn, NJ) as a solvent control.
**In vivo AUB Exposure**

Mice were anesthetized with an i.p. injection of a mixture of ketamine (45 mg/kg) and xylazine (8 mg/kg) and exposed to either 50 µl PBS or 50 µl AUB (0.5 mg in PBS) on days 0, 3 and 6 via aspiration challenge as previously described \(^{31}\). Briefly mice were placed on a 60° incline, the tongue was extended to prevent swallowing and 50 µl aliquots of AUB or sterile PBS were instilled into the oral cavity. The nose was pinched gently during instillation to improve aspiration efficiency.

Anti-GM-CSF (MP1-22E9) and the IgG2a isotype (GL117) hybridoma cell lines were kind gifts from Dr. George Deepe (University of Cincinnati College of Medicine) and Dr. Fred Finkelman (Cincinnati Children’s Hospital Medical Center), respectively. Antibodies were obtained from ascites in Pristane-primed athymic nude mice and purified using salt precipitation and ion-exchange chromatography, as previously described \(^{79}\). Mice received 1 mg of antibody in 100 µl sterile saline via tail vain injection 24 hours before (Day -1) and after (Day 1) initial AUB aspiration challenges.

**Determination of DC Phenotype In the Lungs**

Twenty-four hours following AUB challenge, the lungs were excised, minced and incubated in 6 ml of media containing RPMI 1640, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 mg/ml liberase CL (Roche Diagnostics, Indianapolis, IN) and 0.5 mg/ml DNase I (Sigma, St. Louis, MO) for a period of 45 – 60 minutes at 37°C. Digested tissue was processed through a 70-micron cell strainer, following which erythrocytes were lysed with ACK lysis buffer (Invitrogen, Carlsbad,
CA) for 5 minutes. Lysis was stopped using 10 ml RPMI supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Lung cell suspensions were analyzed on the LSRII flow cytometer (BD Biosciences, San Jose, CA). Populations were gated on forward scatter, side scatter and 7AAD fluorescence to exclude debris and dead cells. Myeloid DCs (CD11c^{hi}, CD11b^{+}, Gr1^{-}) and pDCs (CD11c^{low}, CD317^{+}, Gr1^{+}) were identified using APC, Pacific Blue or Alexa Fluor 647 conjugated anti-CD11c (clone: N418), anti-CD11b-PECy7 (clone: M1/70), (eBioscience, San Diego, CA), anti-Gr1-Alexa Fluor 750 (clone: RB6-8C5) and anti-CD317-Alexa Fluor 488 (clone: 120g8). All pre-conjugated antibodies were purchased from eBioscience (San Diego, CA) with the exception of anti-CD317, a kind gift from Giorgio Trinchieri (National Cancer Institute, Fredericksburg, MD), which was conjugated to AF488 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Compensations of spectral overlaps were performed using the BD FACSDiva (BD Bioscience, San Jose, CA) and FlowJo (Treestar, Inc., Ashland, OR) analysis software.

**Determination of BAL GM-CSF Levels**

Lungs were lavaged three times with 1ml of cold Hanks’ balanced salt solution without calcium or magnesium (Mediatech Inc., Herndon, VA). The recovered lavage fluid (70 to 80%) was centrifuged (300 x g for 8 minutes) as previously described. Supernatants were removed and stored at -80°C until growth factor levels were assayed. GM-CSF levels were determined via ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's recommendations.
Generation of Bone Marrow Derived DCs (BMDDCs)

Bone marrow cells (1.5×10^7) were harvested from 6–8 week old A/J mice. Following harvest, red blood cells were lysed for 5 minutes in ACK lysis buffer. The remaining cells were cultured in media containing 5 ng/ml of recombinant mouse GM-CSF (PeproTech, Rocky Hill NJ). Fresh GM-CSF was added on day 3 and 6. By day 6 DC cultures are 95–98% myeloid in nature, expressing high levels of CD11c and CD11b. On day 7, cells were harvested and plated using a density of 1×10^6 cells/well. BMDDCs were exposed to 50 µg/ml AUB, RWG, ASP or media for 24 hours. Culture plates were centrifuged at 1600 RPM for 6 minutes and cell-free supernatants were obtained and stored at -80°C for the detection of T-cell skewing cytokines [IL-12p70, IL-12p40, IL-6, IL-1β, TGFβ, IL-10 (R&D Systems, Minneapolis, MN) and IL-23 p19/p40 (eBioscience, San Diego, CA)]. ELISA assays were preformed to determine cytokine levels according to the manufacturer's instructions.

Western Blot

BMDDCs (3×10^6) were serum starved for twenty-four hours prior to treatment with AUB (50 µg/ml) for 0, 15, 30, 45, 60 and 120 minutes. Cell lysates were harvested and resolved on NuPage® Novex Bis-Tris gels (Invitrogen, Carlsbad CA), as previously described, incubated with anti-IκB-α (C-21, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and signals were amplified and visualized using Amersham™ ECL Western Blotting Detection Reagents (Amersham Biosciences, Pittsburgh, PA).
**Electrophoretic Mobility Shift Assay (EMSA)**

BMDDCs \((1 \times 10^7)\) were plated in 100 mm\(^2\) tissue culture plates and treated with AUB \((50 \mu g/ml)\) for 90 minutes. Cell supernatants were removed, nuclear proteins extracted on ice and an EMSA was performed as previously described \(^{80, 81}\). Antibody binding of p65 (Rel A), p50 (NF-κB1) or Rel B to a radiolabeled oligonucleotide NF-κB probe (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was compared to unlabeled NF-κB or AP-1 probes.

**Isolation and Culture of Primary Tracheal Epithelial Cells**

Tracheas were surgically removed from the larynx and main bronchi under sterile conditions and placed in ice-cold Ham’s F12 medium containing penicillin \((100 \text{ U/ml})\) /streptomycin \((100 \mu g/ml)\) as previously described \(^{82}\). Connective tissue was carefully dissected away from the trachea under a stereomicroscope and cut open longitudinally. Tracheas were incubated in Ham’s F12 media containing 1 mg/ml pronase (Roche-Applied Science, Indianapolis, IN) overnight at 4 °C, after which cells were washed from fibrous layers and centrifuged at 350 x g for 10 minutes. The cells were then resuspended and cultured on Primaria\(^{\text{TM}}\) (Falcon, Becton Dickinson, Franklin Lakes, NJ) plates for a period of 3-4 hours under 5% CO\(_2\) at 37 °C to remove adherent fibroblasts. Non-adherent cells were centrifuged \((350 x g \text{ for 10 minutes})\) and resuspended in MTEC/Plus [MTEC: Dulbecco’s modification of Eagle’s medium and Ham’s F-12 in a 1:1 vol/vol mix (Mediatech, Hedron VA), 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 10 μg/ml insulin, 5 μg/ml transferrin, 0.1 μg/ml cholera toxin, 25 ng/ml epidermal growth factor,
30 μg/ml bovine pituitary extract, 5 % FBS, and freshly added 0.01 μM retinoic acid], then counted using a hemocytometer. The average yield of tracheal epithelial cells was 1 – 2 × 10⁵ cells/ trachea. Cell viability was greater than 90% as determined by trypan blue staining. 7.5 x 10⁴ cells were plated on transwell membranes (0.4 μM pores, Costar®, Corning® Life Sciences, Lowell, MA) coated with 0.1 mg/ml type-1 rat-tail collagen (BD Biosciences, Bedford, MA), and cultured under immersion conditions for 6 days. On day 6 epithelial cell confluence was determined by the observation of Trans Epithelial Electric Resistance measurements greater than 1000 ohms using the EVOM™ Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL).

Results

In vivo AUB exposure induces a Th2/Th17 cytokine profile that is associated with an increase in the percentage of DCs.

To determine the nature of the T-cell response to in vivo AUB challenge, we measured T-cell associated cytokine production in whole lung cell suspensions from mice exposed to PBS or AUB in vivo. It is important to note that although ex vivo restimulation is not a direct measure of in vivo cytokine production, unpublished studies in our laboratory show that the allergen-induced cytokine profile, as determined after whole lung cell restimulation, directly correlates with in vivo allergen-induced increases in whole lung cytokine mRNA expression. AUB exposure primed cells to generate a mixed Th2/Th17 cytokine response (IL-4, IL-5, IL-13, IL-17A) upon restimulation, with no effect on the production of the Th1 cytokine IFNγ (Figure 1A). As T-cell skewing and activation
requires the contribution of professional antigen presenting cells, we determined the effects of AUB exposure on the numbers of CD11c⁺ cells in the lung (Figures 1B-C). Exposure to AUB significantly increased the numbers and percentage of CD11c⁺ cells in the lung. Although such studies do not show the direct migration of DCs into the lung, we have shown that AUB exposure directly increased the presence of pulmonary CCL20 (Chapter 3), which supports the hypothesis that AUB exposure induces pulmonary DC recruitment.
Figure 1. AUB primes pulmonary cells to produce a mixed Th2/Th17 cytokine profile that is associated with an increase in CD11c$^+$ cells in mice. (A) Concanavalin A stimulated lung cell production of Th2 cytokines (IL-4, IL-5, IL-13), a Th17 cytokine (IL-17A) and a Th1 cytokine (IFN$\gamma$), 24 hours after the last of 3 AUB inoculations administered over 6 days. (B) Representative flow cytometry histogram of mouse pulmonary CD11c$^+$ cells before and after PBS or AUB challenge. (C) The percentage of pulmonary CD11c$^+$ cells. Data are representative of 2 individual experiments. N = 10 mice/group. Values are mean ± SEM. *p < 0.0001, †p < 0.05, versus PBS.
*In vivo* AUB exposure alters the balance of pulmonary mDCs and pDCs.

To test the hypothesis that AUB exposure results in the recruitment and activation of pulmonary DCs, we examined the impact of multiple AUB inoculations on the percentages of lung mDCs (CD11c\(^{hi}\), CD11b\(^{+}\), Gr1\(^{-}\)) and pDCs (CD11c\(^{low}\), CD317\(^{+}\), Gr1\(^{+}\)). Exposure to AUB significantly (p <0.0001) increased the absolute number and percentage (Figure 2 A-B) of mDCs in the total lung digest and significantly (p <0.05) reduced pulmonary pDC numbers and percentages (Figure 2A, C) as compared to PBS exposed controls. Taken together these results demonstrate that AUB exposure induces a preferential increase in the percentages of pulmonary mDCs with a reduction in pDCs.

To determine if AUB-exposure differentially activates pulmonary mDCs and pDCs, we assessed the percentage of cells expressing the costimulatory molecules CD80 and CD86 in the lungs of PBS- or AUB-inoculated mice. AUB exposure significantly increased the percentages of both cell types expressing CD80 (Figure 2D and E) and CD86 (Figure 2F and G) as compared to DCs analyzed from mice exposed to PBS. Taken together these studies show that *in vivo* AUB exposure activates, or induces the migration of activated mDCs and pDCs in the lung.
Figure 2. AUB recruits and activates lung DCs.

(A) Representative flow cytometric plots of mDCs (top panel, GR1⁻, CD11c\textsuperscript{high}, CD11b⁺) and pDCs (lower panel, GR1⁺, CD11c\textsuperscript{low}, CD317⁺) in the lung before and after AUB exposure. The percentage of mDCs (B) and pDCs (C) as well as the percentage of cells expressing the costimulatory molecules CD80 (D and E) and CD86 (F and G) among mDC (D and F) and pDC (E and G) populations were determined in the lung. Data are representative of two individual experiments. N = 7-8 mice per group in each experiment. Values are mean ± SEM. *p < 0.0001, †p < 0.05, versus PBS.
Figure 2.
**AUB exposed DCs exhibit a Th2/Th17 skewing cytokine profile**

As DC derived cytokines influence the nature of T-cell responses, we assessed the ability of AUB (50 µg/ml) to stimulate BMDDC T-cell skewing cytokine (IL-12p70, IL-12p40, IL-6, IL-1β, IL-23, TGFβ, IL-10) production in comparison to equivalent concentrations of the purified allergens *Aspergillus fumigatus* (ASP) and ragweed (RGW). At these concentrations, AUB was found to be a much more potent stimulator of IL-12p70, IL-12p40, IL-6, IL-1β and IL-23 (Figure 3A-E) than the allergens. On the other hand, AUB did not significantly increase TGFβ above baseline levels (Figure 3F). IL-10 levels remained below the assay detection limit (data not shown). Because the levels of IL-12p70 were substantially lower than those of IL-6, IL-1β and IL-23, these results suggest that AUB drives a cytokine pattern that would favor the differentiation of Th2/Th17 cells.
Figure 3. AUB exposure increases BMDDC T cell differentiating cytokine production

BMDDC production of IL-12p70 (A), IL-12p40 (B), IL-6 (C), IL-1β (D), IL-23 (E) and TGFβ (F) twenty-four hours after exposure to equivalent concentrations (50 µg/ml) of AUB, aspergillus (ASP) or ragweed (RGW). Values are mean ± SEM, N = 3 wells/treatment. *p < 0.0001, †p < 0.05, versus media control.
AUB induced DC cytokine production is MyD88 dependent

AUB is comprised of multiple biologically active substances (bacterial endotoxin, fungal components, transition metals, PAHs) that may activate DCs through a variety of pathways. As several of these components have been shown to activate the TLR family of PRRs, we sought to determine whether AUB could activate DCs via TLR pathways. To do this, we assessed the production of cytokines from DCs derived from MyD88−/− mice, which lack one of the primary signaling components for most TLRs \(^{54, 83, 84}\). Our results show that a deficiency in MyD88 strongly suppressed AUB-induced production of IL-12p70 (Figure 4A) and partially reduced IL-12p40, IL-6, IL-1β and IL-23 (Figure 4B-E). No effects were seen on TGFβ production (Figure 4F). These data indicate that MyD88 signaling plays at least a partial role in AUB-induced DC cytokine production.

The transcription factor NF-κB is induced by TLR activation and mediates TLR induced pro-inflammatory cytokine production. Because the DC cytokine production was partially TLR-dependent, we investigated the role of NF-κB in AUB-induced cytokine production. To this end, we first examined the ability of AUB to induce NF-κB nuclear translocation in BMDDCs. AUB induced a time-dependent degradation of IκBα (Figure 5A). In addition, using an EMSA we show that nuclear proteins from the lungs of AUB-exposed mice bind to a NF-κB oligonucleotide probe and the co-incubation with the NF-κB p50 protein induced a DNA binding complex supershift (Figure 5B). Therefore, in agreement with reports of PM-induced NF-κB activation in other cell types \(^{85}\), we show that AUB induces NF-κB activation in BMDDCs. To confirm that NF-κB signaling was involved in the production of DC cytokines, we inhibited NF-κB signaling prior to AUB exposure with the chemical inhibitor isohelenin (10 μM). We show that isohelenin
completely abrogated the production of the T-cell skewing cytokines IL-12p70, IL-6, IL-1β and IL-23 (Figure 5C, E-G) and partially reduced the production of the cytokines IL-12p40 (Figure 5D) and TGFβ (Figure 5F). These results indicate that AUB-induced DC cytokine production is triggered predominantly through an NF-κB-dependent signaling pathway. Because DC cytokine production was partially MyD88-dependent, our results suggest that another, as yet unidentified NF-κB-dependent pathway, must also contribute to AUB-induced DC cytokine production.
Figure 4. AUB-induced cytokine production is partially MyD88 dependent.

BMDC production of IL-12p70 (A), IL-12p40 (B), IL-6 (C), IL-1β (D), IL-23 (E) and TGFβ (F) 24 hours after exposure AUB (50 µg/ml) in MyD88−/− mice, as compared to their C57BL/6 controls (WT). Data are representative of 2 individual experiments. Values are mean ± SEM, N = 4 wells / treatment in each experiment. *p < 0.0001.
Figure 5. AUB-induced DC cytokine production is NF-κB dependent.

(A) Western blot and quantification of the time-dependent degradation of IκBα in BMDDCs after AUB (50 µg/ml) exposure. Data are graphed as the percentage of total IκBα, as compared to the untreated control. (B) NF-κB EMSA of nuclear extracts from AUB treated BMDDCs. Supershift analysis was performed with antibodies against p65, p50, c-Rel, RelB. Specificity for NF-κB is shown by competing with unlabeled AP-1.

BMDDC production of IL-12p70 (C), IL-12p40 (D), IL-6 (E), IL-1β (F), IL-23 (G) and TGFβ (H) 24 hours after AUB exposure (50 µg/ml) in the presence of the NF-κB inhibitor isohelein or the vehicle control (DMSO). Values are mean ± SEM, N = 3 wells / treatment. *p < 0.0001, †p < 0.05.
Figure 5.
AUB-induced a GM-CSF dependent shift in DC phenotype and activation

As GM-CSF is implicated in the selective expansion of mDC populations and inhibition of pDC expansion, we hypothesized that the AUB-induced pulmonary mDC/pDC imbalance (Figure 2 A-C) may result from increased pulmonary GM-CSF levels. To test this hypothesis, we first determined the kinetics of AUB-induced GM-CSF secretion in the lung. A/J mice were exposed to AUB or PBS and sacrificed 3 hours (3hr) and 24 hours (D1) after a single dose, or 24 hours after 3 challenges (D7), for the determination of BAL GM-CSF levels. AUB exposure induced a significant (p < 0.0001), early increase in GM-CSF that was not detected after multiple challenges (Figure 6A). The kinetics of GM-CSF production in the lung is consistent with a role for GM-CSF in the early expansion of pulmonary DCs.

To determine whether AUB induced GM-CSF production is responsible for changes in the lung mDC/pDC balance, we analyzed the DC subset phenotype following in vivo GM-CSF neutralization. Because GM-CSF production peaked early after a single AUB exposure (Figure 6A), antibodies against GM-CSF (MP1-22E9, 1mg/mouse), or isotype control antibodies were administered by intravenous (i.v.) injection 24 hours before and after the initial AUB inoculation. When compared to isotype controls, GM-CSF neutralization significantly (p <0.0001) reversed the AUB-driven increase in lung mDCs (Figure 6B) with no effect on pDCs (Figure 6C). Furthermore, GM-CSF neutralization decreased the percentage of mDCs expressing the costimulatory molecules CD80 and CD86 (Figure 6D, F), with no significant effects on the percentage of pDCs expressing costimulatory molecules (Figure 6E, G). These data indicate that AUB
exposure induced a GM-CSF dependent shift towards the recruitment and activation of pro-asthmatic pulmonary mDCs.
Figure 6. AUB-induced increases in lung mDCs are GM-CSF dependent

(A) GM-CSF production in the BAL of mice exposed to PBS or AUB for either 3 (3 hr) or 24 hours after a single exposure (D1), or 24 hours after 3 exposures (D7). The percentage of mDCs (B) and pDCs (C), as well as the percentage of cells expressing the costimulatory molecules CD80 (D and E) and CD86 (F and G) among mDC (D and F) and pDC (E and G) populations, were determined in the lung after administration of the IgG2a isotype (GL117) or anti-GM-CSF (MP1-22E9) antibodies. Values are mean ± SEM, N = 7-8 mice/group. *p < 0.0001, †p < 0.05, versus PBS.
Figure 6.
AUB-induced T-cell cytokine production is partially GM-CSF dependent

Although previous studies have shown that GM-CSF is important in allergen-induced Th2 cytokine production, the role of GM-CSF in regulating T-cell cytokine production to environmental PM has not been previously explored. To this end, we examined the effect of in vivo GM-CSF depletion on T-cell cytokine production in whole lung cell suspensions from mice exposed to PBS or AUB in vivo. Interestingly, we see that GM-CSF neutralization partially reduced the increases seen in the Th2 cytokines IL-4, IL-5 and IL-13 following AUB exposure (Figure 7A), while it almost completely abolished the production of the Th17 cytokines IL-17A and IL-17F (Figure 7B). In contrast, GM-CSF neutralization did not alter the levels of the Th1 cytokine, IFNγ (data not shown). Taken together, these results indicate AUB-induced GM-CSF production contributes to a selective increase in the percentages of pulmonary mDC populations, which prime naïve T cells to produce a mixed Th2/Th17 pulmonary cytokine profile.
Figure 7. AUB induction of a mixed pulmonary Th2/Th17 cytokine profile is GM-CSF-dependent

(A) Th2 (IL-4, IL-5, IL-13) and (B) Th17 (IL-17A, IL-17F) cytokine production from concanavalin A restimulated lung cells from mice exposed to three AUB challenges (Day 7) and treated with either an anti-GM-CSF mAb (2 mg) or an isotype control mAb (2 mg). Values are mean ± SEM, N = 15-16 mice. *p < 0.0001, †p < 0.05.
AUB-induced mTEC GM-CSF production is oxidative stress dependent

As the airway epithelium is the initial site of contact with inhaled pollutants, we hypothesized that the increase in pulmonary GM-CSF observed after AUB exposure may be derived from the airway epithelium. Furthermore, because previous reports indicate that PM may regulate GM-CSF production through oxidative stress pathways, we treated AUB-induced primary mouse epithelial cells with the general antioxidant, NAC (10mM) and examined GM-CSF protein levels. We observed that AUB-induced GM-CSF is significantly reduced when cells are exposed in the presence of NAC, indicating that AUB-induced GM-CSF secretion is dependent upon oxidative stress (Figure 8). NAC did not significantly impact the baseline levels of GM-CSF (data not shown).
**Figure 8.** AUB induced epithelial cell GM-CSF secretion is oxidative stress dependent. *In vitro* mTEC GM-CSF protein levels in supernatants 24 hours after exposure to AUB (50 µg/ml) in the presence or absence of the antioxidant NAC (10 mM). Values are mean ± SEM, N = 3-12 replicates/group. *p < 0.0001.
Discussion

As we have previously found that in mice AUB exposure resulted in the development of T cell-dependent AHR, and that T cell responses are dependent upon signals provided by innate immune cells such as DCs, we sought to determine the effects of ambient PM on the recruitment, phenotype, and function of pulmonary DCs \textit{in vivo}. We found that \textit{in vivo} AUB exposure resulted in a dramatic increase in the percentages of DCs in the lungs of mice. Moreover, these newly expanded DC populations are highly activated as evidenced by an increase in the percentage of DCs expressing the costimulatory molecules, CD80 and CD86. Moreover, there is a selective recruitment/expansion of mDCs, while the percentages of tolerogenic pDCs are actually reduced following \textit{in vivo} AUB exposure. Because previous studies in allergen models have shown that antigen presentation by mDCs is necessary and sufficient to drive allergic airway immune responses, while pDCs promote immune tolerance \textsuperscript{19, 65, 86, 87}, our results suggest that AUB exposure skews the DC balance towards a more immunogenic phenotype. Of note, our finding that the percentages of pDCs are actually reduced appears to be unique to AUB exposure as the numbers of pDCs in allergen models generally increase, albeit to a lesser extent than do mDCs.

The importance of DCs in instructing naïve T cell differentiation is well established and this instruction is thought to be largely dependent upon local cytokine secretion at the immunological synapse. Therefore, we examined the effects of AUB on DC cytokine production. Because many of the DC cytokines of interest (IL-6, TGFβ) are also made by multiple other cell types in the lung, limiting our ability to differentiate the direct effect of AUB on DCs as opposed to secondary DC activation from surrounding
PM-activated cells, we utilized an in vitro model of GM-CSF derived BMDDCs to determine the direct effects of AUB on DC cytokine production. BMDDCs cultured in this manner are predominantly myeloid (CD11c\textsuperscript{high}, CD11b\textsuperscript{+}) in nature, mimicking the skewing we see in vivo with AUB. Using this system, we observed that AUB exposure significantly increased DC production of cytokines necessary for the skewing of naïve T cells toward Th1 (IL-12p70, IL-12p40), Th2 (IL-6) and Th17 (IL-6, IL-1β, IL-23, IL-12p40) differentiation. Although the p40 subunit, which is shared between IL-12 and IL-23, was highly induced by AUB, the levels of IL-12p70 relative to those of IL-23 (1:10), suggest that p40 preferentially partners with p19 to drive IL-23 production in this instance. Although we do not see increased production of TGF-β, it is important to note that substantial levels of this cytokine were measured at baseline. The constitutive levels of TGF-β are likely sufficient to combine with the elevated levels of IL-6 and IL-1β observed after AUB exposure, to drive Th17 differentiation. In summary, although previous studies have shown that mDCs activated by PM produce a pattern of cytokines that promote both Th1 and Th2 differentiation \cite{70}, our studies have uncovered the previously unappreciated ability of AUB to also induce DC production of a pattern of cytokines favoring Th17 differentiation.

Classically, Th2 cytokines are thought to be the major effector molecules driving the development of allergic asthma in both humans and mice \cite{3,88}. Surprisingly, our results suggest that AUB induces the production of a mixed Th2/Th17 cytokine profile in the lung. The involvement of a mixed Th2/Th17 cytokine profile in asthma pathogenesis is supported by human studies showing increased production of IL-17A in the lungs of asthmatics, particularly those with more severe disease \cite{11,89}. For example, IL-17A levels
in induced sputum correlate with bronchial hyperactivity in asthmatics and increased levels of IL-17 were detected in bronchial biopsies of individuals with moderate to severe asthma, as compared to mild asthmatics or healthy controls. Furthermore, aerosolized organic dust induced IL-17A production in human BAL lymphocytes. In mouse models, IL-17 played a significant role in ozone-induced and allergen-driven AHR, as well as Th2 cytokine production. IL-17 also synergized with Th2 cells to enhance AHR over that induced by Th2 cells alone. Yet, the role of IL-17 in asthma remains controversial because previous reports indicate that the antibody depletion of IL-17 exacerbated allergen-induced bronchial Th2 cytokine production with no impact on AHR and that exogenous IL-17 reduced allergen-driven AHR. As we observed that AUB exposure induced the production of IL-17A and IL-17F, we speculate that Th17 cells play a pathogenic role in our model. However, this awaits formal demonstration.

As GM-CSF has previously been shown to mediate mDC differentiation and expansion as well as drive Th2 and Th17 responses, we examined the contribution of GM-CSF to AUB-induced responses in vivo. Our studies provide several lines of evidence that GM-CSF plays an important role in the responses of the lung to AUB exposure. First, we demonstrated that in vivo exposure of mice to AUB induced the production of GM-CSF as early as three hours post-exposure, suggesting that GM-CSF production preceded the recruitment of DCs to the lung. Secondly, the in vivo antibody depletion of GM-CSF suppressed AUB-induced increases in the percentages of activated pulmonary mDCs. Lastly, blockade of GM-CSF suppressed the AUB-induced increases in the levels of both Th2 and Th17 cytokines, but does not have a significant effect on the levels of the Th1 cytokine IFN-γ (data not shown). Interestingly, GM-CSF blockade
completely suppressed IL-17A production, but only partially inhibited Th2 cytokine 
production, suggesting that GM-CSF may play a more important role in regulating Th17-
inducing factors in the lung. Our results are supported by previous reports by 
Sondereggar et al. 95 who show that the administration of exogenous GM-CSF 
significantly increased DC production of the Th17-skewing cytokines IL-6, IL-12p40, 
and the transcription of IL-23p19, while GM-CSF deficient mice have impaired APC IL-
6 and IL-23 production and Th17 cellular differentiation. Although we did not directly 
assess the role of GM-CSF in AUB-induced AHR and airway inflammation in our study, 
Ohta et al. 32 have previously shown that in vivo depletion of GM-CSF reduced diesel 
exhaust-induced AHR. Although we have not ruled out the production of GM-CSF by 
other cells in the lung, our studies with primary mouse tracheal epithelial cells 
demonstrated that GM-CSF is produced following treatment with AUB. These results 
suggest that the airway epithelial cells are a primary source of GM-CSF in the lung 
following AUB exposure. This is consistent with studies by Bleck et al. 96 which showed 
that PM-conditioned epithelial cells induce immature monocyte derived DC maturation 
and T-cell stimulating capacity in a GM-CSF dependent manner. Taken together, these 
studies suggest that epithelial cells rapidly produce GM-CSF following AUB exposure, 
which in turn serves to activate mDCs to drive Th2/Th17-mediated immune responses in 
the lung.

Numerous studies indicate that the biological effects of PM may be the result of 
PM-induced oxidative stress that is predominantly mediated through transition metals and 
PAHs 97, 98. Indeed, previous studies have shown that AUB-induced oxidative stress is 
important in driving DC activation and cytokine production 71. However, the exposure of
DC’s to PM in the presence of a thiol antioxidant N-acetyl-cysteine only partially reduced AUB-induced cytokine production, indicating that non-oxidant dependent pathways are also involved.

It is well recognized that “danger signals” conveyed through PRRs are required for DC cytokine production. Because AUB contains low levels of bacterial endotoxin and fungal components, we determined whether AUB induction of DC cytokine production was dependent upon TLR signaling by examining the effects of AUB on BMDCs from MyD88 deficient mice. We observed that IL-12p70 production was completely abolished in DCs from MyD88 deficient mice, whereas IL-12p40, IL-6, IL-1β and IL-23 were only partially suppressed in MyD88 deficient mice. These results suggest that IL-12p70 production is completely dependent upon TLR (1, 2, 4, 6-9, 11) signaling, whereas Th17-skewing cytokine production is either dependent upon the TLR4 MyD88 independent pathway or a TLR3 dependent pathway. However, it is unlikely to be TLR3-mediated, as the TLR3 ligand is thought to be double-stranded viral RNA. Alternatively, AUB-induction of Th2/Th17-skewing cytokines may be due to signals conveyed through other non-TLR PRRs such as dectin family members, which are activated by fungal antigens. Lastly, MyD88-independent signals may be mediated through AUB activation of oxidative stress pathways. The near complete abrogation of AUB-induced DC cytokine production after NF-κB inhibition, despite only a partial reduction of cytokine production in MyD88 deficient mice, supports the conclusion that AUB exposure induces DC cytokine production through multiple pathways culminating in NF-κB activation. As these studies did not evaluate the in vivo role of NF-κB or MyD88, further studies are
still required to elucidate the role of these pathways in vivo in AUB-induced DC and T cell activation.

In summary, our studies provide new insights into the mechanisms by which exposure to environmental particles drive the development of allergic inflammation. Specifically, our data supports a model in which AUB induces the production of GM-CSF, presumably by the airway epithelium in an oxidant dependent manner, which sets into motion a cascade of events which drives the preferential recruitment, and activation of mDCs expressing high levels of co-stimulatory molecules, and producing a Th2/Th17-promoting cytokine profile (IL-6, IL-23, IL-1β). Moreover, AUB activation of these innate immune pathways, occurs through both MyD88-dependent and independent pathways involving downstream NF-κB-dependent signaling pathways. Taken together, these studies provide a plausible sequence of events by which exposure to inhaled environmental particles leads to the development of adverse health effects.
References


Particulate Matter and House Dust Mite Induce Unique and Overlapping Gene Expression Patterns in Airway Epithelial Cells
Abstract

**Background.** We have shown that exposure to PM induces the recruitment and activation of pulmonary DCs. Therefore we sought to begin to determine the mechanism(s) by which PM initiates DC recruitment and activation at the airway surface.

**Objective:** To this end, we compared the effects of exposure to either PM (AUB, 50 µg/ml) or house dust mite (HDM, 100 µg/ml) on the transcriptional profiles of mouse tracheal epithelial cells utilizing the Affymetrix GeneChip mouse genome 430 2.0 array.

**Results.** Our analysis revealed that 155 transcripts out of the approximately 45,000 probes on the array were modulated (<0.67 or >1.5 fold) by both exposures, 576 transcripts were uniquely regulated by AUB, and 100 were uniquely induced by HDM. Similarly expressed genes fell into several major categories including chemokines (Ccl20), cytokines (Tnf), oxidative stress (Ncf4), cellular metabolism (Vnn1), and innate immune mediators (C2, Cfb). Of particular interest were genes in the oxidative stress pathways. As our data implicated oxidative stress and, more specifically, an increase in the expression of NADPH oxidase genes, in AUB-induced mTEC activation, we used pharmacological inhibition of NADPH oxidase pathways and mice lacking NADPH oxidase signaling to demonstrate a role for NADPH oxidase activity in AUB-induced CCL20 production. Finally, we show that AUB-exposed epithelial cells can indeed condition DCs to preferentially induce the pro-asthmatic cytokine IL-6. **Conclusion.** Our results show that the airborne pollutant, AUB, may initiate allergic inflammation at the mucosal surface through pathways that are similar to those induced by the common aeroallergen, HDM. Further study of these candidate genes will provide valuable insight.
into the gene-environment interactions driving susceptibility to AHR and asthma and may lead to the development of new therapies for asthma.

**Introduction**

Asthma is a chronic inflammatory disease of the airways that has been on the rise in recent decades. Although the factors driving this increase are not well understood, exposure to ambient PM has been consistently associated with increases in asthma prevalence and morbidity.\(^1\)\(^,\)\(^2\) In support of this association, we\(^3\)\(^,\)\(^4\) and others\(^5\) have previously reported that exposure of mice to ambient PM collected in urban Baltimore directly induces the major pathophysiological features of asthma including AHR, eosinophilic inflammation, and mucus hypersecretion. Similar to what has been observed in allergen exposure models\(^6\)\(^-\)\(^8\), we have shown that the development of AHR in response to AUB exposure is dependent upon T cell differentiation and activation (Chapter 4). Moreover, we have demonstrated that the T cell response is likely driven by mDCs recruited to the airways following AUB exposure (Chapter 5). However, the factors produced in the local lung environment that drive the recruitment and activation of DCs following allergen and pollutant exposures are not well understood.

Recent studies suggest that airway epithelial cells, which are the point of initial contact with inhaled substances, may play an important role in the initiation of immune responses through the production of chemokines and cytokines capable of recruiting and activating immune cells such as DCs and T cells\(^9\)\(^-\)\(^23\). Both allergens and pollutants have been shown to induce the production of a variety of inflammatory mediators (IL-6, IL-1β, TNF) and chemokines (CCL20, KC) from the airway epithelium that may potentially
recruit pulmonary DCs. Indeed, we have recently shown that HDM exposure induces the production of the immature DC chemokine, CCL20, by human airway epithelial cells.\textsuperscript{19} Likewise, Reibman et al.\textsuperscript{21} have shown that ambient PM induces CCL20 production in human bronchial epithelial cells. Although these studies suggest that PM may initiate allergic inflammation through mechanisms similar to those of allergens, the exact factors underlying the initiation of immune responses at the airway surface by these two triggers of allergic inflammation are not well understood. Thus, to gain further insight into the mechanisms underlying the ability of AUB to initiate innate and adaptive immune responses at the airway surface, we compared the effects of AUB and HDM exposure on gene expression patterns in primary mouse tracheal epithelial cells utilizing a comprehensive Affymetrix Microarray approach.

**Materials and Methods**

**Mice**

Male and female A/J mice, Cybb\textsuperscript{tm1Din/J} (gp91\textsuperscript{phox-/-}), C57BL/6 mice (6 to 10 weeks old, Jackson Laboratories, Bar Harbor, ME) and MyD88 deficient (MyD88\textsuperscript{-/-})\textsuperscript{24} mice were housed in an environmentally controlled, specific pathogen-free facility at Cincinnati Children’s Hospital Medical Center. The mice received access to food and water ad libitum. Mice were handled in accordance with the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee.
PM and Allergen Preparations.

Ambient PM was collected in Baltimore as previously described. Briefly, collection of PM was conducted in urban Baltimore using a high volume cyclone that was continuously operated for periods of three months. The cyclone was operated at a flow rate of 0.6 \( \text{m}^3/\text{minute} \), which allowed for a theoretical size cutoff of 0.85 \( \mu \text{m} \) aerodynamic diameter. PM size determination was performed using light microscopy and the median particle size was determined to be 1.78 \( \mu \text{m} \) with a standard deviation of 2.21 \( \mu \text{m} \). Endotoxin levels in PM were determined using the Limulus amebocyte lysate (LAL) assay, and averaged 10 EU/ml endotoxin. Microscopic examination revealed the presence of fungal hyphae. HDM was purchased from Greer Laboratories (Lenoir, NC). NAC (10 mM) and apocynin (100 \( \mu \text{M}, 1 \text{mM} \)) were purchased from Sigma (St. Louis, MO).

Isolation and Culture of Primary Tracheal Epithelial Cells

Tracheas were surgically removed from the larynx and main bronchi under sterile conditions and placed in ice-cold Ham’s F12 medium containing penicillin (100 U/ml) and streptomycin (100 \( \mu \text{g/ml} \)), as previously described. Connective tissue was carefully dissected away from the trachea under a stereomicroscope, after which the trachea was cut open longitudinally. Tracheas were incubated in Ham’s F12 media containing 1 mg/ml pronase (Roche-Applied Science, Indianapolis, IN) overnight at 4 °C, following which cells were washed from fibrous layers and centrifuged at 350 x \( g \) for 10 minutes. The cells were then resuspended and cultured on Primaria™ (Falcon, Becton Dickinson, Franklin Lakes, NJ) plates for a period of 3- 4 hours under 5% CO\(_2\) at 37 °C to remove adherent fibroblasts. Non-adherent cells were centrifuged (350 x \( g \) for 10 minutes) and
resuspended in MTEC/Plus [MTEC: Dulbecco’s modification of Eagle’s medium and Ham’s F-12 in a 1:1 vol/vol mix (Mediatech, Hedron VA), 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, plus 10 µg/ml insulin, 5 µg/ml transferrin, 0.1 µg/ml cholera toxin, 25 ng/ml epidermal growth factor, 30 µg/ml bovine pituitary extract, 5% FBS, and freshly added 0.01 µM retinoic acid], then counted using a hemocytometer. The average yield of tracheal epithelial cells was 1 – 2 × 10^5 cells / trachea. Cell viability was greater than 90% as determined by trypan blue staining. 7.5 x 10^4 cells were plated on transwell membranes (0.4 µM pores, Costar®, Corning® Life Sciences, Lowell, MA) coated with 0.1 mg/ml type-1 rat-tail collagen (BD Biosciences, Bedford, MA), and cultured under immersion conditions for 6 days. On day 6 or upon reaching confluence, as determined by the observation of Trans Epithelial Electric Resistance measurements greater than 1000 ohms using the EVOM™ Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL), cells were treated on the apical layer with AUB (50 µg/ml) or HDM (100 µg/ml) in a volume of 0.5 ml for 24 hours.

**RNA Extraction and Microarray Analysis.**

Total RNA was extracted with the TRIZol® (Invitrogen, Carlsbad, CA) reagent according to the manufacturer’s recommendations, resuspended in diethylpyrocarbonate-treated water, then purified using the RNeasy® mini kit (Quiagen, Valencia, CA) and quantified spectrophotometrically. Double-stranded cDNA was synthesized from 20 - 50 nanograms of total RNA. The Ovation RNA Amplification System Version 2.0 (Nugen) was used to prepare target cDNA that was then fragmented and biotin labeled using the
FL-Ovation cDNA Biotin Module V2 (Nugen). Complementary DNA was hybridized to Affymetrix GeneChip® mouse genome 430 2.0 arrays containing 45,101 oligonucleotide probes in the GeneChip Hybridization Oven 640 (Affymetrix) according to Affymetrix protocols, processed by the Affymetrix GeneChip Fluidics Station 450, and scanned with an Affymetrix GeneChip Scanner 3000 7G. Overall microarray quality was determined by taking into consideration the percentage of “present” internal control genes, GAPDH and β-actin, the 3’/5’ signal ratio for control genes and the average background signal, as recommended.

Analysis of the gene expression profiles for each sample was conducted using the GeneSpring GX software (Agilent Technologies). The identification of genes altered in response to AUB and HDM was determined based on the expression levels of genes. With the 45,101 probe sets on the MOE 430 2.0 array a two-pronged statistical approach was used to analyze the microarray data comparisons of PBS, AUB and HDM including: 1) the robust multiarray average (RMA) method that utilizes only perfect match information and 2) the Benjamini-Hochberg False Discovery rate multiple testing correction.

Genes that were statistically different after exposure were determined using an ANOVA, with a p-value cutoff of 0.05, between either AUB or HDM treated cells and PBS controls.

**Validation of Gene Expression Patterns by RT-PCR**

To confirm the microarray results, the expression of 8 representative genes from the several major gene categories was measured by RT-PCR in epithelial cell samples
from the RNA not used for the microarray. Briefly, RNA was isolated as described above and treated with 1 unit/µl DNase I (Invitrogen, Carlsbad, CA) at 65°C for 5 minutes. Reverse transcription was carried out on 1 µg of RNA in a 20 µl reaction containing 1X reverse transcriptase buffer, 10mM dNTP, 0.1M DTT, 40 units/µl of RNase out (Invitrogen, Carlsbad, CA), and 200 units of Superscript II Reverse transcriptase. RT-PCR reactions were prepared using the iQ™ SYBR® green Supermix (Bio-RAD, Hercules, CA) and were analyzed on the iCycler (Bio-RAD, Hercules, CA). RT-PCR primer sequences were obtained from the qPrimerDepot, quantitative PCR primer database (http://mouseprimerdepot.nci.nih.gov/), or using the online PrimerBank 26, 27. Sample ratios were normalized to the expression of the housekeeping gene β-actin.

**Generation of Bone Marrow Derived DCs (BMDDCs)**

Bone marrow cells (1.5×10^7) were harvested from 6 – 8 week old A/J mice. Following harvest, red blood cells were lysed for 5 minutes in ACK lysis buffer. The remaining cells were cultured in 5 ng/ml mouse GM-CSF (PeproTech, Rocky Hill NJ). Fresh GM-CSF was added on day 3 and 6. By day 6 DC cultures are 95 – 98% myeloid in nature, expressing high levels of CD11c and CD11b. On day 7, cells were harvested and plated at a density of 1×10^6 cells/well. BMDDCs were exposed to 50 µg/ml AUB for 24 hours. Culture plates were centrifuged at 1600 RPM for 6 minutes and cell-free supernatants were obtained and stored at -80°C for the detection of the T-cell skewing cytokines IL-6 and IL-12p40 (R&D Systems, Minneapolis, MN). ELISAs were performed to determine cytokine levels according to the manufacturer's instructions.
Results

Comparison of PM and allergen-induced gene expression patterns

To determine the commonalities between PM and allergen-induced epithelial cell gene expression, we compared the gene expression patterns of tracheal epithelial cells exposed to AUB (50 µg/ml) or HDM (100 µg/ml) for 24 hours using a microarray approach. An overall analysis of variance that included both AUB and HDM treated cells revealed that 3391 sequence tags were significantly altered (<0.67 or >1.5 fold) out of the approximate 45,000 gene probes on the GeneChip mouse genome 430 2.0 array. From this list we see that 731 probes were differentially expressed by a factor of 1.5 fold in AUB exposed cells in comparison to normalized untreated controls, whereas 255 probes were modulated by HDM. Using Venn analysis of these independent lists, we observe that 155 probes were altered by both AUB and HDM treatment (Figure 1A-B), 576 genes were uniquely altered by AUB (Figure 1A) and 100 were uniquely altered by HDM (Figure 1A). Hierarchical clustering of the overlapping genes revealed that the majority of genes were altered in a similar manner between both exposures, however in some cases AUB and HDM induced directionally different changes in gene expression.
Figure 1. Change in mouse tracheal epithelial cell gene expression after pollutant (AUB, 50 µg/ml) or allergen (HDM, 100 µg/ml) treatment. (A) Venn analysis of changes in gene expression showed that 155 genes are altered after both exposures, 576 genes are uniquely altered after AUB exposure and 100 are uniquely altered after HDM exposure. (B) Hierarchical clustering of gene transcriptome changes after both AUB and HDM exposure as compared to untreated control groups.
Patterns of gene expression between AUB and HDM

Using current asthma literature and the gene ontology classification system provided in the analysis software, we separated genes of interest into 11 categories (Table 1). To further eliminate non-specific changes only genes that were altered by a factor of two were categorized. Gene families altered by both AUB and HDM included those involved with inflammation (Saa3, C3, Cfb, Chi3l1), chemokines (CCL20), cytokines (Tnf), regulators of peptidase activity (Serpin3g), solute carriers (Slc5a1) and those involved in carbohydrate metabolism (Lzp-s). Of particular interest we observed that genes involved in cellular metabolism (Vnn1, Vnn3) and NADPH oxidase activity (Noxo1, Ncf4) were highly induced. We show that both exposures decreased the expression of structural genes such as cadherin (Cdh17), which may reflect the loss of epithelial cell barrier function. Interestingly, AUB upregulated the expression of Tgfbi, yet HDM exposure resulted in a suppression of the expression of Tgfbi.

In addition to genes highly regulated by both exposures, we observed unique AUB- or HDM-induced changes in gene expression. The genes most highly increased among those uniquely altered by AUB (Table 2) were categorized as epithelial cell products (Muc4), purinergic receptors (P2ry6), apoptotic genes (Ank2), peptidases (Klk10), cell signaling and communication (Trim30), oxidative stress (Cyp1b1), structural genes (Krt6a), cell metabolism (Acab), ion transport (Slcola5) and ion binding (Anxa10). Consistent with the decrease in Cdh17 seen after both exposures, we see a pattern of AUB-induced suppression of structural genes (actins, cadherin, procollagens, fibulins, myosin, transgelin) suggesting that AUB may alter the phenotype of airway epithelial cells.
On the other hand, the gene expression most highly induced by HDM exposure (Table 3) included a structural gene (Cldn1), genes involved in cytokine signaling (Ili203, Csf1) and a gene involved in cellular metabolism (Megf10). Moreover, unique to HDM we observed a significant decrease in surfactant protein (Sftpb) and aldehyde dehydrogenase (Aldh1a7) gene expression.
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## TABLE 1. (Cont’d) Shared AUB and HDM-induced gene expression patterns in mouse tracheal epithelial cells

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Values are mean fold changes as compared to untreated controls. N = 2 independent samples/group. Differences in gene expression were determined by ANOVA (p < 0.05) with the Benjamini-Hochberg False Discovery rate multiple testing correction.
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Values are mean fold changes as compared to untreated controls. N = 2 independent samples/group. Differences in gene expression were determined by ANOVA (p < 0.05) with the Benjamini and Hochberg False Discovery Rate multiple testing correction.
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Values are mean fold changes as compared to untreated controls. N = 2 independent samples / group. Differences in gene expression were determined by ANOVA (p < 0.05) with the Benjamini-Hochberg False Discovery rate multiple testing correction.
Validation of AUB- and HDM-induced changes in gene expression

In order to determine the validity of the changes in gene expression we observed following AUB and HDM treatment of epithelial cells on the Affymetrix array, we determined the expression of genes from multiple categories using real-time PCR analysis. Among those genes that overlapped between AUB and HDM, we determined the expression levels of the inflammatory markers C2, C3 and Saa3 (Figure 2A- C), Chi3l1 (Figure 2D), Ncf4 (Figure 2E) and Vnn1 (Figure 2F). In addition we validated the gene expression pattern of genes uniquely expressed by AUB (Cyp1b1, Figure 2G) and HDM (Csf1, Figure 2H). We observe that both AUB and HDM increased the expression of C2, C3 Saa3, Chi3l1, Ncf4 and Vnn1 as compared to untreated controls. We confirmed that AUB uniquely increased the expression of Cyp1b1, whereas HDM uniquely increased the expression of Csf1. In addition, we examined protein levels of the immature DC chemokine CCL20 in epithelial cells exposed to either AUB (50 µg/ml) or HDM (100 µg/ml). As we observed on the array, both AUB and HDM induced significant increases in CCL20 levels as compared to untreated controls (*p <0.0001, Figure 3).
Figure 2. Validation of genes altered by AUB or HDM treatment of mouse epithelial cells. RT-PCR analysis showing C2 (A), C3 (B), Saa3 (C), Chi3l1 (D), Ncf4 (E), Vnn1 (F), Cyp1b1 (G) and Csf1 (H) gene expression from mTECs treated with AUB (black bars) or HDM (grey bars) in comparison to untreated controls (white bars). (A-G) Genes chosen for validation were taken from multiple subsets in gene lists showing AUB and HDM overlapping gene expression (A-G), gene expression uniquely altered by AUB (G) and gene expression uniquely altered by HDM (H). Values are mean ± SEM, N = 2-3 replicates/group. *p < 0.0001, †p < 0.05.
Figure 3. Pollutant and allergen exposures stimulate mTEC secretion of CCL20.

CCL20 protein levels in mTEC supernatants after a 24 hour *in vitro* treatment with AUB (50 µg/ml) or HDM (100 µg/ml). Values are mean ± SEM, N = 3 replicates/group. *p < 0.0001, versus untreated controls.
AUB-induced mTEC CCL20 production is oxidative stress dependent

Because the genes most highly upregulated by both HDM and AUB were members of oxidative stress pathways (Noxo1, Ncf4, Cybb), we hypothesized that the induction of CCL20 may be oxidative stress dependent. To test this hypothesis, we treated AUB-induced primary mouse epithelial cells with the general antioxidant, NAC (10 mM) and examined CCL20 protein levels. We observed that AUB-induced CCL20 is significantly reduced when cells are exposed in the presence NAC, indicating that AUB-induced CCL20 secretion is dependent upon oxidative stress (Figure 4A). Of particular interest, we observed that AUB induced significant expression of genes in the NADPH oxidase family (Cybb, Ncf4). For this reason, we sought to establish a role for AUB-induced NADPH oxidase activation in mTEC CCL20 production. The increases in CCL20 levels seen after AUB were reduced in a dose dependent manner when the cells were treated with the NADPH oxidase inhibitor apocynin (100 µM, 1 mM, Figure 4B). As apocynin is a non-specific inhibitor of NADPH oxidase activity, we sought to confirm the role of NADPH oxidase in CCL20 production. To this end, we examined CCL20 production in mTECs derived from mice lacking NADPH oxidase activity (gp91phox/−). Consistent with the pharmacological blockade experiments, we observed that AUB-stimulated CCL20 levels were significantly reduced in the gp91phox/− cells when compared to cells from wildtype controls (Figure 4C). Taken together these results demonstrate that AUB induces CCL20 production at the airway surface through a novel NADPH oxidase pathway.
Figure 4. AUB induced epithelial cell CCL20 secretion is oxidative stress dependent.

(A) **In vitro** mTEC CCL20 protein levels in cell supernatants 24 hours after exposure to AUB (50 µg/ml) in the presence or absence of the antioxidant NAC (10 mM). (B) AUB-induced CCL20 protein levels in the presence or absence of the NAPDH oxidase inhibitor, apocynin (100 µM, 1mM). (C) CCL20 production from mTECs derived from C57BL/6 (WT) mice or those lacking NADPH oxidase activity (gp91phox−/−). Values are mean ± SEM, N = 3-12 replicates/group. *p < 0.0001, †p <0.05.
AUB-stimulated epithelial cell supernatants activate DCs

Because it has been suggested that factors produced in the local lung environment regulate the activation and cytokine producing ability of DCs recruited to the airway wall, we asked whether AUB-exposed epithelial cells are able to modulate DC function. To explore this hypothesis, we examined the ability of mTEC supernatants exposed to AUB, in the presence or absence of NAC, to induce BMDDC production of IL-6 and IL-12p40. Interestingly, we find that AUB-treated mTEC supernatants significantly induced the production of the Th2/Th17 skewing cytokine, IL-6, in non-stimulated BMDDC (Figure 5A, p < 0.0001) as compared to supernatants from untreated epithelial cells. Moreover, the mTEC-stimulated IL-6 production was inhibited when AUB-stimulated epithelial cells were pre-treated with NAC. No changes were seen in the BMDDC levels of IL-12p40 after stimulation with supernatants from AUB treated mTECs as compared to untreated mTEC controls (Figure 5B). These results suggest that AUB induced the release of, as yet undefined mediators, by the epithelium, which skew DC cytokine production towards a pattern favoring either Th2/Th17 differentiation through an oxidative-stress dependent manner.
Figure 5. AUB-exposed mTECs modulate DC production of T-cell skewing cytokines through an oxidative stress-dependent mechanism. (A) IL-6 or (B) IL-12p40 production from bone marrow derived DCs exposed to supernatants taken from mTECs after exposure to AUB (50 µg/ml) in the absence (black bars) or presence (grey bars) of the antioxidant NAC (10 mM). Values are mean ± SEM, N = 4 replicates/group. *p < 0.0001, †p < 0.05.
Discussion

To begin to identify the pathways that may underlie the ability of pollutants to recruit DCs and activate T cells, thereby inducing experimental asthma, we compared gene expression patterns induced by the real-world pollutant AUB and by the allergen HDM in primary mouse epithelial cells utilizing an Affymetrix gene-profiling approach. We found that AUB and HDM induced both unique and overlapping gene expression patterns in mouse epithelial cells. Transcripts present in this group included those previously associated with innate pulmonary defense and asthma. Specifically, several components of the complement activation pathway (Cfb, C2, C3) were upregulated by both AUB and HDM. Our findings are consistent with previous studies indicating that susceptibility to PM- and allergen-induced AHR is dependent on the complement factor 3. Indeed, airborne pollutants such as ozone, cigarette smoke and organic dust activate the complement pathway and the anaphylatoxin C3a has been described to promote a pulmonary Th2 phenotype.

In accordance with our previous reports showing that AUB exposure induces the recruitment of neutrophils (Chapters 3-4), we observed that both AUB and HDM induced the expression of two chemokines (Cxcl1 and Cxcl5) known to be important in neutrophil recruitment. In addition, previous reports support a role for these chemokines in asthma as Cxcl1/Cxcl5 gene expression is increased in animal models after primary allergen challenge, Cxcl5 expression is increased after exposure to the inhaled pollutant ozone and pulmonary Cxcl1 expression is increased by inhaled PM. Taken together these data suggest that the induction of these chemokines by AUB in airway epithelial cells is important in the recruitment of neutrophils to the airway mucosa.
Among those genes uniquely induced by AUB was the gene encoding the cytochrome P450 enzyme Cyp1b1, which metabolizes PAHs. These findings are consistent with previous reports that indicated that urban dust increases production of the cytochrome P450 enzymes (CYP1B1, CYP1A1)\(^{42}\). Furthermore recent reports indicate that a genetic polymorphism in CYP1B1 (CYP1B1 variant, V432L), which may potentially increase CYP1B1 activity\(^{43}\), is associated with asthma in a Russian cohort\(^{44}\). Taken together these reports indicate that PM may initiate allergic airway responses through the activation of PAHs.

In addition to upregulating epithelial cell genes, AUB also significantly reduced the expression of several genes including aquaporin1 (Aqp1), a transmembrane water channel. Our finding that AUB reduced the expression of Aqp1 is consistent with previous reports that the expression of several aquaporins (Aqp1, Aqp4, Aqp5) are reduced after antigen (OVA) sensitization and challenge\(^{45}\). Moreover, mice that are deficient in aquaporin-5 exhibited increased ACh-induced bronchoconstriction\(^{46}\).

Of particular interest is the identification of a novel family of genes induced by both AUB and HDM in epithelial cells. We found that both stimuli significantly elevated the expression levels of the Vnn1 and Vnn3 genes in epithelial cells. To our knowledge, this gene family has not been previously associated with susceptibility to PM- or allergen-driven AHR. Vanin genes encode a pantetheinase involved in the production of cysteamine\(^{47}\), a known antioxidant\(^{47}\) that is also regulator of gamma glutamylcysteine synthetase (γGCS) activity, which is necessary for the production of GSH\(^{48}\). Previous reports indicate that, through its effects on γGCS, Vanin-1 limits the production of the GSH\(^{48}\). Vanin-1/-/- mice have shown GSH-induced resistance to whole-body gamma-
irradiation- or paraquat-induced oxidative injury, apoptosis and inflammation \(^{48}\). Thereby we see that a lack of Vanin-1 protects against oxidative injury through its effects on the cycling of GSH. Thus an increase in Vanin gene expression appropriately corresponds with an increase PM- or HDM-induced oxidative stress. Interestingly, it has been shown that a nonsense mutation in the Vnn3 promoter in A/J mice, which leads to a truncated protein and lack of pantetheinase protein activity, is partially responsible for the susceptibility of A/J mice to malaria \(^{49}\). Moreover, Vanin-1 deficient mice also exhibited increased IL-10 and arginase expression \(^{50}\), which is associated with alternative macrophage activation, suggesting a role for Vanin-1 in Th2 immune responses. Taken together these observations suggest that the induction of Vanin genes by both HDM and AUB may be involved in their ability to regulate oxidative stress pathways or to induce the development of maladaptive Th2 immune responses, as typically seen in asthma.

We also observed that both AUB and HDM increased the expression of Ccl20, a chemokine known to recruit immature DCs, \(^{51}\) as well as Th17 cells \(^{52}\), which supports our data showing that AUB induced an increase in the percentage of pulmonary mDCs (Chapter 5) and Th17 cells (Chapter 3 – 5). Our data are in support of previous reports that indicate that both AUB \(^{21}\) and HDM \(^{19}\) induce the production of CCL20 in human airway epithelial cell lines. CCL20 has an established role in the development of allergic asthma and is significantly increased in the BAL of asthmatics after allergen provocation \(^{53}\). In addition CCR6-deficient mice, which lack the receptor for CCL20, exhibit reduced pulmonary DC and CD4+ T-cell recruitment in response to cigarette smoke \(^{54}\) as well as abrogated cockroach antigen-driven pulmonary IL-5 production and eosinophilia \(^{55}\).
These reports indicate that epithelial cell-derived CCL20 may play a key role in modulating T-cell driven airway disease.

Of particular interest, we showed that both AUB and HDM induced the production of genes that support NAPDH oxidase pathway activation (Noxo1) and increased activity (Ncf4), however this induction is significantly higher in AUB exposed mTECs as compared to HDM exposed cells. In addition, we observed that AUB uniquely induced the expression of Cybb, a gene encoding one of the major NAPDH oxidase catalytic subunits. Because previous reports indicated that the induction of oxidative stress is necessary for PM-induced epithelial cell pro-inflammatory cytokine production, we asked whether the induction of CCL20 by AUB was oxidative stress dependent. Indeed, we show that AUB induced mTEC CCL20 production is dependent on oxidative stress. Interestingly, using both pharmacological inhibition of NADPH oxidase pathways and epithelial cells derived from mice lacking NADPH oxidase activity (gp91^phox^-/-), we established a unique role for NADPH oxidase activity in PM-driven epithelial cell CCL20 production.

Finally, as the goal of this study was to determine the underlying mechanisms by which PM initiates asthmatic responses, we wanted to confirm that PM-exposed epithelial cells influence DC production of T cell skewing cytokines. We examined the ability of supernatants from AUB exposed mTECs to induce DC IL-6 (Th2/Th17) and IL-12p40 (Th1) production. It is important to note that although previous reports indicate that higher levels of PM can induce epithelial cell IL-6 production, at the chosen dosage (50 µg/ml AUB) IL-6 levels remain at or below the detection limit of our ELISAs (~30 pg/ml, data not shown). We observed that soluble factors produced by AUB exposed
mTECs stimulated a significant increase in IL-6 as compared to mTEC supernatants from untreated cells. This response is dependant on oxidative stress. Interestingly, IL-12p40 levels we not statistically altered. As we have seen that the direct treatment of DCs with AUB is a potent stimulator of DC IL-12p40 production (Chapter 5), we can conclude that AUB stimulated mTECs promote the development of pulmonary Th2/Th17 responses rather than Th1.

In summary, our studies strongly suggest that airway epithelial cells play a pivotal role in the induction of PM-induced allergic inflammation. Specifically, we observed that AUB induced epithelial cell expression of numerous pro-asthmatic genes that are also induced after allergen challenge. We show that AUB may drive a response similar to that seen in allergic asthma through CCL20 driven recruitment of DCs in a NADPH oxidase dependent manner. Moreover, AUB activated epithelial cells may promote the induction of Th2/Th17 responses by promoting DC production of Th2/Th17 skewing cytokines. Taken together these studies suggest that PM induced airway epithelial cell activation is a conceivable mechanism by which exposure to ambient PM may induce features similar to those seen in allergic asthma.
References


Chapter 7

Summary and Future Directions
To date, little is known about the exact mechanism(s) by which environmental PM may lead to the onset or worsening of respiratory health in susceptible individuals. Thus the goal of this thesis was to evaluate mechanisms by which real-world PM exposure may contribute to the increase in the incidence and the prevalence of asthma. To this end we tested the hypothesis that ambient PM collected in urban Baltimore (AUB) activates the airway epithelium, which leads to the recruitment and activation of DCs and subsequent activation of CD4+ T cells in the lung.

In a kinetic analysis of the effects of AUB exposure on airway function and inflammatory indices of asthma in mice, we show that AUB induces a gradual increase in AHR that is associated with a shift from a predominantly neutrophilic inflammatory response towards an eosinophilic and lymphocytic response, concomitant with increases in mucus containing cells in the airway wall. These pathological findings are temporally associated with a gradual shift from a pulmonary Th1/Th17 milieu towards a mixed Th2/Th17 cytokine response. The shift towards Th2/Th17 cytokine production is concomitant with the increase in pro-Th2/Th17 pulmonary chemokine production (MCP-1, CCL20). Moreover, these changes are associated with early and sustained changes in the oxidative/antioxidant balance in the lung, which favor the generation of pro-oxidant pathways. Taken together these studies support our overall hypothesis that AUB alone can induce the hallmark features of asthma including AHR, eosinophilic inflammation, and mucus hypersecretion. In addition, the kinetics of this response may explain why single PM challenges in healthy individuals generally fail to induce significant changes in pulmonary function parameters, despite substantial epidemiological evidence that supports a role for PM in the increase in asthma prevalence.
As our initial studies suggested that the pathophysiological features of asthma induced by AUB were temporally related to T cell cytokine production, we next tested the hypothesis that the adverse effects of AUB exposure were dependent upon lymphocytes. We show here for the first time that PM-induced AHR, eosinophilia, mucus production and Th2/Th17 cytokine production is lymphocyte dependent. Although we believe that this response may be dependent on conventional CD4 cells, a caveat of these studies is our use of Rag1-/- mice, which lack all lymphocyte lineages to explore the role of T cells in AUB-induced AHR. Thus, we are unable to differentiate between the contribution of T-cells, B-cells or NKT cells. However, the physiological changes did track with the changes in cytokines which are not known to be produced by B cells, but may be produced by NKT cells or conventional CD4+ T cells. Furthermore, despite the accepted role of Th2 cells in asthmatic responses, we do not directly show a role for Th2 or Th17 family members in the development in AUB-induced asthma. Thus further studies utilizing either antibody blockade or mice genetically deficient in IL-4, IL-5, IL-13 or IL-17 are necessary to identify which cells/cytokines are critical for AUB-induced asthma. In addition we show that AUB primed pulmonary cells toward the production of an early Th1/Th17 phenotype that shifts toward a Th2/Th17 phenotype with multiple AUB inoculations. However we are aware that these studies do not directly measure in vivo production of pulmonary T cell cytokines. Thus, the determination of T cell cytokines in the BAL of AUB exposed is necessary.

Based on our findings that lymphocytes mediate the development of AUB-induced AHR and airway inflammation, we explored the impact of AUB exposure on DC recruitment and activation, as these professional antigen-presenting cells are critical
regulators of T cell differentiation. Interestingly, we see that AUB shifts the pulmonary mDC/pDC balance toward a predominantly mDC and thus immunogenic, phenotype. In addition AUB exposure results in DC activation as exhibited by the in vivo increase in the numbers of DCs expressing CD80/CD86 and the direct in vitro DC production of T-cell skewing cytokines. Consistent with our earlier findings, we found AUB induced DC production of cytokines that are known to be important in directing the differentiation of naïve cells towards Th2/Th17 differentiation, suggesting that these cells are critical in the AUB-induced asthma. Interestingly, the recruitment and activation of mDCs was partially regulated by the cytokine GM-CSF. This is consistent with our observation that GM-CSF is produced early after AUB exposure, suggesting that it may precede the AUB-induced DC influx and thus differentiate and expand pulmonary mDCs. Moreover, we demonstrate that mDC production of a Th2/Th17-skewing pattern was completely dependent on NF-κB signaling, while only partially dependent on MyD88 signaling suggesting that AUB exposure regulates pro-allergic functions of DCs through a combination of both MyD88-dependent and independent pathways. Further investigations into MyD88 independent signaling pathways are necessary. The exposure of DCs derived from MyD88/TRIF mice to AUB could be performed to fully delineate the contribution of TLRs to AUB-induced DC production of T-cell cytokines. Additional studies in individual TLR deficient mice may point us to the exact TLRs involved, if any, in airway responses to AUB exposure. In addition, pretreatment of DC’s with inhibitors of oxidative stress (NAC, GST, apocynin) or with AUB treated with β-glucanases to remove fungal moieties could be performed to identify the contribution of non-TLR pathways to AUB-induced DC production of T-cell skewing cytokines. As AUB is a
complex mixture containing many potential PAMPs, bacterial endotoxin, fungal hyphae, PAHs, and transition metals, we expect to see the activation of multiple pathways. Identification of the exact components involved in immune activation by PM will greatly inform the design and implementation of air quality standards to reduce the adverse effects of ambient PM exposure on human health.

Because signals in the local environment can drive the recruitment and activation of DCs, we sought to determine the factors that initiate the development of AUB-induced asthma. As the airway epithelium is the initial contact site with environmental PM, we assessed transcriptome changes in AUB-exposed purified tracheal epithelial cells as compared to cells exposed to HDM, an allergen that can directly induce allergic asthma. In support of previous reports on gene changes in allergic asthma, we see that AUB and HDM increased the expression of genes involved in innate immunity such as complement components (C2, C3, Cfb), acute phase reactants (Saa3), cytokines (TNF) and chemokines (Cxcl1, Cxcl5, Ccl20). Of particulate interest, both AUB and HDM increase the production of the immature DC chemoattractant, CCL20, which is consistent with the \textit{in vivo} increase in DCs we observed in the lungs of AUB-exposed mice (Chapter 5) and the potent recruitment of Th17 cells. Of interest we see that both AUB and HDM induce genes that support NADPH oxidase activity (Noxo1, Ncf4). However, as compared to HDM, AUB induced higher expression of these genes, in addition to uniquely inducing the expression a gene encoding the major enzymatic subunit of the NADPH-oxidase family (Cybb). We went on to show that indeed, CCL20 production is dependent upon NADPH oxidase pathways suggesting that AUB recruits mDC to the lung through increasing CCL20 release from the epithelium through its ability to activate NADPH
oxidase. This data is in support of our previous data showing that AUB exposure alters
the antioxidant-oxidant balance in the lung (Chapter 3) as well as previous reports
suggesting that NADPH oxidase activity is enhanced in mouse models of allergic asthma
\(^1,^2\). Nonetheless, further studies are needed to formally demonstrate a role for both
CCL20 and NADPH oxidase in PM-induced AHR and airway inflammation. In addition
to genes previously implicated in asthma, we also identified several new pathways that
have not been studied in the context of allergic disease, but have been implicated in Th2
immune responses, including the vanin pathway (Vnn1, Vnn3). These genes are ideal
candidates for further investigation.

Taken together the studies reported in this thesis strongly support the
epidemiological studies that suggest an association between PM exposure and the rise in
asthma prevalence. Moreover, they provide considerable insight into the mechanisms by
which PM may contribute to the increases observed in the prevalence of asthma.

Specifically, we demonstrate that the airway epithelial cell can initiate the development
of experimental asthma through the oxidative stress dependent production of mediators
that recruit (CCL20) and expand (GM-CSF) immunogenic DCs. These DCs are primed
toward a pro-asthmatic myeloid phenotype in the presence of pulmonary GM-CSF and
activated by AUB to preferentially produce a Th2/Th17 skewing profile of cytokines.
Through the downstream effects of Th2/Th17 cytokines, AUB may drive the
pathophysiological symptoms of asthma (AHR, eosinophilia, mucus production). Overall
these studies support a role for exposure to increased levels of PM with the increase in
asthma prevalence in modernized countries. Importantly, these studies strongly argue that
greater regulation of PM levels in the US and worldwide are needed to prevent adverse health effects.
Figure 1. Summary of the mechanisms by which AUB can induce allergic asthma.

Airway epithelial cells act as sensors of PM and preferentially recruit and activate pro-asthmatic mDCs in response to particulate antigens. Once DC subsets are conditioned by either PM-exposed epithelial cells or through direct PM-activation these cells can activate lymphocyte-dependent adaptive immune responses.
References
