ABSTRACT

MADISON, SHARON LYNN. The effects of PM$_{2.5}$ on allergic inflammation in mast cell deficient mice. (Under the direction of Bruce Hammerberg.)

Animal models of asthma have confirmed epidemiological findings that exposure to fine particulate matter (PM$_{2.5}$) can enhance asthmatic symptoms, including eosinophilic inflammation and airway hyperresponsiveness. Critics have dismissed the possibility that these studies utilizing artificial exposure scenarios, like intratracheal instillation (i.t.), can be legitimately extrapolated to human risk largely due to the fact that the doses required for this type of model exceed the normal ambient concentrations of PM$_{2.5}$. In order to improve the credibility of the findings from previous animal studies utilizing the i.t. method for delivery of aqueous particle suspensions to the lung, and to determine the biological mechanisms responsible for the observed enhancement of allergic inflammation following PM$_{2.5}$ exposure, large-scale air samplers have been developed making it possible to directly expose wild type (WT) and genetically altered mice to fine, concentrated ambient particles (CAPs). In this study allergic asthma was modeled in both WT and mast cell deficient (MCD) mice by local (L) or systemic (S) sensitization to ovalbumin (OVA). Two weeks later mice were challenged with OVA (day 0) and then exposed to CAPs (day 0 & 1) with numerous endpoints collected (day 0-2). Overall, there was a temporal difference in the bronchoalveolar lavage cell profile between L and S sensitized mice, and the contribution of mast cells (MC) to this differential response was best observed for neutrophils at day 0 and day 1. Compared to air exposed mice, CAPs depressed total inflammatory cell infiltrates in the bronchoalveolar lavage fluid at day 0 and day 1 after OVA challenge for all groups. This overwhelming difference of limited cellular infiltration of
monocytes and neutrophils in the bronchoalveolar lavage fluid following CAPs exposure, and the significant difference between the L and S sensitization protocols, confound interpretation for all of the factors examined. However, the specific finding that CAPs can enhance eosinophil recruitment by day 2 after OVA challenge indicates that the results from previous animal studies utilizing i.t. PM$_{2.5}$ exposures do in fact support the epidemiological associations linking PM$_{2.5}$ exposures with the enhancement of allergic inflammation indicative of the asthmatic phenotype. Given the strict regulation of immunological tolerance at mucosal surfaces like the lung, the genetic variability of different mouse strains, and the daily changes in ambient PM$_{2.5}$ composition, the findings of this study prompt many unique questions. However, the bottom line is that this study demonstrates that ambient PM$_{2.5}$ does alter Th2-like responses in mice by enhancing pulmonary BAL eosinophils in the late phase response (day 2), and that mast cells are critical to their recruitment.
THE EFFECTS OF PM$_{2.5}$ ON ALLERGIC INFLAMMATION IN MAST CELL DEFICIENT MICE

BY

SHARON L. MADISON

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APPROVED BY:

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DEDICATION

Wesley Scott Madison

This thesis was inspired by Max Fischer, and is dedicated to my husband Wesley.
The author, my daughter Sharon, was born on February 22, 1971 at Fort Bragg, NC. At the age of 4 months she experienced the first of many relocations that are associated with life as an Army dependant. I feel that it was this vagabond lifestyle above anything else that contributed to her development as an adaptable, amicable, and creative individual.

Early on, I suspected that Sharon would pursue a career in a technical field, possibly engineering. As a baby, she would quietly amuse herself in her playpen for hours, disassembling, examining, and re-assembling her tinker toys. However, nearing the age of four, we quickly decided that a position in the medical field would be a good fit for Sharon’s interests. At that time, her father was pursuing his Masters Degree in Psychology, and of course had a variety of textbooks lying around the house at any given time. Sharon has always been intrigued by books, and was especially fond of my husband's developmental psychology textbook. The "brain book" became one of her favorites. Seeing as how she couldn’t read yet (a sore subject between Sharon and myself), she would sit alone and make-up stories for only the most interesting pictures of dissected brains. Do you think Einstein started out this way?
Moving along into the school days, Sharon finally began her academic pursuits in the first grade. After her first day in class, she came home in tears. She was angry with me, because I hadn’t taught her to read. I allowed her to scold me momentarily, but quickly rebutted telling her that I had given her numerous opportunities to learn how to read, those of which she neglected to pursue. Once she calmed down, she made her wishes known...she was going to know how to read before she returned to class the next day. Especially since, "Everyone in her class already knows how to read!" That afternoon, and into the evening hours, Sharon surrounded herself with storybooks, and began associating words with pictures, and managed to achieve substantial improvement. At school, she started out in the "slow" reading group, but by the end of the year was in the top group, and the following year enrolled in an advanced program for exceptional students. To add to her early literary accomplishments, she later won a writing contest in the second grade about, "What it means to be an American." Little did she know how much her perseverance would benefit her future literary endeavors as a graduate student.

Academically, Sharon was quite successful, as long as it didn’t interfere with her social life. She graduated from Pine Forest Senior High School in Fayetteville, NC in 1989, and that fall entered Appalachian State University as a freshman majoring in Business...no wait, History...no wait, well, you’ll find out. She was awarded an ROTC scholarship and had intended to follow in her father’s footsteps. As a cadet she was the commander of her college drill team, she graduated from Airborne School, and was the first female member of the Appalachian State ROTC Ranger Challenge Team. While she was very successful in the ROTC program, two events occurred which quickly stole
Sharon’s heart, pulling her away from her aspirations of becoming an Army Officer. Those two things were her newfound love of science, and of course, a guy.

First, she took biology during summer school so that she could avoid having to take those long, three-hour labs during the regular semester. As we all know, that kind of commitment can really take a toll on your social schedule. Unfortunately, she fell in love with science and spent the remainder of her college career in the laboratory, day in and day out. Next, she met a guy that would later make her Mrs. Wesley S. Madison. However, it took him two, long years to convince her of this fact. He finally wore her down and I am happy to report that they have been married for seven years now, and have all the makings for a wonderful life together, barring another year of writing this thesis.

As for her professional background, Sharon worked as a cooperative education student at Burroughs Wellcome during her senior year in 1994. Once she graduated from Appalachian State University with her BS in Biology, she was offered a position as a Biological Science Laboratory Technician for the ORD/NHEERL-H Experimental Toxicology Division of the U.S. Environment Protection Agency in the Research Triangle Park, NC. She enjoyed nearly five years there as an employee, and then left work to become a graduate student at NC State University to pursue a Masters in Immunology and Toxicology.

Upon the completion of this degree, Sharon will continue building on her experience as a scientist, and plans to continue challenging herself by pursuing a
certification in computer programming and database management. She also has aspirations of writing a successful novel or textbook, and of one day running her own business.
ACKNOWLEDGEMENTS

To date, this has been the most challenging project that I have ever undertaken. Given this level of achievement, I owe a great deal of gratitude to a host of mentors. First and foremost, I would like to thank my previous supervisor and primary advisor from the U.S. Environmental Protection Agency, Steve Gavett, for guiding me through this process while at the same time allowing me the freedom to work independently. We spent many of long hours in the lab together, and I feel that Steve has played a big part in helping me to develop from a naïve college graduate into a working professional. For this, there is not enough that can be said to his benefit. During this process I have found scientific writing to be far more challenging than I had ever expected. Steve has helped me through this process with his thorough evaluations of my manuscripts, and has taught me the value of showing attention to detail. Finally, it has been Steve’s flexibility, which has allowed me to determine my own level of success in writing my thesis, a process that has given me a great deal of personal satisfaction.

Secondly, I would like to thank the faculty and students of the Immunology Department at NCSU’s College of Veterinary Medicine for their influence on my perspective. I have had the good fortune to meet some amazing faculty members and
students, both of which have humbled and inspired me. One point that cannot go without mentioning for any student completing the rigors of a degree in Immunology at NCSU is Immunology Journal Club. While it oftentimes felt more like an interrogation from Napoleon himself rather than a collaborative discussion of the current literature, journal club has taught me a lot about myself and the value of being well prepared. As I did not perform my research at the Vet School, I had few opportunities to really get to know many of the faculty. However, in choosing my committee members, journal club served as a forum for selection, as both Sue Tonkonogy and Bruce Hammerberg influenced me due to their objective participation in the weekly discussions. For that I thank you.

Thirdly, I would like to thank Ken Adler for his participation with my committee. I chose Dr. Adler, because he is an adjunct Professor to the Toxicology Department, and much to my benefit, was familiar with the type of research that I performed. His familiarity with the subject matter can be attributed to his relationship with the US EPA where he serves as the primary coordinator for all of the activities related to the Cooperative Training Program established between the university and the EPA. This training grant provided me a stipend for living expenses, and the opportunity to continue working at the EPA for the remainder of my graduate research. Thank you for making this unique training possible for me and others.

Last but certainly not least, I have to thank my family and friends for suffering with me through my servitudism to this project. At times, it has truly been a grueling experience, and without their love and support I might have dropped everything to flip burgers at McDonald’s. I include in my list of family members all of those great people out at the
EPA. When I started there in 1995, I was the youngest person in the group, so there are numerous people there that I consider to be parental figures in addition to their role as professional colleagues. I have listed several people below, but this list is by no means all inclusive. To all of you, I say, “Thank you.”

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Sherry & Jim Shaw
John G. Shaw
Tony & Helen Steimle
Tony & Regina Steimle
Ken & Sandy Shriver

The Steimle and Shriver Cousins
Dr. Katie Wu
Mette & Linh Schladweiler
Julie, Raymund & Karen Nolan
Dorinda & Dave Brockway
Dr. Marsha Ward
Dee Sailstad & Family
Debbie Andrews & Family
Elizabeth Boykin
Lori Jimmerson

Dr. Allen McAlexander & Family
Najwa Coates
Dr. Amy Lambert
Dr. Matthew Campen

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Dr. Jean Wiester
Dr. Dan Costa
Dr. Penn Watkinson
Rick Jaskot
Lenny Walsh
Hassell Hilliard
Paul Evansky
Ed Lappi

Dr. Linda Birnbaum
Dr. Jan Dye
Dr. Ian Gilmour
Dr. Mary Jane Selgrade
Joanne Cook
Darrell Winsett
Annette King
April Jones
Craig Earl
Michelle Taylor
A. Daigger & Co.
Mark Miller
Ludwig Teran
Max Fischer
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<td>airway epithelial cell</td>
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<td>AHR</td>
<td>airway hyperresponsiveness</td>
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<td>AM</td>
<td>alveolar macrophage</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>B7RP</td>
<td>B7 regulated protein</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<td>C x T</td>
<td>concentration times time</td>
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<td>CAA</td>
<td>Clean Air Act</td>
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<td>CAPs</td>
<td>concentrated ambient particles</td>
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<td>CCR</td>
<td>C-C chemokine receptor</td>
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<td>c-kit</td>
<td>c-kit proto-oncogene</td>
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<td>CLP</td>
<td>caecal ligation puncture</td>
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<td>CMD</td>
<td>count median diameter</td>
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<td>CNS</td>
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<td>CO</td>
<td>carbon monoxide</td>
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<tr>
<td>CTLA</td>
<td>cytolytic T-lymphocyte associated antigen</td>
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<td>EAE</td>
<td>experimental autoimmune encephalitis</td>
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<td>EAR</td>
<td>early asthmatic response</td>
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<td>EC</td>
<td>elemental carbon</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>ET</td>
<td>total respiratory elastance</td>
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<td>FBS</td>
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<td>FcR</td>
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<td>GM-CSF</td>
<td>granulocyte and macrophage colony</td>
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<td>H&amp;E</td>
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<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.t.</td>
<td>intratracheal</td>
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<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<td>ICOS</td>
<td>inducible costimulatory molecule</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>ICP-AES</td>
<td>inductively coupled plasma-atomic</td>
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<td>intraepithelial mast cell</td>
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<td>interferon</td>
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<td>interleukin</td>
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<td>JAK-STAT</td>
<td>Janus Kinase Signal transducer and</td>
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<td>L</td>
<td>local sensitization</td>
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<td>LAR</td>
<td>late asthmatic response</td>
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<td>LDH</td>
<td>lactate dehydrogenase</td>
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<td>lymphocyte function-associated antigen</td>
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<td>mouse mast cell protease</td>
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<td>NAAQS</td>
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<td>N-acetyl-b-d-glucosaminidase</td>
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<td>NFAT</td>
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<td>natural killer cells</td>
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<td>O.D.</td>
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<td>OC</td>
<td>organic carbon</td>
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<td>ovalbumin</td>
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<td>proteinase activated receptor</td>
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<tr>
<td>Pb</td>
<td>lead</td>
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<td>platelet-endothelial adhesion molecule</td>
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<td>P-selectin glycoprotein</td>
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LITERATURE REVIEW
AIR POLLUTION

HISTORICAL ACCOUNTS OF NOTABLE AIR POLLUTION EPISODES

In order to more clearly understand the evolution of scientific knowledge concerning air pollution and asthma, it is important to briefly review the origins of the problem in modern society. The Western World was forever changed by the evolution of the average consumer. The mid-eighteenth century period referred to as the Industrial Revolution marks the beginning of this cultural, political and economic phenomenon characterized by the invention of power-driven machines, which automated the repetitive tasks performed during the production of tangible goods. Increased productivity lowered the price of goods, enabling the middle-class, or average consumers, the luxury of products previously unavailable to them [1, 2].

The major power source for the industrial machinery of the mid-eighteenth to nineteenth centuries was the steam engine. As the name implies, steam engines were run by the kinetic energy produced when water was boiled into steam. The primary fuel source for boiling water was obtained by burning coal, which at this time was the most
efficient fuel available [2, 3]. Coal burning was widespread in Europe, and utilized in both industry and domestic dwellings for heat [4]. Unfortunately, burning of coal results in the production of sulfur dioxide, a pungent gas that forms acidic aerosols when combined with water in the atmosphere, and one that is currently known to adversely affect lung function [5, 6].

Even prior to the Industrial Revolution, the adverse effects of burning coal were well recognized. The problem was so severe that on September 14, 1661, the well-respected essayist, John Evelyn, publicly addressed Parliament with his *Fumifugium*, a formal recognition of the air pollution problem in London, and its adverse effects on public health. The following quotation directed to King Charles II by Evelyn, describes the very instance in which he was inspired to write and dedicate his *Fumifugium* for the benefit of his Majesty’s health, while simultaneously providing a vivid image of the horrendous air quality of the times (Figure 1) [7-9]. While validating public concern and spurring an interest in the cause and effect relationship between air pollution and adverse health effects, little improvement was actually accomplished in the years that followed Evelyn’s pro-environmental publications related to air pollution.

Nearing the twentieth century, as industrialization progressed from Europe to the Americas, the growing fear and reality of coal sources becoming depleted [10], along with advances in hydraulic power and improvements in fuel efficiency, lead to the invention of the internal combustion engine by Rudolph Diesel [11]. This new engine required the use of liquid for fuel, and spawned the incorporation of diesel and gasoline powered automobiles into everyday life. This pivotal change in engineering soon made petroleum the most sought after energy source [3], and with a new breed of fuel came a
new breed of air pollution composed of a complex mixture of reactive gases and particulate matter [12].

Although it was suspected, by Evelyn and others, even prior to the Industrial Revolution that combustion by products in the air, whether they originated from coal or some other source, were responsible for causing adverse health effects [4, 7], little proof had been produced prior to 1965. In London alone, between the years of 1873 to 1962, eleven fog episodes had been recorded resulting in excess mortality [13-23]. Of these acute pollution episodes, the London Fog of 1952 was the most dramatic case documented, since the days that followed the oppressive fog, claimed approximately 4,000 excess deaths attributed to cardiopulmonary failure. Despite the devastation of the episodes in London and other cities (Table 1) it was not until 1965, 13 years after the London Fog and 300 years since the *Fumifugium*, that a scientifically sound and causal relationship between air pollution and human health was established. The primary reason for this was a relative lack of control data necessary to prove or disprove a hypothesis by the scientific method [12, 24, 25].

**ESTABLISHMENT AND REVISIONS TO THE NAAQS**

Holland and Reid are the first to be credited with accomplishing the task of demonstrating through the scientific method, a causal relationship between air pollution and adverse health effects [12, 26]. Their study was published in the *Lancet* in 1965 and was designed to quantitatively compare the lung function of postal workers in the metropolis of London (the exposed population) with that of postal workers in smaller...
cities (the control population). All things being relatively equal, aside from the
differences in air pollution concentrations, the findings of their study demonstrated that
the postal workers in the polluted city of London had decreased lung function in
comparison to those in the smaller, less-polluted cities. This pivotal study, quantitatively
established the differences between an exposed versus a control population, and finally
confirmed the plausible suspicions surrounding excess morbidity due to air pollution
exposure. Furthermore, consequential strides in the science of epidemiology arising from
this study and others eventually legitimized public concerns surrounding the detrimental
effects of environmental pollutants [27-31], and ultimately prompted the enlistment of
government agencies in the U.S. to regulate pollution levels [32-34].

In order to safeguard the American population against the hazards environmental
pollutants originating from, and continually released into the air, water and soil by
anthropogenic sources, the U.S. government created the U.S. EPA (United States
Environmental Protection Agency), a federal agency specifically tasked to protect the
public from the adverse effects of hidden environmental contaminants. The EPA
formalized the previously state regulated Clean Air Act (CAA) of 1963 and began to
legally enforce monitoring and emission standards for the most widely dispersed airborne
pollutants known to pose a threat to human health [35-37]. The foundation for the
original and the current standards comes from epidemiologic studies as well as clinical
and controlled exposure studies in humans and animals, respectively. The standards are
determined based on the premise that the maximum limits set for a given air pollutant
should be sufficiently stringent, such that both healthy and susceptible subpopulations are
protected by an adequate margin of safety. In order to ensure the highest level of
protection, the CAA is periodically reviewed and amended by the US EPA based on the most recent and credible scientific data available. Most notable among the early amendments (1971), was the mandated enforcement of the National Ambient Air Quality Standards (NAAQS), which specifically state the limits for six major air pollutants based on health endpoints [38, 39]. The list has expanded since the 1971 amendment, but the current “criteria pollutants,” are sulfur dioxide (SO₂), nitrogen dioxide (NO₂), carbon monoxide (CO), lead (Pb), ozone (O₃), and particulate matter (PM). Maximum limits for these standards are listed in Table 2.

Enforcement of the NAAQS standards has improved air quality by significantly lowering air pollutants. For instance, from 1988 to 1995 the weighted annual average for PM in New York was reduced by 24% [40]. Despite this success, the prevalence of and severity of diseases like asthma continue to grow at an alarming rate, and some speculate that air pollution is partially to blame [12]. Recently, attention has been focused on the standards for O₃ and PM, since it was determined upon periodic review in 1996 that neither of these standards was adequate in protecting human health [41-44]. The NAAQS was eventually revised for both O₃ and PM, however the circumstances surrounding these decisions were somewhat different.

In the case of O₃, controlled exposures in healthy humans [45-47] and individual-level epidemiologic studies [28, 48] concluded that while there are acute physiologic effects associated with short term O₃ exposure at levels reflective of the 1979 standard [(0.12 ppm, 1-hour average), 49], there are also long term effects, including bronchoconstriction and inflammation, that are strongly associated with a total daily exposure [41, 42]. Based on the risk assessment paradigm and risk management models
set forth by the CAA, a new standard for O₃ was adopted to include not only a 1-hour average, but also an 8-hour average of 0.08 ppm to protect against its acute inflammatory and lung injury effects which results from sustained, but moderate elevations in O₃ levels (Table 2) [50]. Since the studies leading to the development of the standard were performed in healthy humans, the results add credence to the likelihood that the original standard was not protecting susceptible populations such as those individuals with asthma and other chronic obstructive pulmonary diseases [51-54].

Revision of the PM standard was somewhat different from the O₃ NAAQS revision. The major reason for this is that the revision of the 1996 O₃ NAAQS was in direct accord with quantitative health endpoints such as pulmonary function testing in healthy volunteers using an epidemiologic model which observes effects at the individual-level, rather than population based. The advantage of an individual-level study is that the dose-response can be clearly identified for a single person, thereby increasing the statistical power of the collected findings as a whole [28]. In contrast with O₃, the technologic advances in the characterization and environmental monitoring for PM were still in their infancy in the 1980s, making direct dose-response assessments for PM impossible. Still, population based studies appeared to show an association between morbidity and mortality [12]. It took advances in several disciplines including atmospheric chemistry, pulmonary physiology, aerosol science, and epidemiology in order to provide better sampling information and statistical analyses for revision of the PM standard. Empirical data collected from these disciplines in the 1970’s and 1980’s was synthesized to improve the strategies for determining the true nature of the risk to PM exposure [12]. This is a very complicated matter and since there is no single study
that can thoroughly represent the evolution of the PM standard, it is important to appreciate the findings of the independent disciplines that lead to the current regulations.

According to the 1970 NAAQS amendment, PM was measured as a total suspended particulate (TSP), which includes particles less than 50µ in mean mass aerodynamic diameter (MMAD). Following this amendment, atmospheric chemists demonstrated that within the realm of TSP there are two different modes of particles distinguished by the particle’s MMAD. The two modes are called the coarse mode and the fine mode (Figure 2). The coarse mode (>2.5µ) generally originates from the mechanical breakdown of windblown crustal materials and is composed mainly of silica, magnesium, potassium and sodium. The fine mode of particles (< 2.5µ) is formed by the nucleation and aggregation of gaseous and ultrafine combustion by products from anthropogenic sources. Depending upon the source, these particles can contain a variety of constituents including sulfates, metals and carcinogenic organic species (Figure 3) [55].

Concomitant to the new findings in atmospheric chemistry, investigations pursuing the relationship between respiratory mechanics and particle deposition relative to the particle’s size and hygroscopicity lead to particle classifications based on the location of deposition in the respiratory tract as shown in Figure 2 [56-59]. Generally, coarse particles greater than 10µ are not inhalable and impact the respiratory tract within the nose, while coarse particles less than 10µ (PM10) are capable of entering the lung and depositing by impaction on the tracheobronchial portion of the lung. Fine mode particles less than 2.5µ (PM2.5) are capable of traveling deep into the alveolar spaces of the lung, and deposit by sedimentation rather than impaction [60, 61]. Studies relating varied
levels of respiration to the physical properties of particles showed that decreased respiratory rate or increased tidal volume increases the deposition rate of fine mode particles [61-65]. Additionally, since it was demonstrated that particle deposition in a diseased lung is enhanced by 50-100% when compared to a healthy lung [66, 67], and that the deposition pattern in a diseased lung is usually concentrated in focal regions of the segmental bronchi [68-71], it was reasonably speculated that smaller particles would increase the risk of adverse health effects [72]. In terms of the asthmatic lung, airway hyperreactivity has also been associated with enhanced regional deposition of particles, further increasing the risk for susceptible populations like asthmatics [73-77].

**THE PM$_{10}$ AND PM$_{2.5}$ STANDARDS**

By the 1980’s, epidemiologic studies clearly showed a strong correlation between TSP and adverse health effects [12, 78-85]. However, improvements in fine mode PM collection techniques accompanied by the aforementioned deposition data, and advanced mathematical modeling used to calculate and relate the contribution of different PM fractions to health endpoints, collectively demonstrated that smaller particles were more strongly associated with hospital admissions [86-94], decreased pulmonary function [95-100], and respiratory symptoms [88, 96, 97, 100-102]. The most convincing evidence in support of the new PM$_{10}$ benchmark was a study published by an economist at Brigham Young University, Arden C. Pope III, that clearly established a relationship between PM$_{10}$ levels and adverse health effects [103].

The landmark Utah Valley study was made possible due to an economic incident in a town near Salt Lake City. A local steel mill had been operating there since the
1940’s, serving as a point source for greater than 50% of the total Utah Valley PM$_{10}$ emissions. Due to a labor strike, the mill was shut down in 1986 and re-opened 13 months later when a new owner took over the mill. Since the study population was largely Mormon, indicating strong genetic similarities and a low rate of cigarette smoking, and since other pollutants such as O$_3$ were not nearly as prevalent as PM in this geographical location, Pope was provided with a unique opportunity to examine the effects of air pollution on the health of the local population. This study correlated PM$_{10}$ levels with hospital admissions, emergency room visits, and school absences. It demonstrated that during the time in which the steel mill was closed, the general health of the population improved based on the aforementioned endpoints. This study and others confirmed that air quality is an important contributor to the quality of life in the general population, but more importantly, that PM$_{10}$ is more detrimental to the lung function of susceptible groups. Asthmatics are recognized as a group susceptible to PM exposures, and other studies show that PM can enhance morbidity associated with asthma [66, 104-109]. While this study did not specifically address the effects of PM$_{10}$ on asthma alone, asthma was an endpoint under consideration, demonstrating a 4.2% increase in asthma and bronchitis hospital admissions [103, 110, 111]. Since it is estimated that more than 17 million Americans suffer from asthma [112] causing an economic burden in excess of 6 billion dollars (1990), due to lost school days and time from work, as well 43% of the total cost for emergency department visits [113, 114], the significance of these
epidemiologic studies is apparent, especially when one considers that the prevalence of asthma has increased by 160% in children under the age of four since 1980 [9].

Following the enforcement of the PM$_{10}$ standard, numerous studies continued to demonstrate excess morbidity and mortality even at levels below the current PM$_{10}$ standard [115, 116]. It was purported that the respirable fraction, PM$_{2.5}$, may be more important in the induction of adverse health effects simply by virtue of the fact that this fraction has the greatest and deepest deposition pattern in the lung and is likely to act as a surrogate for those particle components linked to morbidity and mortality [43, 44]. In order to provide an adequate margin of safety for everyone exposed to PM the US EPA decided to regulate both PM$_{10}$ and PM$_{2.5}$. (Table 2) [117]. The standard for PM$_{10}$ is an annual mean of 50 $\mu$g/m$^3$ with a 24-hour maximum of 150 $\mu$g/m$^3$. The PM$_{2.5}$ standard is an annual mean of 15 $\mu$g/m$^3$ with a 24-hour maximum of 65 $\mu$g/m$^3$. Major debate followed the EPA’s decision, due to discrepancies in methodology between the epidemiologists, and due to the substantial economic burden placed on industry for meeting regulatory compliance [12, 118, 119]. Some of the controversy stemmed from the fact that many of the PM$_{2.5}$ studies were performed with retrospective calculations of PM$_{2.5}$ from TSP concentrations rather than active quantitative measurements [12]. Some also believe that there was a relative lack of evidence of morbidity in susceptible groups associated with PM$_{2.5}$ exposure [12, 118, 119]. Nonetheless, in an effort to protect all individuals that are potentially at risk, the standards were passed and are currently in effect today.

Despite the reservations by some, Dr. Douglas Dockery from Harvard University’s School of Public Heath was able to provide important evidence that there is
a dose dependent risk of mortality following PM$_{2.5}$ exposure [115] with no detectable threshold [120, 121]. The publication of this data is fittingly referred to as the Six Cities Study, since the mortality rates and PM$_{2.5}$ levels were measured in 6 different metropolitan areas showing a convincing trend towards PM$_{2.5}$ associated mortality. While these results were an important component in determining current regulation, other studies have shown a lack of causality between PM$_{2.5}$ exposure and mortality, but instead an increase in respiratory symptoms due to PM$_{2.5}$ exposure [95, 106, 122-130].
ASTHMA

DEFINITION

In the past 20 years, the prevalence of asthma has increased by 75% [9]. Non-fatal attacks in medicated asthmatics can be quite severe as evidenced by the fact that nearly half a million hospitalizations in 1993 were primarily due to asthma [9]. By contrast, the number of people with self-reported asthma in the U.S. significantly increased from 10.4 to 14.6 million people over the course of four years (1990-1994), indicating that there are varying degrees of disease severity, some of which go untreated (Figure 4) [9]. While classification and treatment of the disease can be quite complex, the disorder is generally nondiscriminatory affecting individuals from different cultural and genetic backgrounds with symptoms that are quite varied from person to person, and highly dependent upon environmental exposures. For this reason the National Heart Lung and Blood Institute (NHLBI) has provided an operational definition of the disease regardless of patient classification, disease severity, allergic status or exposure (Figure 5) [131].

Clinically speaking, individuals suffering from recurrent episodes of wheezing, shortness of breath, coughing and bronchoconstriction may be positively diagnosed with
asthma following the reversal of these symptoms [132], and historically, treatment of asthma relied on controlling the obvious respiratory symptoms associated with an attack by administering bronchodilators such as a short-acting β-2 agonist [133]. While this type of treatment regiment provides protection against a potentially fatal physiologic condition, and is still an important part of asthma maintenance, it does not treat the underlying chronic inflammation, which as mentioned in the definition, promotes disease symptoms [131]. It has been well recognized that chronic inflammation is central to perpetuation of the disease, and it has been hypothesized that tissue remodeling following the damaging effects produced by toxic eosinophil products [134] and other proinflammatory mediators, such as cytokines and growth factors, leads to eventual alterations in lung architecture and ultimately function [135]. Confirmation of this hypothesis has been achieved based on the fact that eosinophils recovered from the lungs of asthmatic patients have been correlated with disease severity [136-143]. Given this, I have provided a more academic definition of the disease, which more closely describes the immunologic phenomenona, which must take place in order to produce the hallmark pulmonary eosinophilia associated with the asthmatic phenotype (Figure 6).

This simplistic and somewhat biased description of asthma asserts that the promulgation of the physiologic symptoms, by which asthma is clinically recognized, is the net effect of an inflammatory cascade specifically geared towards eosinophil recruitment. The mechanism by which this occurs, in the absence of an ongoing infection, have yet to be determined. For this reason, animal models of asthma are often employed in order to decipher those pathways critical for eosinophil development, homing, and activation. The purpose of this section is to describe how the use of a
vaccination protocol, or rather a sensitization protocol, induces the allergic phenotype in a mouse model, and to emphasize the complexities associated with this model due to variations in T helper cell priming and the costimulatory signal elicited via different routes of sensitization.

ASTHMA MODELS IN MICE

The allergic mouse model described herein is commonly employed by researchers to study asthma-like symptoms including airway hyperresponsiveness and pulmonary eosinophilia, to name a few. The rationale for the use of this model stems from the fact that the most important risk factor for the development of asthma begins with the induction of allergies [144]. An allergic reaction is defined as a response to innocuous environmental antigens due to pre-existing T cells or antigen-specific immunoglobulins (Ig), particularly IgE. While there are different mechanisms of allergy [145], mediated by different classes of antibodies, the type I hypersensitivity reaction is of particular importance in asthma, since this mast cell-mediated reaction is responsible for the physiologic symptoms experienced during the early asthmatic response (EAR). Furthermore, the events elicited during EAR have been linked to the severity of the T cell-mediated, late asthmatic response (LAR), which is most commonly associated with substantial pulmonary eosinophil infiltration and severe decrements in lung function which can last for several hours [146-148].

Given this, it is not surprising that in order to mimic asthma by inducing a type I hypersensitivity reaction, animal modelers have capitalized on the wealth of information pertaining to vaccine development that has been accumulated over the past century. A
vaccination is an adaptive immune response that is deliberately provoked by introducing an antigen into the body leading to long-lived, protective immunity to the antigen in question in the form of Ig, or rather antibody production. The methods for testing a typical vaccination protocol are quoted above in Figure 4 from the textbook entitled *Immunobiology: The Immune System in Health and Disease* written by noted immunologists Dr. Charles A. Janeway and Dr. Paul Travers. This description is analogous to the sensitization protocol used in this study (Figure 7) [149].

The “candidate vaccine” (Figure 7) in this allergic model is to chicken egg ovalbumin (OVA), an allergenic protein that is major histocompatibility complex class II (MHC II) restricted, and thus presented to, and conversely detected, by CD4+ T helper cells (Th) through their T cell receptor:CD3 complex (TCR). Under normal circumstances, the “immune response elicited” by the administration of OVA displays the Th2 phenotype characterized by the release of specific interleukins (IL); IL-4, IL-5, IL-6 and IL-13 [150]. Successful activation, proliferation and differentiation of Th cells into memory and effector Th2 cells is referred to as the sensitization phase (OVA-sensitization), and is dependent upon the nature of the signals directed from the antigen presenting cells (APC) to the naïve Th cell during MHC II:OVApeptide:TCR engagement, as discussed later in this section. When the Th2 cells become effector cells they can then provide “T cell help” to B cells for isotype switching to the allergic antibody isotypes, IgG1 and IgE. It takes antigen-specific B cells approximately two weeks to complete their isotype switch and affinity maturation to the OVA epitopes, but once OVA-specific Igs are systemically disseminated, i.e. IgE bound to the high affinity IgE receptor (FcεRI) on mast cells, and increased serum levels of IgG1, the first-line of
defense against future exposures to OVA (i.e. arming of mast cells for the type I hypersensitivity reaction) is established indicating that the sensitization phase is complete.

The second step occurs when the OVA-sensitized mice are “challenged” (Figure 7) by inhalation with OVA, thus initiating the first line of defense, i.e. the type I hypersensitivity reaction. The full development of the inflammatory cell recruitment, including eosinophilia, can be severely delayed in mice lacking mast cells [151-162]. FcR-triggered mast cell activation is an important initiator of inflammation in this model, and plays a prominent role in eosinophil recruitment. Mast cell activation has been well characterized and is described in brief here: cellular activation occurs when antigen crosslinks the FcεRI, surface bound IgE molecules on the mast cell, or when IgG1 attached to the antigen binds to the FcγRIII on the surface of the mast cell, leading to the release of bioactive mediators, including histamine, prostaglandins, leukotrienes and cytokines, all of which promote inflammation [163, 164]. It should be duly noted here that varying the ratio of IgE:IgG1 can alter the outcome of type I responses as demonstrated by the specific immunotherapy in humans to bee venoms and aeroallergens where IgG1 titers are intentionally and dramatically increased in order to sequester antigen before it can activate mast cells [165-171]. Additionally, animal studies have elucidated an alternative mechanism diverting the type I response by demonstrating that the FcγRII severely downregulates the FcR-mediated mast cell activation, since mice deficient in this receptor did not respond as quickly to passive cutaneous anaphylaxtic reactions as wild type mice [172]. These facts stress the importance for IgE production rather than IgG1 in achieving the theoretical response expected for a type I
hypersensitivity reaction, especially since the genetic predisposition to produce IgE, a condition referred to as atopy, is one of the predictors for the development of allergy and asthma (Figure 8). Additionally, the clinical success of specific immunotherapy demonstrates the pivotal role mast cell regulation plays in mediating allergic responses.

Finally, the “prevalence and severity” (Figure 7) of the allergic reaction is determined by the number of eosinophils that can be recovered from the lungs by bronchoalveolar lavage (BAL). An absolute distinction between the OVA-sensitized and non-sensitized mice should be evident in that non-sensitized mice have no eosinophils in their BAL fluid, owing to the fact that they do not possess OVA-specific IgE, and therefore cannot induce a type I hypersensitivity reaction. In the past, some have argued that IgE-mediated mast cell activation is not required for eosinophil recruitment [173-182], however, firm conclusions in these studies were arrived at without a full appreciation of the redundant nature of the immune response. Successful sensitizations are not always easily attained. Slight procedural and genetic variations can affect the outcome of OVA-sensitization in mice, specifically with respect to the route of sensitization [183, 184].

ACTIVE IMMUNITY REQUIRES Th CELL ACTIVATION

In humans, a major feature to the development of allergic sensitization to pollen and house dust mite in the mucosal surfaces of the lung is due to a defect in immunologic tolerance to repeated exposure of these antigens [185, 186]. Much remains to be learned, however, on how mucosal tolerance is retained in healthy humans, while in others,
tolerance is broken leading to allergic sensitization and increasing the risk for the development of asthma. Defining the modalities of mucosal tolerance is central to determining the relationship between allergies and the development of asthma in humans. Unfortunately, it is the same protective mechanism that has hampered the practical application of mucosal sensitizations in the allergic animal models, which are typically employed by researchers to study the various aspects of the inflammatory cascade in asthma [185-187]. In the past, T cell tolerance was believed to be due to TCR engagement on Th cells in the absence of a costimulatory signal from the apposing professional APC [188-190]. However, another level of complexity to the current understanding of mucosal tolerance has recently been modified due for the following reasons:

1) The discovery of new accessory molecules on both the T cell and the APC [191, 192].

2) The determination that the differential expression of adhesion molecules on both the T cell and the apposing APC may alter the efficiency of the costimulatory signal [188, 193-200].

3) Two newly described classes of Th cells alter the convention of the long-standing Th₁ vs. Th₂ paradigm in both humans and mice [201-205].

Furthermore, these new findings stress the importance of the innate immune response, the ontogeny of the APC, the microenvironment of antigen processing and presentation, and the temporal index of cytokine production by the APC during Th cell priming.
**Route of Antigen Administration**

In order to achieve the endpoints, which serve to define allergic asthma in humans (IgE antibody production, eosinophils, and airway hyperresponsiveness), the mouse model utilized in this study is critically dependent upon the clonal expansion, and differentiation of naïve Th cells into memory and effector Th2 cells. Until recently, mucosal administration of OVA (local sensitization) was avoided in the allergic mouse model due to the potential for T cell tolerance rather than Th2 cell polarization, as observed in rats following repeated exposure to OVA [185, 187]. Typically, researchers have relied on a fool-proof method for sensitization involving intraperitoneal injection (i.p.) of OVA plus an adjuvant (systemic sensitization) in order to ensure appropriate Th cell priming [206-208]. However, given the wealth of knowledge gained in the science of immunology over the past decade, and the long-standing awareness that different routes of antigen administration can lead to different outcomes in a given sensitization protocol [209], a renewed interest in developing techniques for local sensitizations, rather than the artificial, systemic sensitization protocols, is currently being pursued [210-212].

In order to fully appreciate the specific requirements leading to Th2 cell polarization, it is important to start with the current knowledge of the signals required for naïve Th cell priming leading to OVA-sensitization. Sensitization is a multi-step process, but we will begin at the point where a naïve Th cell encounters an APC in a secondary lymphoid tissue bearing the appropriate MHCII:OVApeptide complex, which complements the TCR of that cell. Following recognition of the MHCII:OVApeptide complex, the naïve Th cell can be activated through type I cytokine receptor signaling to undergo clonal expansion, and differentiation [150, 213]. Activation of the Th cell leads to limited differentiation into an immature Th0 cell characterized by its secretion of IL-2,
interferon (IFN)-\(\gamma\) and IL-4 at very low levels. Next, the Th\(_0\) cell undergoes several rounds of proliferation, while progressively maturing and differentiating in accordance with the cytokine signals received throughout the progression of the cell cycle [214]. In general, when TCR triggering is coupled with signaling through the IL-4 receptor (IL-4R), Th\(_2\) cell development is the outcome. By contrast, TCR triggering and signaling via the IL-12 receptor (IL-12R), enhances the production of IFN-\(\gamma\), promoting Th\(_1\) cell development [215, 216]. Successive triggering of the TCR, plus increased expression of T cell growth factors, and a dynamic co-stimulatory signal all are necessary to stimulate Th\(_0\) cells to develop into effector cells capable of responding to OVA.

The first notable difference between the systemic and local routes of sensitization occurs during clonal expansion of the Th\(_0\) cell. Specifically, the lymphoid organs draining the exposed tissue, and the T cell growth factor mediating clonal expansion are different. In the systemic sensitization protocol, the primary location for T cell priming is in the spleen with IL-2 serving as the major T cell growth factor. By contrast, local sensitization occurs in the bronchial associated lymphoid tissues, and the predominant T cell growth factor is IL-4 [217]. Reinforcing the role of IL-4 as a T cell growth factor, comes from the work by Miner describing the development of the prototypical Th\(_0\) cell. These studies revealed that in addition to the prototype, there is a subset of Th\(_0\) cells which secrete both IL-4 and IFN-\(\gamma\), but not IL-2 [218]. The implications resulting from this difference in T cell growth factors, both of which may lead to Th\(_2\) cell polarization, have yet to be determined, but allude to a role for a specific subset of Th cells which distinctly develop in response to IL-4 and serve to protect mucosal tissues from overt inflammatory responses.
**Growth factors IL-2 and IL-4**

Before the demonstration that the outcome of Th cell activation is specifically influenced by the lymphoid compartment of antigen encounter, dogma stated that IL-2 is absolutely necessary for Th₀ cell priming, and that the production of IL-2 is dependent upon the costimulatory signal delivered by ligation of CD28 [213]. IL-2 signaling is a well-characterized process, and is summarized here in order to highlight some of the important processes involved in the IL-2-mediated, systemic sensitization protocol. Once the naïve Th cell is stimulated through its TCR, a signal is generated leading to the expression of the IL-2 receptor-α chain (IL-2Rα, or CD25). The significance of this step is that addition of CD25 to the pre-existing intermediate-affinity IL-2R, which is composed of a β-chain (IL-2Rβ) and the common gamma-chain (γc), leads to expression of the high-affinity IL-2R (IL-2Rβ + γc + IL-2Rα) which serves to amplify the Th cell’s responsiveness to IL-2. Furthermore, signaling through the high-affinity IL-2R serves as a checkpoint for proliferation and amplifies the expression of other pro-inflammatory cytokines [209].

The protein scaffold for intracellular IL-2 signaling, as well as for all type I cytokine receptors, is grounded in the activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway [219]. Once IL-2 has bound the high-affinity IL-2R, the receptor-associated JAKs, 1 and 3, phosphorylate the β-chain in order to provide a docking station for STAT molecules [220-222]. Once the STAT molecules, –3, 5a, or 5b, [223, 224] have associated with the receptor, the JAKs phosphorylate the STATs consequently increasing their affinity for one another and inducing homo- or hetero- dimmers or tetramers in various combinations [225, 226]. The dimerized STAT
molecules can then translocate to the nucleus and bind the DNA in the promoter region designated by specific sequences called IL-2 response elements [IL-2RE, 227, 228, 229]. Genes containing an IL-2RE serve as the template for the production of numerous molecules involved in progression into the cell cycle, the expression of CD25, and the secretion of IL-2 [230, 231].

**CD28 Costimulation**

As mentioned earlier, another important checkpoint in Th cell priming is the ligation of CD28 on the surface of the Th₀ cell by one of its ligands, B7-1 (CD80) or B7-2 (CD86). It has been demonstrated in numerous in vitro and in vivo studies, including the allergic mouse model of asthma, that the adaptive immune response is critically dependent on CD28 signaling in order to achieve antigen-specific Th cell proliferation [190], Th₂ cell differentiation [232, 233], pulmonary eosinophilia, and airway hyperresponsiveness [234-236]. The main function of CD28 signaling is to amplify the positive feedback loop for IL-2 proliferative signals [237-240] by stabilizing IL-2 mRNA [241]. Similar to the IL-2 responsive genes, the connection between receptor signaling via CD28 and gene transcription is attributed to sequences in the promoter region called CD28 response elements [CD28RE, 242]. CD28REs are involved in the transcription of pro-inflammatory cytokines including IL-6 and the chemokine, IL-8 [243].

**Inhibition by IL-10**

In contrast to the efficiency of CD28-mediated Th cell priming, compelling evidence has recently linked Th cell tolerance, not simply due to a lack of CD28 signaling during TCR engagement, but rather due to an active suppression of CD28 signaling by IL-10. It would seem that IL-10 assumes a contradictory role in adaptive
immunity, since IL-10 production is often likened to an active Th\(_2\) phenotype. However, IL-10 is now known to produce an immunosuppressive effect during Th cell priming by virtue of the physical association of the IL-10 receptor (IL-10R) with CD28 on the surface of Th cells [165, 244]. During Th cell priming, IL-10 can be produced in large quantities by monocytes and NK-T cells. Signaling through the IL-10R effectively blocks the CD28 costimulatory signal through dephosphorylation [165]. This blockade inhibits the IL-2 positive feedback loop and can potentially lead to T cell tolerance [245]. Enhancement of the tolerogenic effect of IL-10 can also be attributed to its downregulation of the costimulatory molecules of the B7 family and the downregulation of MHCII expression by the APC [246-252].

Interestingly, the induction of tolerance is reversible in some instances, and may be circumvented altogether when a sufficient number of TCRs is triggered during MHC:OVApeptide:TCR engagement. This implies that while CD28 may be critically involved in Th cell priming, signaling through other cell surface receptors operating via concurrent signal transduction cascades will either enhance, or impede CD28 responsiveness through competitive binding interactions with the multitude of second messengers operating in parallel signal transduction cascade. In this respect, minor modifications in a sensitization protocol, leading to differential expression of cell surface molecules could easily lead to variable results.

*Enhancement of Costimulation*

To circumvent Th cell tolerance, appropriate CD28 signaling can be ensured in the IL-2-mediated, systemic sensitization protocol since it often relies on an adjuvant to increase the likelihood of Th cell priming. An adjuvant is any substance that enhances
the immune response to an antigen with which it is mixed. Historically, adjuvants have been used in human vaccines by adding cellular components expressed by pathogenic organisms, which are known to stimulate the antigen non-specific, or rather, an innate immune response. The natural benefit of this innate resistance, to bacteria for example, includes, but is not limited to, the activation of complement and the induction of the acute phase response [253]. The innate response is characterized by the release of tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8, and IFN-α. It is the signal transmitted by these cytokines which induces the expression of costimulatory molecules on the surface of the APC, and in turn, it is the nature of the activated phenotype of the APC which obligates the outcome of the adaptive immune response [253].

One example of a strong adjuvant is bacterial lippopolysaccharide (LPS). Expressed on Gram negative bacteria, LPS efficiently activates APCs by binding to their cell surface molecules called pattern recognition repeat receptors (PRR-receptors). For LPS, these include the LPS binding protein (CD14), the integrins, CD11b/CD18 or CD11c/CD18, and the recently discovered Toll receptors [254, 255]. This receptor-mediated activation of the APC efficiently induces the innate immune response and activates the APC to initiate bactericidal killing, antigen processing, migration to the lymph nodes and maturation into an efficient stimulator of naïve Th cells.

For this model, instead of mimicking bacterial infection, as with LPS, aluminum hydroxide (alum) is used as an adjuvant. The advantage of using alum instead of bacterial components is that while it can also initiate an innate immune response, there is an added benefit due to the fact that when injected, alum forms a gelatinous, insoluble,
antigen-complexed, precipitate which is retained in the gut for long periods of time [256].

The added benefit of this trait is that the likelihood for antigen uptake is increased due to prolonged retention, and alum also induces a systemic acute phase response due to its accumulation in the liver [257]. Unfortunately, the specific details comparing the innate responses elicited by alum versus other adjuvants, is difficult to attain. In the end, both types of adjuvants will stimulate the APC to release early-acting, antigen non-specific cytokines, thereby ensuring the conditions required for Th cell priming and differentiation. Specifically, APC-derived IL-1β and IL-6 released during naïve Th cell priming promote the differentiation of the naïve Th cell into the Th₀ phenotype, which in conjunction with the CD28 costimulatory signal and IL-4, results in the maturation of a functional Th₂ cell secreting high levels of IL-5 and IL-13 [232].

ICOS

The costimulatory signal cannot be solely attributed to CD28. Costimulation can be more accurately described as a competitive interaction between overlapping signal transduction cascades running in parallel. Therefore, it is actually an integrated signal influenced by the phenotypical expression of cell surface molecules during the initial phases of TCR engagement. One such parallel pathway is initiated by the newly identified, inducible costimulatory (ICOS) molecule. ICOS has been shown to provide additional signaling to the CD28 pathway leading to preferential Th₂ cell development [191]. It is expressed on activated T cells, and like CD28, has been identified as a key mediator to allergic airway inflammation in a mouse model [258]. Some speculate that CD28 is the critical factor for Th cell priming, while ligation of ICOS with B7-regulated
protein 1 (B7RP-1) is more important for Th2 effector cell responses [259, 260]. While ICOS promotes clonal expansion using IL-4 as the major T cell growth factor, this molecule has not been linked with tolerance induction at mucosal surfaces. By contrast, the counter-regulator of CD28, the cytolytic T lymphocyte-associated antigen 4 (CTLA-4) is thought to be important to tolerance induction due to its inhibitory effect on Th cells whereby ligation of CTLA-4 inhibits the IL-2 positive feedback loop and increases the Th cell’s production of TNF-α [259].

B7-H1

Another newly identified accessory molecule related to the CD28 signaling pathway, but expressed on the APC, is B7-H1. The information gathered for this molecule thus far has indicated that it induces a tolerogenic effect during Th cell priming due to its ability to enhance IL-10 and IFN-γ production, while simultaneously blocking the production of IL-4 [192, 261]. Incidentally, even though B7-H1 appears to tolerize Th cells during priming, it does not limit their proliferation, and may therefore serve to expand a population of tolerogenic cells. The expression of B7-H1 introduces another point of divergence between the local and systemic routes for sensitization, since B7-H1 mRNA can be detected in mucosal tissues, like the lung, and immune privileged sites, like the brain, but not in the spleen. [192].

Adhesion Molecules

Adhesion molecules have also been shown to influence the costimulatory signal. For example, lymphocyte function-associated antigen-2 (LFA-2), or rather CD2, is well
known for its adhesive properties sustained during MHCII:OVApEpTeid:TCR engagement, and for its role in partially activating Th cells when it binds Lck during the early stages of TCR signaling. However, the long-term ligation (30-40 minutes) of CD2 to its ligand on the APC, CD58 (LFA-3), results in the direct activation of STAT-1, and the subsequent expression of tumor growth factor-beta (TGF-β), a cytokine well known to inhibit active immune responses [262]. Furthermore, STAT-1 is involved in the regulation of IFN-γ signaling, a known antagonist to Th2 cell development. STAT-1 may interfere with the ability of STAT-3 and STAT-5 to activate IL-2 responsive genes through their competitive binding and antagonistic relationship with one another [263, 264]. By contrast, crosslinking of CD2 with CD3 promotes IL-2 production in T cells, independent of CD28 signaling, through the activation of Shc and Cbl, two second messengers noted to upregulate the activation of the IL-2, IL-4 and IL-5 genes [265].

Reinforcing the idea that adhesion molecules are part of the integrated costimulatory signal, it has been demonstrated that in addition to its transient adhesive properties during TCR engagement, CD11a/CD18 (LFA-1) ligation by intracellular adhesion molecule-1 (ICAM-1) increases intracellular calcium concentrations [266, 267]. Intracellular calcium can activate the second messengers, protein kinase C (PKC) and calcineurin, which in turn activate the transcription factors, nuclear factor-kappa B (NF-kB) and nuclear factor of activated T cells (NFAT), respectively [219]. Another class of adhesion molecules expressed on T cells, the α4β1 integrins, can participate in CD3-mediated T cell activation [268-273]. For instance, very late antigen-5 (VLA-5) binds fibronectin, and ligation has been shown to provide a costimulatory signal inducing IL-2 gene transcription via activation of the transcription factor, AP-1 [274]. These findings indicate that T cell activation could potentially be altered due to changes in the signal
transduction cascade elicited by differential expression of adhesion molecules on the
APC, and on the extracellular matrix (ECM) in the microenvironment during
MHC:OVAnpeptide:TCR engagement [273].

ACTIVE IMMUNITY VERSUS
TOLERANCE

Recently, two new Th cell subsets have been identified whose functions resolve
some of the questions related to the mechanisms involved in the development of T cell
tolerance.

Tolerogenic Th cells

The T regulatory cell 1 (Tr1), found in both mice and humans, is an activated and
differentiated Th cell identified due to its production of high levels of IL-10 during
MHC:OVAnpeptide:TCR engagement [203]. In humans, the onset for development of Tr1
cells is believed to be due to the APC-mediated innate immune response to antigen,
which precedes Th cell priming. It has been demonstrated that activation of the APC
leading to the production of IFN-α promotes Th1 cell development by virtue of its ability
to upregulate the inducible β-chain of the IL-12R, a necessary step for IFN-γ
responsiveness and Th1 cell development [275-277]. However, in mucosal tissues, where
Th cell development is already skewed towards a Th2 phenotype, IFN-α serves to initiate
IL-10 production by the APC [202]. The action of the IL-10 during Th cell priming
blocks CD28 costimulation leading to limited proliferation and an immunosuppressive
by-stander effect on neighboring Th cells [165, 245, 278]. Consequently, Tr1 cells
produce virtually undetectable levels of IL-2, proliferate poorly, and they do not produce
IL-4 [203]. In addition to the blockade of CD28 phosphorylation [245], IL-10 signaling results in activation of STAT-1 and STAT-3 [279, 280], while IFN-α activates STAT-4 and STAT-5 [281, 282], potentially deterring the dimerization of STAT-6 with STAT-3 or STAT-5 which is required for IL-4 production and Th₂ differentiation [283-287]. A definite potential for the action of Tr₁ cells promoting tolerance in this allergic mouse model comes from a study which indicates that despite the low numbers of Tr₁ cells produced in vivo, Th₀ cells primed with OVA in the presence of Tr₁ clones, in vitro, fail to secrete IL-2, IL-4, IL-5 or IFN-γ following re-stimulation [203].

The other newly described class of immunosuppressive Th cells are found in the draining lymph nodes from mucosal surfaces and have been named Th₃ cells. They differentiate in response to IL-4 and are characterized by their secretion of TGF-β, an immunosuppressive cytokine responsible for the isotype switch to IgA secretion by antigen-specific B cells [201]. Th₃ cells were discovered due to their enhancement of oral tolerance to myelin basic protein, the autoantigen responsible for the Th₁-mediated pathology of experimental autoimmune encephalitis [EAE, 204, 288-292]. Prior to the discovery of these TGF-β, immunosuppressive, regulatory T cells, the mechanism by which oral tolerance was thought to protect from EAE was due to Th₂-mediated immune deviation away from the Th₁ phenotype. However, since the induction of oral tolerance is still observed in IL-4 deficient mice, it is clear that T cell tolerance cannot simply be equated to the antagonistic relationship between Th₁ and Th₂ cells, but rather a separate classification for Th cells [293]. When activated by antigen, Th₃ cells secrete IL-4, IL-10 and increasing amounts of TGF-β due to a positive feedback loop regulating its production [205, 294]. Interestingly, the differentiation of Th₃ cells is enhanced by the
use of adjuvants such as lipopolysaccharide and subunit B of the cholera toxin, two strong adjuvants that are typically associated with a costimulatory signal leading to the upregulation of the IL-2 responsive genes, rather than TGF-β [289]. By contrast, IL-13, a cytokine with similar functions to IL-4, is not involved in Th₃ cell development and may serve as an important pathway to the development of allergic sensitization [294].

**Current state of Th cell Tolerance**

The bottom line is that variations in the integrated costimulatory profile and cytokine milieu can influence Th₀ cell priming and differentiation, since the expression of complementary ligands on the surface of the APC obligates the Th₀ cell to signal through specific pathways. Furthermore, APCs from different tissue compartments can vary in their cell surface expression and capacity to release specific cytokines. In a natural setting, the induction of immunity or tolerance may be largely under the control of the APC based on its origin of ontogeny. In general, APCs of lymphoid origin induce tolerance, while APCs of myeloid lineage promote active immunity [295]. For these reasons, it is easy to appreciate how immune responses may differ with respect to the anatomical location of the APC and the form in which the antigen is administered. Furthermore, what appears to be an anergic state in T cells repeatedly stimulated via mucosal surfaces, is actually an antigen-specific, active inhibition of IL-2 responsiveness and Th₀ cell priming by long-lived, regulatory T cells [202, 203]. Another important factor to the development of allergic sensitization versus tolerance is the T cell growth factor utilized in the draining lymph nodes where the antigen is originally presented. Historically, IL-2 has been described as the only T cell growth factor. While it is a T cell growth factor, its primary role in Th cell priming is to initiate proliferation for active
immune responses. IL-4 is now recognized as a T cell growth factor, and in the future, IL-13 may be considered in this capacity as well.
MAST CELLS

THE MAINTENANCE OF HEALTH

Health is not a static condition, but represents a dynamic state of physical and emotional well being, which is continually subjected to internal and external modifications including emotional stress, changes in temperature, mechanical injury, and infection. The body must rapidly accommodate these changing conditions by altering communication between the affected tissue and those organ systems, like the central nervous system, the endocrine system, and the immune system, which collectively mediate the response to internal and external modifications [296]. Those mechanisms which are prescribed as vital to the maintenance of health include [297, Webster's Dictionary, 298]:

1) The maintenance of the internal fluid and temperature homeostasis
2) The adaptation to stress
3) Defense against biotic organisms
4) Repair and regeneration of damaged tissues or cells
5) Hemostasis for the maintenance of blood volume and pressure
Mast cells may be best thought of as part of a dynamic network of cells communicating information from the local tissue environment to those systems within the body responsible for maintaining normal health. They have been referred to as a monocellular endocrine system since they participate in each of the five mechanisms listed above [299]. However, they are most frequently recognized for their participation in immunologic defense and tissue repair.

**CELL - CELL COMMUNICATION**

Since mast cells are positioned in barrier tissues including the skin, the lung and the gastrointestinal tract (gut), all of which are continually battling stresses from the external environment, their participation in the defense against infection and tissue repair are of particular importance. They are in physical contact with many different kinds of cells (heterotypic cell to cell contact) in the tissues, and amass a multitude of pre-formed, and newly synthesized bioactive mediators. Mast cell dysregulation has been linked to the progression of fibrosis, cancer, and tissue remodeling during disease [135, 300-312]. Since no one cell type can account for the complex chain of events leading to the maintenance of health or the pathogenesis of a disease, it is important to compare the contribution of mast cells to other cells of similar origin and function (particularly hematopoietic cells from the same lineage) in order to clearly distinguish the unique functions of mast cells relative to their anatomical location. Of particular interest, is the fact that despite the mast cell’s prominent role in Th2-mediated adaptive immune responses, they are of myeloid lineage, the members of which are often associated with innate immune responses [253]. Mast cells differentiate from the same precursor as
macrophages and granulocytes, but are unique in the fact that they reside for long periods of time in the peripheral tissues in close contact with nerve fibers, fibroblasts, epithelial cells, mucous secreting cells and endothelial cells [313]. Their anatomical disposition enables them to participate in homeostatic balance at a variety of levels, as well as providing immunologic defense mechanisms during both innate and adaptive immune responses [314].

**INNATE RESISTANCE**

The specific tissue of interest in this thesis is the lung. However, it should be noted that since the lung and the gut are both mucosal barriers sharing similar properties, future reference to parasitic infection models in the gut are included for the sake of analogy translating potential mast cell functions in the lung [315-317]. Albeit with the realization, that much of this information has yet to be confirmed, unless otherwise stated. The value of these parasite infection models is that they provide evidence for mast cell activation and eosinophil recruitment for non-allergic, innate resistance responses. This is a feature often overlooked for these cells, since parasitic infections in humans are rather rare in westernized society, and the role for mast cells and eosinophils has seemingly shifted towards allergic reactions rather than host defense [318]. Nonetheless, their innate functions may foreshadow the onset of allergic sensitization leading to the Th2-mediated immune response where mast cell hyperplasia and eosinophilia are commonly observed together [319-329]. Similarly, primary infections in these models provide a foundation for understanding the fundamental mechanisms responsible for the differential initiation of the innate immune response, whether it be
predominantly neutrophilic or eosinophilic, which ultimately forecasts the nature of the adaptive immune response.

**ONTOGENIC ORIGIN**

*Mast cells, granulocytes and monocytes/macrophages*

As mentioned earlier, mast cells, granulocytes and macrophages each develop from the same myeloid progenitor cell [316, 330-342]. This confers to each cell type the capacity for phagocytosis [343-345], antigen presentation [346-348], the presence of granules in their cytoplasm [342, 349, 350], bactericidal activity [347, 351], and mobility in response to a chemotactic gradient [352, 353]. The first bifurcation on the road towards lineage commitment occurs in the bone marrow when the myeloid precursor commits to either the monocytic or granulocytic cell line [354]. Granulocytes are short-lived white blood cells lacking the inherent ability to migrate from the bloodstream into tissues. However, during a local inflammatory response, neurogenic and endocrine signaling to the bone marrow rapidly induce granulocyte production, while at the same time local cytokine production upregulates the expression of adhesion molecules, and provides a chemotactic gradient for granulocyte recruitment into the affected tissue [355-357].

Granulocytes and mast cells share an associative relationship during the early immune response. Mast cell mediators are critical to the upregulation of adhesion molecules, which allow for the passage of granulocytes into the inflamed tissue. A bilateral communication between granulocytes and mast cells has also been proposed as a
survival mechanism during chronic inflammation [358-360]. It has however, been
difficult to elucidate the extent of their interdependence for one another, and remains a
controversial subject with respect to eosinophil recruitment, since IgE-mediated
eosinophil recruitment can be demonstrated in mast cell deficient mice [173-176, 178-
182].

Downstream from granulocyte development, a phenotypically immature cell
recognized as a monocytic, agranular, CD34+/c-kit^{hi}/Thy-1^{lo}/CD13^{+}/CD14^{+}/CD23^{-} cell
exits the bone marrow, and subsequently enters into a peripheral tissue for maturation
into either a macrophage or a mast cell [330]. Commitment of this precursor to either
lineage is attributed to the differential expression of growth factors in the peripheral
microenvironment throughout the course of their migration.

The major differentiating factor between mast cell versus macrophage lineage
commitment in vivo is SCF, a widely expressed growth factor acting on c-kit positive
cells [361]. The integration of SCF-c-kit signaling with other cytokines in the bone
marrow provides an early differentiating signal for the immature monocytic cell,
however, in the periphery, the SCF-c-kit ligand interaction is recognized as a selective
growth and survival factor for mast cells, but not for macrophages [362, 363]. The
CD34^{+}/c-kit^{hi}/Thy-1^{lo}/CD13^{+}/CD14^{+}/CD23^{-} cell was first confirmed as a mast cell
precursor by in vitro culturing of mouse fetal blood and liver with IL-3 [364-366] and
SCF [367], and later confirmed by differentiation of the same precursor in human blood
[368-370], and through a reconstitution study using the mast cell deficient mice [371]. In
addition to SCF and IL-3 [365], optimal culture conditions for mast cell development
include IL-6 as a comitogen, and IL-10 [331, 372, 373]. Mast cell precursor have been
shown to be bipotential, since they can be manipulated in culture with IL-10 to differentiate in macrophages [330, 331, 368, 372-374].

The CD34+/c-kithi/Thy-1lo/CD13+/CD14-/CD23- precursor cell develops into a macrophage not only in response to IL-10, but also requires signaling from GM-CSF and M-CSF for maturation in the periphery [375, 376]. While not discussed in detail here, dendritic cells resemble macrophages, and may also originate from this lineage [377]. They constitute a diverse family of antigen presenting cells with their functionality determined based on their ontogenic origin from either the myeloid or lymphoid precursor [295]. For this reason, the mention of dendritic cells will be limited to this excerpt with the understanding that dendritic cells are a critical component to the inflammatory response, but are not recognized as having a unique relationship with mast cells.

**Mast cells require cell to cell contact for growth and differentiation, macrophages do not**

The ECM is an abundant source of SCF, and consequently, mast cells tend to be located in the submucosa of the lung in direct contact with fibroblasts, blood vessels, nerve fibers, mucous secreting cells and the airway epithelium (Figure 9). Tactile stimulation may be key to mast cell survival, since overlaying mast cells on fibroblasts in culture extends their survival time, while fibroblast conditioned media alone is inadequate [378, 379]. The lack of tactile stimulation may account for the results obtained in studies where mast cell precursors, which have seemingly committed to the mast cell lineage, were manipulated to differentiate into macrophages in the presence of IL-10. While they are adherent cells, macrophages are easily passed in culture indicating a relative lack of need for heterotypic cell to cell contact for survival. The fact that
macrophages tend to reside in extracellular spaces with only moderate contact with neighboring cells, reinforces their virtual independence from the ECM, and thereby distinguishes the macrophages functionality from that of the mast cell (Figure 9).

**MAST CELL SURVIVAL AND PHENOTYPE**

Gauging the contribution of peripheral mast cells to inflammatory responses, in vivo, is a difficult task due to their relationship with macrophages, their spatial separation, vast heterogeneity, and limited numbers. Furthermore, since the mast cell’s peripheral survival is dependent upon the availability of SCF from neighboring cells, as evidenced by their anatomical location in mucosal surfaces, and their special co-culture requirements, it is difficult to distinguish mast cell function from other confounding factors in the microenvironment. Fortunately, the non-promiscuous, virtual dependence of SCF-c-kit signaling for mast cell survival in vivo has allowed for the formation of a variety of spontaneous mutants which completely lack peripheral mast cells due to a defect in this signaling pathway [380].

*Mutations at the W or Steel locus account for mast cell deficiency*

Mast cell deficient mice (MCD mice) develop when one of numerous mutations occurs in the genes mapping to the dominant white spotting locus (*W*), or the Steel locus (*Sl*) in the mouse genome. The gene products from these loci, the c-kit proto-oncogene, and the Steel gene, respectively, are necessary for providing the molecular signaling pathway for constitutive mast cell development [381-384]. The normal product of one of
the two alleles of the c-kit gene is a transmembrane tyrosine kinase receptor called by the same name, and widely expressed on the surface of a variety of different cells [381, 385]. The ligand for c-kit is the product of one of the two alleles of the Sl gene found at the Steel locus [382, 386, 387]. Through alternative splicing, the normal Sl gene encodes both a soluble and membrane bound form of the c-kit ligand, called stem cell factor (SCF). SCF-mediated signaling via c-kit (SCF-c-kit) provides an important growth and survival signal for many cells and developmental processes including hematopoiesis, melanogenesis, spermatogenesis, cognitive development, peripheral mast cell maturation, and survival. Of note, is the fact that homozygous mutations occurring on both alleles of either c-kit or SCF results in a lethal mutation.

**Sl/Sld mice**

A heterozygotic mutation of the Steel gene results in mast cell deficiency. While not discussed in great detail here, the Sl/Sld mutant has a point mutation in a membrane targeting region of its cytoplasmic domain, thereby decreasing both membrane bound and soluble release of SCF [388]. This mutant is considered a model for mast cell deficiency, because progenitor mast cells may develop in the bone marrow, but never mature in the tissues, since the membrane bound form of SCF is required for efficient SCF-c-kit signaling in the periphery.

**W/Wv mice**

The specific mutant utilized in this study, called the W/Wv mutant, results from a heterozygotic mutation in the tyrosine kinase domain of the c-kit receptor [389]. Specifically, this mutation blocks the required ability for c-kit molecules to autophosphorylate upon dimerization with its ligand, SCF. The benefit of using this
mutant strain over the $Sl/Sp^d$ mutant, is that when supplied with mast cell progenitors, the $W/W^v$ mouse can restore peripheral mast cell expression and survival, since the SCF gene is intact and the wild type progenitor mast cells express a normal c-kit receptor [384, 390]. This technique has proven to be a useful tool, and a common practice, for validating the results obtained with the $W/W^v$ mouse.

*Mast Cell Heterogeneity*

Membrane bound SCF is the predominant growth factor for mast cells in the peripheral tissues [391-393]. While mast cells do have the capacity for autocrine SCF-c-kit ligand signaling, the expression of membrane bound SCF on neighboring cells represents the key to mast cell survival. This varied interaction of mast cells with its neighbors also confers a unique functionality to every mast cell, and accounts for the vast amount of heterogeneity within mast cell populations [390, 394] to include differences in proteoglycan, histamine and protease content in the secretory granules [317, 395-405]. Alterations in the composition of the secretory granule content is dependent upon the cytokine milieu in the local microenvironment, and the strength of the adhesive interactions between the mast cell and its neighbors. The significance of the differential expression of cytokines in the microenvironment is that the mast cell may change both phenotypically and functionally depending upon the nature of an ongoing inflammatory response, and therefore, may not be appropriately represented in animal models that do not clearly represent a chronic inflammatory state, as is the case with the allergic mouse model. For this reason, the classification of mast cells has become an important point of interest in the investigation of allergic disease.
Mast Cell Classification

Metachromatic staining

Historically mast cells were classified based on their location and staining properties as either the connective tissue-type, or the mucosal-type mast cell [406]. Recently though, in host defense models for parasitic infections, the conventional mast cell classifications have been reevaluated. In the gut, connective tissue mast cells can be more adequately described as a submucosal mast cell (SMMC), and the mucosal mast cell is actually an intraepithelial mast cell (IEMC). Under normal conditions, both resident SMMCs and IEMCs can be identified in the gut, but in response to helminthic infection, SMMCs can migrate from the submucosa into the epithelium, thereby complicating mast cell classification according to anatomical location [407].

T cell dependence

SMMCs and IEMCs are T cell-independent and –dependent, respectively [316, 408], as determined by worm clearance studies following *T. spiralis* infections. This discovery was deduced based on the fact that IEMC hyperplasia is a necessary requirement [407] for the elimination of adult worms during *T. spiralis* infection, as are Th₂ cells. The finding that Th₂ cells are required for worm clearance came from earlier observations in T cell deficient mice, which simultaneously revealed that athymic, nude mice completely lack IEMCs [409], despite the fact that their SMMCs repertoire is normal. Therefore, it logically follows that IEMCs require T cell-derived, Th₂ cytokines in order to survive in the periphery, since the simultaneous absence of IEMCs and T cells leads to an increased worm burden. Specifically, IL-3 and IL-9 in combination with SCF are prerequisites to the constitutive development, survival and expansion of IEMCs [410, 411]. A similar process occurs in the lung, and epithelial-associated mast cells (i.e.
IEMC) and submucosal mast cells have been shown to be Th2-dependent and – independent, respectively [408, 412]. Recently, the mechanism by which mast cells migrate from the submucosa into the epithelium has been identified for human asthma [413], indicating a similar change in mast cell phenotypes in the lung.

Protease expression

The identification of a family of mast cell-specific neutral proteases has allowed for differential classification of mast cells based on their granule content [414]. Alterations in protease expression occurs due to the influence of cytokine signaling, and changes throughout the migratory process during development and inflammation. This collection of neutral proteases is now known to comprise an extensive family of active enzymes of similar structure and function, but each with a unique specificity. Some of their functions include the activation of proteinases found on the surface of neighboring cells [300, 415], degradation of the ECM, and anti-coagulant activity through the cleavage of fibrinogen into fragments [416].

In the mouse, the most commonly reported proteinases are mouse mast cell protease (mMCP) -1, -2, -5, -6, -7, and –9 [417, 418]. The mMCP-6 and –7 display trypsin-like activity and are referred to as tryptases [419]. The mMCP -2, -3, -4, -5, and –9 display chymotrypsin-like cleavage capabilities and are categorized as chymases [420, 421]. Another secretory granule protease, mouse mast cell carboxypeptidase A (mMC-CPA) is an exopeptidase involved in liberating active enzymes from their association with the granule matrix during activation-mediated exocytosis [422, 423]. Both chymases and tryptases have been implicated in human disease. Tryptase serves as a specific marker for mast cell activation and is currently considered to be a prime target for treating inflammatory diseases like asthma, since the structure for the enzymatically
active form has been identified, holding promise for this tetramer as a potential target for therapeutic intervention [147, 424-428].

Tryptases actually comprise a family of neutral, serine proteases which are stored in the secretory granules and released upon mast cell activation to serve as an anticoagulant, to degrade neuropeptides, facilitate tissue remodeling by degrading fibronectin, and to enhance fibrinogenesis in wound repair. There are currently two major forms of the tryptase molecule, the α and the β are the major isoforms [429], each of which can be further separated into several distinct isoforms within the same class. For example, there are currently three different forms for β-tryptase labeled βI, βII, and βIII [430]. Determining the functional role for each of these proteases in situ has proven to be a difficult challenge due to their limited quantity in vivo and unknown specificity, however recombinant DNA technology currently provides a mechanism for artificially producing recombinant forms of the human models for further study [431].

Extracellularly, tryptase is stabilized by heparin [416], and is resistant to deactivation by all endogenously known proteinase inhibitors [425], which may account for its longevity allowing for participation in both the EAR and the LAR [146, 147, 416, 428, 432]. Tryptase inhibitors can completely abolish the LAR indicating a prominent role for mast cells in both the EAR and LAR [428]. A natural tryptase inhibitor, lactoferrin, is released by neutrophils and has also been shown to abolish the LAR in allergic sheep [427]. Furthermore, tryptase expression can be enhanced by cysteinyl leukotrienes, another mast cell product of the EAR [433].

In tissue remodeling, tryptase cleaves proteinase activated receptor-2 (PAR-2) on epithelial cells [415], which in turn activates matrix metalloproteinase-9 responsible for
degrading the ECM. Cleavage of PAR-2 from the surface of fibroblasts stimulates their proliferation [300]. Additionally, tryptase can directly cleave fibrinogen into fibrinogen fragments thereby releasing blood clot formation which may be formed during tissue injury and must be removed for tissue repair [416].

**MAST CELL ACTIVATION**

*Exocytosis versus secretion*

In addition to the IgE-mediated mast cell activation utilized in this study, there are a variety of other mechanisms for mast cell activation, which may result in the differential expression of mast cell mediators. Mast cells can release pre-formed and newly synthesized mediators via exocytosis and secretory pathways, respectively. Even during exocytosis, the extent of mast cell granule release may be increased or decreased depending on the nature of the inflammatory response, indicating that the mast cell can operate at a variety of levels in the inflammatory response.

*Artificial stimuli*

Several drugs can activate mast cells and have been used extensively for studying mast cell activation in both humans and animal models. Compound 48/80 induces mast cell activation and is often used experimentally to deplete the contents of mast cells granules. This technique is useful when attempting to determine the contribution of mast cells to a specific physiologic or immunologic response [350, 434]. Given the early identification of these mast cell activators (1970’s), it is not surprising that a variety of drugs have been developed to inhibit mast cell activation. Specifically, cromolyn derivatives are known to be the most efficient drugs available for the maintenance of
asthma, but not for acute episodes. It acts as an agonist to substance P-mediated mast cell activation [435, 436]. Other experimental manipulations utilized to activate mast cells in humans include the inhalation of hyperosmolar saline, the inhalation of cold air, and the inhalation of histamine which not only activates smooth muscle fibers, but can also act on mast cell histamine receptors to induce their degranulation. [433, 437].

**Physical stimuli**

As noted above, the inhalation of cold air, hyperosmolar saline aerosols, and acidic solutions like SO₂ can trigger mast cell activation. Each of these mechanisms individually represents a change in temperature, pulmonary surface tension and pH, respectively [433, 438, 439]. For this reason, the mast cells can be thought of as a rheostat in the lung responding to the physical changes induced by exposure to the external environment. However, their activation in this capacity is likely secondary to the activation of sensory nerve fibers, whose neuropeptides are known to directly stimulate mast cells. Additionally, metabolically stressful situations, such as exercise or hypoxia, cause the release of a metabolic by-product, adenosine, which is known to directly cause mast cell activation and to promote inflammation [440].

**Biologic stimuli**

Viruses and bacteria can activate mast cells in a non-IgE-dependent fashion [441-443]. As in macrophages, bacterial binding to PRR receptors on the surface of the mast cell causes activation [444, 445] inducing histamine release [446] and increased epithelial permeability [447]. *Escherichia coli* induces histamine release from mast cells which can be differentially secreted depending upon local conditions including pH and temperature
Bacterial infection in conjunction with treatment with bradykinin increases the release of serotonin from mast cells [448].

Vasoactive peptides

The most potent natural activator of mast cells are the complement fragments C3a and C5a, also known as anaphylatoxins [449-451]. Receptor binding of these fragments results in the massive release of preformed histamine, and other mediators which causes a severe drop in blood pressure leading to death. On a less dramatic scale, complement-mediated activation of mast cells is linked to the induction of the inflammatory cascade through the induction of vasodilation to increase local blood flow, increases permeability of the vascular endothelium leading to edema, and initiates the chemotaxis of neutrophils through the proteolytic activation of chemokines like, IL-8 [450, 452, 453]. Another vasoactive peptide known to activate mast cells, bradykinin, is a member of the kinin-kallikerin system, which is known to strictly regulate vascular pressure, thereby alluding the intimate relationship of the mast cell to the maintenance of hemostasis [395, 454, 455].

Endothelins are a family of ubiquitously expressed vasoconstrictive peptides released by a variety of cell types. They possess diverse biological function due to their bilateral communication with the hypothalamic-pituitary-adrenal axis, as evidenced by endothelin-mediated release of both pituitary and adrenal hormones [456], and by the observation that corticosteroids inhibit their production [457, 458]. In addition to endocrine functions, endothelins operate locally in the maintenance of hemostasis [456]. They are a prominent feature in the pathogenesis of hypertension [459], and are extensively described for their contribution to vascular remodeling in ulcerative colitis.
With respect to the lung, they are known to induce bronchoconstriction through a receptor-mediated binding mechanism on bronchial smooth muscle cells [461]. The expression of endothelins are upregulated by the bronchial epithelial cells of asthmatics [462], and asthmatic patients which are medicated with steroids have decreased epithelial cell expression of endothelin as a result of treatment [463].

Endothelins are believed to be the most potent activator of mast cell histamine release, in vivo [464]. Under normal conditions, the relationship between mast cells and endothelin expression may be antagonistic in nature given their hypo- and hypertensive capabilities, respectively. Endothelin-mediated mast cell activation may serve as a balancing mechanism between vasoconstriction and vasodilation in response to physical and metabolic changes. Another similarity between increased endothelin levels and mast cells is in the observation that they each participate in the process of angiogenesis [304, 306, 465]. Specifically, endothelin stimulates the production of vascular endothelial growth factor in vascular smooth muscle cells at levels reflective of those observed during hypoxia [466, 467], while secretion of mast cell-derived histamine and heparin induces the migration of endothelial cells to the ends of growing capillaries [468-471]. This could account for the increased vascularization in the lungs of asthmatics, especially since tryptase, another mast cell mediator has recently been shown to promote angiogenesis as well [426].

Since asthmatic hospitalization have been correlated with an under use of inhaled steroids, an increase in emotional stress and depression, and an increase level of exposure to allergenic triggers [460], a role for the enhancement of mast cell activation by endothelin provides a link between the mast cell and the hypothalamic-pituitary-adrenal
This relationship is not related to the EAR, but probably occurs via secretory mechanisms [463, 473]. This speculation has yet to be tested, but the interaction between the immune system and the endocrine system are of intense interest, as steroid therapy is a common treatment for asthma. Furthermore, given the prominent component of allergen and emotional triggers associated with asthma symptoms, a basis for understanding the intimate relationship between the endocrine, immune, and nervous system are critical to optimizing therapeutic intervention [457, 458].

Neuropeptides

In keeping with the theme of the dynamic interaction of mast cells with neighboring cells, and the effect this interaction may have on mast cell phenotype and function, a brief discussion of two neuropeptides capable of activating mast cells is in order. In response to injury or an axon reflex [474], sensory nerve fibers release tachykinins and nerve growth factor (NGF), both of which activate mast cells to promote the release of histamine [475], serotonin [476], and the selective induction of TNF-α secretion [477, 478]. In the skin neuropeptide release accompanied by mast cell activation is referred to as the wheal and flare reaction [479], and is characterized by redness, swelling, and pain due to vasodilation and plasma extravasation [480, 481].

The differential response to tachykinins in conjunction with mast cell heterogeneity have been demonstrated for substance P [404] [396, 397]. While the direct injection of substance P into normal skin induces the wheal and flare reaction via both mast cell -dependent and –independent mechanisms [482], its effectiveness is not nearly as potent at other anatomical locations. Specifically, greater than one hundred-fold concentrations of substance P are required to activate mast cells from the rat peritoneum.
than from rat skin [479]. In respiratory system, tachykinins have been reported to cause bronchoconstriction by stimulation of postganglionic vagal nerve endings and through mast cell activation [455, 483]. However, these findings are somewhat controversial since it has also been reported that lung mast cells do not respond to substance P at all [400, 484]. These conflicting results demonstrate that the mechanism of mast cell activation is related to its anatomical location. Specifically, nerve endings that stain positively with a monoclonal antibody to substance P can only be identified in close association with the expression of neurokinin receptors in the central airways [485], a location which also coincides with mast cell populations in the lung. This anatomical relationship explains the observation that increased levels of substance P may be recovered from the bronchoalveolar lavage fluid of asthmatic subjects, [486], and that its release coincides with increased histamine levels in both the EAR and the LAR [146].

Nerve growth factor (NGF) is an important intermediate for the elicitation of pain, swelling, and neutrophil recruitment [487, 488]. Like tachykinins, the direct injection of NGF into the skin induces the wheal and flare reaction. NGF causes increased vascular permeability and edema, neutrophil recruitment and the elicitation of both peripheral and central sensitization to the hyperalgesic sensation of pain following mechanical or thermal injury. It has been proposed that the direct activation of mast cells by NGF is the primary mechanism leading to inflammation in the skin. Mast cells are required to provide the major signals leading to the activation and subsequent expression of adhesion molecules on the endothelium, which allow for the extravasation of granulocytic cells into the affected tissue. The mast cell’s relationship to NGF-mediated pain sensitization is reinforced by the observation that mast cell numbers are increased and positively correlated with NGF levels in the urine of subjects suffering from chronic bladder pain,
referred to as interstitial cystitis, where NGF and tryptase levels have been identified as good clinical markers to the diagnosis of disease [489]. Other studies in the skin have indicated that NGF induces the secretion of histamine from mast cells, rather than exocytosis, which may conversely stimulate other peripheral nerve fibers for initializing the sensation of pain [487, 490]. These studies reinforce the bilateral communication observed between the mast cell and its neighbors through the mast cell’s secretory pathways.

While chronic pain is not associated with asthma, increased innervation and sensitivity of nerve fibers decrease the threshold of the asthmatic lung to physical stimuli. This implies that the bilateral communication between mast cells and nerve fibers may be an important feature to the development of asthma. Interestingly, as there is an EAR and LAR observed in IgE-mediated asthmatic responses, which are attributed to mast cells and T cells, respectively, the sensation of pain is also characterized as having an early and late response similarly mediated by mast cells and T cells, respectively. And with respect to repair mechanisms, mast cell numbers are increased during the tissue repair process following injury [491, 492], reinforcing their potential role as modulators in airway remodeling in asthma.

**FceRI crosslinking**

At the high end of the scale for both artificial and natural means for mast cell activation, FceRI crosslinking represents the most reliable mechanism leading to the immediate release of histamine and other preformed mediators. IgE-mediated mast cell activation triggers the EAR, while simultaneously initiating the oftentimes more severe, T cell-mediated, LAR. While the EAR is thought to significantly contribute to the
morbidity associated with asthma, the significance of the mast cell’s participation to the chronic inflammation associated with asthma has been questioned. In humans, not all LARs are preceded by an EAR, and in mice, pulmonary eosinophilia can occur in the absence of mast cells. However, this disparity may represent the individual to individual phenotypic differences observed for such a dynamic process, and reflects back to the overall heterogeneity observed in mast cell populations whereby phenotypic heterogeneity may translate into functional differences. Genetic differences are also known to affect the outcome of IgE-mediated responses in that there are high-IgE producing strains, like the BALB/c and AJ mice, and low-IgE producing strains, like the C57BL/6 [184, 493]. However, as mentioned previously, for those low-IgE producing strains, IgG bound to FcγRIII induces mast cell activation [494] and may serve as an alternative pathway for mice of the C57BL/6 background during type I responses (Figure 9).

LEUKOCYTE RECRUITMENT

The Endothelial-Leukocyte Adhesion Model

The model which describes the process by which leukocytes extravasate from the bloodstream into tissues is called the endothelial-leukocyte adhesion cascade [495]. This process details the sequential binding of leukocyte-associated adhesion molecules with activated endothelium adjacent to sites of tissue infection or injury. The process is dependent upon the sequential completion of four distinct steps [253].

1) Rolling adhesion
2) Tight binding
3) Diapedesis
4) Migration
The mechanisms for both granulocyte and lymphocyte recruitment, into inflamed tissues and lymphoid organs, respectively, have been studied within the context of this the endothelial-leukocyte adhesion cascade. Neutrophil recruitment will be described here in order to illustrate the importance of mast cell activation for inflammatory cell extravasation, and later these mechanisms will be related to eosinophil recruitment. Compelling evidence relating mast cell activation to both neutrophil and eosinophil recruitment are demonstrated by their assistance in what was originally called the “histamine effect”, but what is now recognized as the multifaceted upregulation of adhesion molecules on the endothelium [496-498]. Additionally mast cells have been recognized for their contribution to the generation of a chemotactic gradient necessary for inflammatory cell homing [495, 499, 500], as well as for the degradation of the ECM which is important for extracellular migration of inflammatory cells into the tissues [501-505].

**Selectin-mediated rolling**

The requirements for the first step in this process is the systemic circulation of neutrophils bearing the cell surface molecule referred to as L-selectin (CD62L). Inactive, hematopoietic cells express L-selectin on their cell surface, indicating their state of readiness for recruitment. By contrast, the endothelium has to be activated by pro-inflammatory stimuli in order to initiate this process. During the earliest stages of inflammation, P- and E-selectin are upregulated within minutes, to hours, respectively, on perivascular endothelial cells in response to mast cell-derived mediators. Histamine and TNF-α are the predominant mediators for P- and E-selectin expression, respectively [496, 506-510]. Interaction of the passing neutrophils expressing, L-selectin and P-selectin glycoprotein-1 [PSGL-1, 511], with the endothelial bound selectins, serves to slow the
neutrophil’s velocity through the intermittent binding of these selectin molecules as the neutrophil rolls across the “sticky” endothelium. Differential regulation of P- and E-selectin provides a means for cellular specificity with respect to both the temporal and spatial signals elicited during the inflammatory response.

Expression of both P- and E-selectin is induced by different mechanisms. Pre-formed P-selectin can immediately expressed by endothelial cells following stimulation with complement component C5a, thrombin, bradykinin, and free radicals [450]. However, the mast cell mediators, including leukotriene B4 and C4 have been implicated, with histamine being the predominant factor leading to the expression of P-selectin. In contrast with the rapid expression of P-selectin, the expression of E-selectin requires de novo synthesis which is initialized by mast cell-derived TNF-α, and is also initiated due to signaling via IL-1-like receptors [497, 498, 512, 513]. Mast cells participate in the IL-1 signaling pathway leading to the expression of E-selectin since the release of mast cell chymase cleaves IL-1β to an active form.

Cellular activation can be described in the context of numerous processes. For the neutrophil, activation leading to the release of toxic mediators represents their penultimate state of activation and effector function. However, throughout the course of their recruitment, neutrophils undergo several modifications, each of which lowers their activation threshold for the induction of effector functions in the tissues. The first of these steps is noted during the rolling process, whereby P-selectin bound neutrophils are activated by endothelial and mast cell-derived and platelet activating factor (PAF) and IL-8. This activation step coincides with the shedding of L-selectin, and initiates the expression of another class of adhesion molecules, referred to as integrins, which mediate
the second step in this process [514, 515]. For this reason, the rolling of the neutrophil across the endothelium is a critical step in the recruitment process, and has been demonstrated to be absolutely necessary for neutrophil recruitment [516].

**Integrin-mediated tight binding**

While the rolling step is initiated by activated endothelial cells, the tight binding step is dependent upon three different factors including the neutrophil’s ability to express and distribute integrin molecules, the release of heparin-binding chemokines, and the variable expression of the integrin ligand, ICAM-1, on the endothelium. As mentioned previously, the rolling process initiates the upregulation of the integrins. Specifically, two β2 integrins, CD11a/CD18 and CD11b/CD18, which are commonly referred to as LFA-1 and Mac-1/CR3, respectively [495], and stored in cytoplasmic granules of the neutrophil facilitating their rapid expression during the rolling process. Cytoskeletal rearrangement is another process initiated during rolling, but that is completed due to integrin receptor signaling during the tight binding phase.

Tight binding is an integrin-dependent process which initializes another level of activation for the neutrophil. A calcium-dependent, conformational change in the integrin molecule is induced in order to increase the affinity and avidity of the integrin molecule to its counterligand on the endothelium. This occurs through a mechanism called “inside-out” signaling, which in this case is due to G-protein linked receptor signaling by IL-8 and other C-X-C chemokines. G-protein activation induces the release of intracellular calcium stores, thereby altering the conformation of the integrin molecules on the neutrophil from the “inside–out” [517-519]. In the lung, it has been established that TNF and IL-1 act on pulmonary fibroblasts, epithelial cells and endothelial cells to release chemokines [495, 520, 521], which include, but are not limited to IL-8, GRO-α, and MIP-2 in rodents [522-524]. Cellular activation and integrin
conformation are prerequisite for tight binding to the endothelium, and the ligand primarily responsible for this step is ICAM-1 [518, 525, 526]. Like chemokine production, increased ICAM-1 expression is readily induced by TNF-α and IL-1 signaling [527]. ICAM-1 has been shown to be important for neutrophil recruitment to the lung, as indicated by the slow rate of extravasation when selectin binding provides the primary means for tight binding before diapedesis [528, 529]. Therefore, the differential expression of ICAM-1 can significantly alter the efficiency of inflammatory cell recruitment [530].

**Diapedesis and migration**

The last two steps are not defined in as much detail here, but will briefly be summarized. The third step in the endothelial-leukocyte adhesion cascade is a process called diapedesis, which means the physical translocation of the inflammatory cell through the gaps in the endothelium and into the tissue interstitium [531]. This process involves integrin-mediated binding and cytoskeletal rearrangements. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) mediates the physical interaction between the neutrophil and the endothelial cell, as it is expressed on each cell. Mast cells facilitate the diapedesis process through their release of histamine which opens gaps in the endothelium, followed by TNF-α-mediated localization of the PECAM-1 molecules in between these endothelial gaps [532]. When PECAM-1 on the neutrophil binds to the focally expressed PECAM-1 on the endothelial cell, it is able to pull itself between the endothelial cells and into the tissue [532-535]. Before the cell can squeeze through this narrow gap, it must first become very flat through cytoskeletal rearrangements mediated by the β1 chain of the integrin molecule. Once inside the interstitium, the neutrophil must release enzymes for breaking down the ECM allowing for its progression into the tissue.
Mast cells assist in this degradation process through their release of proteolytic enzymes and collagenases. At this point, the chemotactic gradient shifts from that of a soluble gradient to a chemotactic substrate mediated by adhesive interactions with surrounding cells. Even less is known about those stimuli that account for a shift from a migratory pattern into an effector cell capable of releasing its toxic mediators, but is likely due to a cumulative activating signal throughout the progression of the migratory process.

**Neutrophils**

Mast cell-derived TNF-α is the primary cytokine responsible for upregulating adhesion molecules during the initiation of the endothelial-leukocyte adhesion cascade. Convincing evidence to this fact is provided by studies in mice, using caecal ligation puncture (CLP) as a model for acute sepsis, where neutrophil recruitment is known to be essential for survival. Following CLP in TNFR deficient mice, neutrophil recruitment and survival are severely reduced, indicating a central role for the expression of TNF-α in this process. Furthermore, given the fact that mast cell deficient mice were also highly susceptible to CLP, while complement receptor deficient mice were not, indicates that TNF-α must originate from mast cells [536].

Another study indicating a prominent role for mast cell mediators for neutrophil recruitment was reported following the exposure of mast cell deficient mice to ozone. Ozone activates alveolar macrophages causing the release of PGE₂ and TNF-α. Despite the fact that alveolar macrophages are a primary target during ozone exposure releasing large quantities of TNF-α, it is the complete lack of peripheral mast cells which is responsible for inhibiting neutrophil recruitment after exposure [537]. Some have argued that since mast cell deficient mice have a defect in SCF-c-kit signaling that there may
have been some dysfunction associated with alveolar macrophage development and function [538], and therefore caution should be taken in interpreting the results obtained from these studies. However, it could be argued that a minor debilitation on the part of the alveolar macrophage would unlikely overshadow the complete absence of an entire cell population, leading to such convincing results. Especially since the evaluation revealing a decrease in macrophage numbers was performed in a doubly deficient mouse, the $W/W^v$ mutant and a SHP-1 knock-out. Given the complexities of signal transduction, it would be unreasonable to dismiss the results attained in mast cell deficient mice simply due to moderate anemia.

**Eosinophils**

Eosinophil recruitment from the bloodstream into the tissue requires a series of sequential events that have been described in the endothelial-leukocyte adhesion cascade. As mentioned earlier, rolling is by far the most important step in the process, because it is required to reduce the velocity of the cell while simultaneously activating the integrin expression. Eosinophil rolling is achieved when constitutively expressed PSGL-1 intermittently adheres to P- or E-selectin on the activated endothelium. Studies in gene knockout mice have shown that in the absence of P-selectin, eosinophil recruitment is abated following antigen challenge. Similarly, PSGL-1 blocking antibodies deter eosinophil recruitment following OVA challenge indicating the critical importance of PSGL-1 ligand interactions for initiating the endothelial-leukocyte adhesion cascade. As with neutrophil recruitment, mast cell activation leading to the upregulation of selectin molecules involves the release of histamine, as well as cysteinyl leukotrienes and TNF-α. It has also been demonstrated that IL-4, a mast cell product synthesized de novo upon
cellular activation, can induce the upregulation of P-selectin. Despite the evidence for redundant pathways leading to the upregulation of selectin molecules [450], during an IgE-mediated reaction to antigen, the release of mast cell mediators like histamine would certainly overshadow any other overlapping mechanisms.

As the inflammatory cascade proceeds from rolling to tight binding, there is a great deal of redundancy with respect to adhesion molecules, however simultaneous chemokine and cytokine signaling confers a level of specificity for adhesion of eosinophils versus neutrophils. While IL-8 induces the expression of integrin molecules during rolling on E-selectin [539], IL-5, eotaxin, and SCF enhance this process by facilitating the expression and binding of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, both of which bind to vascular cell adhesion molecule-1 (VCAM-1) and fibronectin [540-544]. The $\alpha_4$ subunit is a prominent feature in antigen-mediated allergic responses [545], with its expression noted to be upregulated on eosinophils in human asthma, and to be positively correlated with disease severity [546]. Similarly, IgE binding to FcεRII on human eosinophils has been shown to enhance integrin affinity in atopic subjects [547]. Recent studies in helminthic infection models, where both mast cells and eosinophils are key to survival, have demonstrated the necessity for the $\beta_7$ integrin chain for both eosinophil recruitment and mast cell hyperplasia in the gut, since $\beta_7$ knock-out mice displayed reduced leukocyte homing and attenuated protective immunity to *Trichinella spiralis* infection [548]. Diapedesis and migration may be mediated through the C-C chemokines eotaxin, eotaxin-2, monocyte chemotactic protein-4 (MCP-4), and RANTES, since inhibition of the C-C chemokine receptor-3 (CCR3) inhibits eosinophil migration [549-551]. To reiterate, the early steps in the inflammatory cascade following antigen exposure rely
heavily on the activation of mast cells with the subsequent upregulation of P-selectin, a necessary signal for eosinophil rolling and adhesion to the endothelium. Without rolling and adhesion, there would be no extravasation into the tissues where the eosinophil would receive a variety of survival signals from adhesion to the ECM and through cytokine signaling from IL-5, IL-3 and GM-CSF.

The known parameters described for eosinophil recruitment in the endothelial-leukocyte adhesion cascade clearly indicate a role for mast cell activation as IL-5, SCF, eotaxin, IL-8, TNF-α and IL-1β are known to participate in this process, and may all be produced or induced by mast cell activation. While these cytokines are synthesized by other cell types, the importance of mast cell activation is evidenced by the fact that the direct injection of eotaxin induces eosinophil recruitment within one hour. However, in the absence of mast cells, while eosinophil recruitment can still occur, it takes twice as long [552]. For example, it has been reported that in antigen challenged, mast cell deficient mice eosinophil recruitment to the lung was found to be normal in comparison with littermate controls. However, one problem with biphasic studies as in allergic reactions is that the time frame in which the endpoints were examined, namely 48 hours after antigen challenge, exceeds the level of sensitivity for adequately determining the contribution of mast cells to the process [173, 174, 176, 179]. By 48 hours, the number of eosinophils allowed to extravasate into the tissue may have been saturated to a point that the effect of mast cell activation on eosinophil recruitment was overshadowed by the T cell-mediated arm of the response. Additionally, eosinophils themselves can produce cytokines like IL-5, eotaxin and GM-CSF which serve to promote survival and the recruitment of more eosinophils to the target tissue, thus perpetuating the response.
Evaluating the effect of mast cell activation at these time points, during the T cell-mediated, late phase reaction does not dismiss the importance of mast cell activation to the recruitment process, but rather confirms the redundancy of the immune response, and identifies a need for determining the specific role of mast cells to the initiation of the inflammatory cascade.

In closing, this introduction has demonstrated that the mast cell activation can affect both acute and chronic inflammatory responses in both innate and adaptive immune setting. Mast cells are important contributors to eosinophil recruitment, and under normal circumstances for an IgE-mediated response, are likely the primary initiators of eosinophil recruitment and extravasation. Mast cell deficient mice do not always reveal the true contribution of mast cells to this response, since downstream, redundant inflammatory processes can compensate for their absence. Still, their central role in the inflammatory response should be held in high regard, despite these obstacles associated with the practical aspects of experimental design.
PURPOSE OF STUDY

As mentioned previously, mast cells are closely related to macrophages. In keeping with the concept that mast cell phenotype and functionality are determined by the local cytokine milieu, it is not surprising that human lung mast cells have been reported to phenotypically resemble macrophages[316, 373, 374]. Mast cells can mature in the absence of one of their distinguishing markers, the expression of FcεRI, which is induced in normal mast cells via IL-4 signaling, and they have been noted to express the monocyte cell surface molecule, CD13 [330, 553, 554]. Both mast cells and alveolar macrophages are regarded as a communication link between the airway epithelium, the immune system, the CNS and the endocrine system. Each possesses diverse functionality serving as the first line of defense in the innate immune response. Namely, they both express a variety of antibody receptors for the detection of opsonized antigens, and like the mast cell, the alveolar macrophage can express the FcεRI for IgE activation in response to allergen exposure.

The major functional difference between mast cells and alveolar macrophages is that macrophages do not constitutively express FcεRI, do not develop secretory granules containing preformed bioactive mediators in the way that mast cells do, and lack the intimate heterotypic cell to cell contact that governs the mast cell’s phenotype and
functionality in the periphery. Instead, alveolar macrophages are found at the surface of the airway epithelium where they are primarily responsible for phagocytizing and opsonizing foreign bodies arriving in the lungs through the airways, and for responding to changes in oxygen tension during respiration. While alveolar macrophages are certainly an abundant source of mediators, evidence supports the fact that during the innate immune response, characterized by the recruitment of granulocytes such as neutrophils and eosinophils, the activation of alveolar macrophages, and other cells, likely initializes mast cell activation. This in turn, causes the release of preformed mast cell mediators, which collectively signal the global orchestration of the immune response by initiating the endothelial-leukocyte adhesion cascade, and by providing the neurogenic, endocrine, and immunologic stimuli necessary for resolving the disruption caused by the offending agent.

Particulate air pollution activates alveolar macrophages and bronchial epithelial cells to release a host of immunologically active mediators. The mechanism by which this innate response occurs is unknown, but is believed to be due to a combination of the physical stimulation produced by inertial impaction of particles in the airways and their subsequent elimination by alveolar macrophages [555], along with the elution of soluble particle components such as transition metals, toxic organic molecules, and acidic sulfates [556] into the lung lining fluid. In the rat, the transition metals iron, nickel, and vanadium have all been attributed to pulmonary toxicity associated with PM exposure as evidenced by the recruitment of both neutrophils and eosinophils [557].

The manner in which the alveolar macrophage responds to the presence of particles in the lung is highly dependent upon dose, since higher doses increase the rate of
particle phagocytosis, thereby providing protection against the induction of an overt inflammatory response. Given the fact that macrophages demonstrate the capacity to modify innate resistance with respect to particle dose, and that this modification is related to, but separately regulated for neutrophil recruitment, indicates that there is some level of control and communication shared between the alveolar macrophage and the mast cell. This is reinforced by the fact that alveolar macrophages exposed to PM release a variety of proinflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-8, and MIP-1α, all of which may modulate the critical communication between the mast cell and its neighboring tissues [558-560].

PM exposure of sensitized mice, which are actively responding to a secondary antigen challenge, display an enhanced level of pulmonary eosinophilia, IL-4 and IL-5 in their bronchoalveolar lavage fluid over the course of several days, indicating that PM can enhance Th2-mediated allergic responses [561]. By contrast, identical PM exposure in normal mice merely produces a neutrophilic infiltration which is quickly resolved within 24 hours. Since PM is known to activate alveolar macrophages in vitro, and recruit neutrophils in vivo, we defer to the fact that PM-induced inflammation is partially mediated by alveolar macrophages, but propose that they likely operate through signaling pathways which are ultimately received by the mast cell through its vast communication network. Support for this notion comes from studies using another irritating pollutant, ozone, where alveolar macrophages are known as a primary target, but mast cell activation is a requirement for neutrophil recruitment [537, 562-564]. Secondly, the observation that alveolar macrophages from allergic mice, but not normal mice, secrete SCF in response to allergen challenge indicates that alterations in gene expression occur
in the allergic lung which may enhance mast cell activation [565, 566]. In our study, we sought to determine whether or not the enhancement of eosinophil recruitment observed in allergic mice following PM exposure can be partially attributed to mast cell activation.
SCIENTIFIC METHOD
EXPERIMENT 1

Hypothesis for Experiment 1:

Mast cells are involved in PM-induced pulmonary eosinophilia and airway hyperresponsiveness in the allergic mouse model of asthma.
MAST CELLS CONTRIBUTE TO ALLERGIC
INFLAMMATION IN MICE FOLLOWING EXPOSURE TO
CONCENTRATED AMBIENT PARTICLES

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Mast cells contribute to allergic inflammation in mice following exposure to concentrated ambient particles. Elevated levels of ambient particulate matter (PM) aggravate symptoms of asthma. We tested the hypothesis that mast cells are involved in enhancement of allergic responses by PM using wild type (WT) and mast cell deficient (MCD) mice. Mice were sensitized with ovalbumin (OVA) locally (intratracheally) or systemically (intraperitoneally). All mice were challenged 2 weeks later with OVA aerosol, and then exposed to concentrated ambient particles (CAPs; average 2245 µg/m³, 3 hr/day) or air for 2 days. One day after the last CAPs exposure, no pulmonary inflammation was observed in sham-sensitized mice. In OVA-allergic mice, systemic sensitization, the presence of mast cells, or exposure to CAPs independently increased numbers of bronchoalveolar lavage (BAL) eosinophils 2-fold in comparison to local sensitization, absence of mast cells, or air exposure. Airway responsiveness to methacholine was significantly greater in allergic WT mice compared to MCD mice, and interactions with systemic sensitization and CAPs exposure enhanced responsiveness. These results show that enhancement of allergic responses by ambient PM is partially mediated by mast cells in mice.
INTRODUCTION

Several air pollutants have been shown to impair pulmonary function and aggravate symptoms of asthma such as cough and wheeze. Exposure to ozone (O$_3$) promotes inflammation and reduces peak expiratory flow in both healthy and asthmatic individuals. Evidence is mounting to implicate particulate matter (PM) as another contributor to the increased human morbidity due to asthma. Increased levels of PM have been correlated with decrements in lung function, increased hospitalizations for asthma, and school absenteeism [567].

A key cell type involved in the pathogenesis of asthma is the mast cell. Classically, the mast cell has been considered to be the primary mediator of type I hypersensitivity reactions, which comprise the early asthmatic response [568]. However, recent studies also indicate a prominent participation of mast cells in innate immune responses to bacterial infections [569], and as a critical modulator of O$_3$-induced pulmonary inflammation [537]. Mast cells are actively recruited to the bronchial epithelium of healthy and asthmatic subjects following exposure to O$_3$ or antigen, respectively [570, 571] and are important for the recruitment of eosinophils into the lung [152]. Exposure of healthy human volunteers to diesel exhaust particles caused significant increases in bronchoalveolar lavage (BAL) fluid histamine and submucosal mast cell numbers, indicating that mast cells may participate in PM-induced lung injury [572].

Recent studies demonstrate a two-way communication via cytokine signaling between mast cells and resident airway or alveolar cells. Mast cell-derived cytokines
such as tumor necrosis factor (TNF-α) and interleukin (IL)-4 may promote the production of stem cell factor (SCF) by alveolar macrophages (AM) and airway epithelial cells [AEC, 566]. SCF in turn can augment mast cell release of histamine [566], leukotriene B₄ [565], and TNF-α [573]. SCF induces recruitment of eosinophils following antigen challenge [565] and promotes airway hyperresponsiveness [AHR, 151]. Given that 1) O₃ can cause the release of pro-inflammatory cytokines and modulate pulmonary inflammation via mast cell activation [537], 2) PM can cause the release of pro-inflammatory cytokines from human airway cells [560, 574] and enhance eosinophil recruitment and AHR in allergic mice [561, 575] and 3) SCF is critical in allergic inflammation [565], we hypothesized that mast cells may also be involved in PM-induced enhancement of pulmonary eosinophilia and AHR. To test our hypothesis, nonallergic and ovalbumin (OVA) allergic mice were exposed to concentrated ambient particles (CAPs). To determine the role of mast cells in response to CAPs, mast cell deficient (MCD) mice lacking the receptor for SCF and wild type (WT) control mice were evaluated. Since route of sensitization can induce different patterns of T cell priming [295, 576, 577], we assessed the effect of OVA sensitization by the systemic route (intraperitoneal) with adjuvant compared to the more physiologically relevant local (intratracheal) route. Accordingly, the objectives of this study were: 1) to determine the effects of CAPs on both nonallergic and OVA-allergic mice, 2) to assess the role of mast cells in responses to CAPs, and 3) to examine the contribution of the route of sensitization on inflammatory endpoints following CAPs exposure. The results of this study show that exposure to CAPs can enhance pulmonary eosinophilia in allergic mice regardless of the route of sensitization, and that this response is at least partially dependent upon the presence of mast cells.
MATERIALS AND METHODS

**Animals.** Eight-week old male WBB6F1/J wild type (WT), and mast cell deficient (MCD) WBB6F1/J-KitW/KitW-v mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in an AAALAC-approved facility, maintained on a 12-hour light/dark cycle, and fed Prolab RMH 3000 (PMI, Brentwood, MO) and tap water *ad libitum*. All animals were held for a minimum of 4 days before treatment. Protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were conducted using national guidelines for the care and protection of animals. A total of 148 mice were used in this study and separated into the following groups: WT=74, MCD=74; Air-exposed=74, CAPs-exposed=74; local sensitization=72, systemic sensitization=76; nonallergic (sham-sensitized)=40, allergic=108. The number of mice in each of the 16 possible combinations ranged from 5 in nonallergic groups to 13 or 14 in allergic groups.

**Ovalbumin Sensitization.** In both local and systemic sensitization protocols, mice were sensitized by administration of a total of 30 µg of OVA (Grade V, Sigma, St. Louis, MO). Local sensitizations were carried out by three separate intratracheal instillations (i.t.) of 10 µg of OVA prepared in 50 µl of sterile phosphate buffered saline (PBS) plus 0.05% Tween-80 (Pierce, Rockford, IL). Sensitizations were carried out 21, 18, and 15 days before OVA challenge ([Figure 10](#)). Control animals received sham-local sensitization with PBS+Tween only. The instillation technique has been described in detail elsewhere [578]. Systemic sensitizations were carried out by two separate i.p. injections of 15 µg OVA in 0.2 ml of aluminum hydroxide gel adjuvant (ADJ;
Alhydrogel, Accurate Scientific, Westbury, NY). Mice were sensitized 20 and 14 days before OVA challenge (Figure 10). Control animals received a sham-systemic sensitization with ADJ only.

**Ovalbumin Challenge and CAPs Exposure.** All mice were challenged two weeks after the final sensitization (or sham-sensitization) as previously described [561]. In brief, mice were challenged by whole body exposure to an aerosol of 1% OVA in saline for one hour (Figure 10). On day 0 beginning 1 hour after OVA challenge and on day 1, mice were exposed to CAPs (2.5 µm size selective inlet; PM$_{2.5}$) in a whole body inhalation exposure system as described by Sioutas et al. [579], using a 4-stage concentrator capable of concentrating PM 100-fold (Figure 11). Control mice were exposed to unconcentrated air at the same temperature, pressure and length of time as mice exposed to CAPs. Since the concentration of PM in the CAPs exposure system is dependent on ambient PM levels and weather conditions, a protocol was devised to produce equivalent total exposure to fine PM over different exposure days despite fluctuating exposure concentrations. Equivalent exposures were achieved by monitoring chamber concentrations with a tapered element oscillating microbalance (TEOM; Rupprecht & Patashnick, Albany, NY), and integrating the recorded concentrations over ten-minute observation periods until a pre-determined level of 6,000 µg•hr/m$^3$ was attained ($\int x \mu g/m^3 \times 0.1667 \text{ hr, Figure 12}$), at which time the exposure was stopped, with a limit of 6 hr per exposure. The final CXT value was determined following exposure by gravimetric analysis of sample filters from the exposure chamber.

**Chemical analysis of CAPs.** Teflon filters (Teflo, 47 mm diameter x 2 µm nominal pore size, Gelman Sciences, Ann Arbor, MI) from each exposure were extracted
as previously described [580]. Particle extracts were prepared by soaking filters in deionized water with mild agitation for one hour. Extract suspensions were then filtered (Autovial Syringeless filter, 0.1 \( \mu \text{m} \) PVDF membrane, Whatman, Clifton, NJ) to remove fine particles and acidified to pH<2 using trace metal grade hydrochloric acid to prevent precipitation and loss of metal ions. Acidified extracts were analyzed for sulfate, manganese, iron, copper, and zinc content using inductively coupled plasma-atomic emission spectroscopy [ICP-AES, 581]). Results are expressed as ng of water-soluble sulfate or metal per \( \mu \text{g} \) PM on the original filter sample. X-ray fluorescence (XRF) spectrometry was used to determine total content of 44 elements in PM on filters [582]. Elemental carbon (EC) and organic carbon (OC) were analyzed on quartz fiber filters by thermal optical analysis (EC/OC, Sunset Labs, Forest Grove, OR).

**Airway Responsiveness to Methacholine.** Two days after OVA challenge, airway responsiveness to i.v. methacholine (Mch) was measured in anesthetized, paralyzed, and ventilated mice. Mice were anesthetized with 1.5-2.0 g/kg urethane, tracheotomized with an 18G cannula and placed on a heating pad maintained at 35° C (Delta Phase, Braintree Scientific, Braintree, MA). Animals were ventilated with constant inspiratory flow (flexiVent, Scireq, Montreal, QC) using hospital grade oxygen, and spontaneous breathing was eliminated by administration of pancuronium bromide (0.8 mg/kg i.p.; Sigma, St. Louis, MO). Breathing frequency (50\( \cdot \text{kg}^{-0.25} \text{min}^{-1} \)) and tidal volume (7.5 ml/kg) were determined by animal body weight [561]. The animal’s heart rate was monitored (SRA-200, MicroMed, Louisville, KY) with a normal range of 400-600 beats/minute. Mice were cannulated via the jugular vein using a 26G needle inserted into PE20 tubing which was attached to an automated syringe pump loaded with a 0.5
mg/ml solution of Mch in saline. Baseline measurements of heart rate, total respiratory resistance (R_T), and elastance (E_T) were taken prior to drug infusion. Bolus doses of Mch were delivered over two seconds every two minutes in half log doses ranging from 31.6 - 3160 µg/kg. Following drug infusion, R_T and E_T measurements were calculated and recorded at 6 s intervals for a total of 1 minute. To standardize lung volumes 30 s before each dose the expiratory port was occluded until airway pressure reached 20-30 cm H_2O. Resistance of the tracheal cannula was subtracted from measured values of R_T. Responses for each dose of Mch were calculated as area under the curve for 1 minute.

**Serum Immunoglobulin ELISAs.** Immediately after measurement of airway responsiveness, blood was collected and serum samples were stored at -80º C. Total serum IgE was assayed by ELISA according to the manufacturer’s instructions (Pharmingen, San Diego, CA; α-mouse IgE, clone R35-72, IgE standard, clone C38-2, biotinylated-α-mouse IgE, clone R35-118). ELISAs for OVA-specific IgG_1 and IgG_2a utilized the same protocol with the following modifications; 1) plates were coated with 5 µg/ml OVA, and the detection antibodies were biotinylated-α-mouse IgG_1 (antigen affinity purified, Zymed, San Francisco, CA) and rat anti-mouse IgG_2a (LO-MG2a-3, Zymed). Insufficient quantity of collected sera prevented measurement of immunoglobulin levels in all mice.

**BAL Fluid Cells, Proteins, and Cytokines.** Mice were lavaged with two aliquots of Ca^{2+}, Mg^{2+}, and phenol red-free Hanks’ balanced salt solution (HBSS; 35 ml/kg, Life Technologies, Bethesda, MD). The BAL fluid was maintained on ice and centrifuged at 360 x g for 12 minutes at 4º C. Supernatants were transferred to a separate tube in order to prepare aliquots for biochemical and cytokine analyses. BAL cells were
resuspended in 1 ml of HBSS and counted (Coulter Z1, Hialeah, FL). Cytospin preparations of BAL cells were made for each sample and stained with Wright’s Giemsa using an automated slide stainer (Hematek 2000, Elkhart, IN). Cell differentials were performed by counting 500 cells per slide. Assays for total protein, albumin, lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAG) were carried out as previously described [557]. Lavage fluid supernatants were supplemented with 10% FBS to prevent loss of cytokines in low protein concentration fluids, and were stored at –80º C until assayed for the presence of IL-4 (clones 11B11 and biotinylated BVD6-24G2) and IL-5 (clones TRFK5 and biotinylated TRFK4) by sandwich ELISA using paired antibodies from Pharmingen (San Diego, CA) according to the manufacturer’s instructions.

**Histological Analysis and Mast Cell Enumeration.** The right lobes of the lung were inflated using phosphate buffered formalin (Fisher Scientific, Pittsburgh, PA) at 20 cm of H₂O and subsequently stored in formalin at room temperature until the tissues were processed. Lungs were embedded in paraffin and sectioned on a full frontal plane exposing both the mainstem bronchi and the distal airways. Histological analysis was performed on hematoxylin and eosin (H&E) stained sections by assessing the presence of inflammatory cells in airways and parenchyma. Formalin-resistant mast cells were enumerated in all groups by counting the total number of toluidine blue positive staining cells on whole sections, although mast cells were generally restricted to the major airways and surrounding connective tissue.

**Statistical Analysis.** Four factors (allergic status - sham vs. sensitized; presence of mast cells - WT vs. MCD; CAPs exposure - air vs. CAPs; and sensitization route -
local vs. systemic) produced sixteen groups of mice (Figure 13). Four-way analysis of variance (ANOVA) was used to test whether any of these factors could independently affect biochemical indicators of lung injury (i.e. all 148 mice were divided into one of 2 groups for each factor). Since nonallergic groups did not show any evidence of an inflammatory response in BAL fluid, only allergic groups (n = 108) were further analyzed by 3-way ANOVA testing for effects of genotype, route of sensitization, and CAPs exposure. In instances where statistical interactions could be identified by 3-way ANOVA, data were further divided into all possible comparisons based on the factors involved and analyzed by 2-way ANOVA. Airway reactivity measurements in allergic mice were also analyzed by 3-way ANOVA for each dose of Mch.
RESULTS

CAPs exposures and chemical characterization. Three separate experiments were conducted consisting of 7 exposure days (Table 3). Exposure chamber concentrations averaged 2,245 µg/m³, while control chamber concentrations averaged 21 µg/m³ (and never exceeded 44 µg/m³), representing approximately a one hundred-fold concentration factor. Exposures reached the TEOM C•T target of 6000 µg•hr/m³ with one exception (9/30/98), when 90% of the targeted TEOM level was achieved in the 6 hr maximum exposure time allowed. TEOM and gravimetric methods of determining concentration were highly correlated, as previously shown in East Coast urban areas in summer months [583]. Gravimetrically determined C•T levels averaged 6,212 µg•hr/m³ for CAPs exposures and 50.6 µg•hr/m³ for air control exposures per exposure day. The average 2-day protocol C•T level for 3 experiments was 12,241 µg•hr/m³. The count median diameter (CMD) of the particles was 1.08 ± 0.04 µm (mean ± SE). The average temperature and relative humidity (mean ± SD) was 76 ± 1.9 °F and 50.9 ± 15.9 % for CAPs exposures and 73.3 ± 2.2 °F and 62.4 ± 3.0 % for air control exposures, respectively.

Approximately 50% of the CAPs composition could be accounted for by XRF and EC/OC analyses. The particles consisted of approximately 26% sulfate and 20% organic carbon (Table 4). Iron, potassium, calcium, magnesium, and zinc were greater than detectable limits in at least 2 of 7 filter samples, but comprised less than 1% of the total mass. Detection of soluble elements by ICP-AES confirmed sulfate levels determined by XRF, but other metals were below detectable limits (data not shown).
**CAPs exposure has no effect on biochemical indicators of lung injury.** By 4-way ANOVA (testing for allergic status, presence of mast cells, exposure to CAPs, and sensitization route), exposure to CAPs had no effect on any biochemical endpoint measured in BAL fluid ([Table 5](#)). Significant differences were observed independently for allergic status, route of sensitization and absence or presence of mast cells. Allergic mice had significant increases in LDH and protein compared to nonallergic controls (independent of mast cells, CAPs exposure, and sensitization route), indicative of increased cytotoxicity and epithelial permeability, respectively. Systemically sensitized mice had significantly greater levels of protein, albumin and NAG than locally sensitized mice (independent of mast cells, CAPs exposure, and allergic status). Surprisingly, levels of LDH and NAG were higher in MCD mice indicating that mast cell products were protective against cytotoxic responses and lysosomal enzyme release, respectively.

**Inflammatory cell infiltration and airway hyperresponsiveness (AHR) are not observed in CAPs-exposed sham-sensitized mice.** In mice which were sham-sensitized by either the local or systemic route, no significant inflammatory responses were observed in either WT or MCD mice, whether exposed to air or CAPs. Alveolar macrophages (AM) accounted for greater than 98% of BAL fluid cells in all sham-systemically sensitized mice and there was no significant difference in total cells among air- or CAPs-exposed nonallergic groups ([Figure 14A](#)). In sham-systemically sensitized mice, CAPs exposure did not affect BAL protein levels ([Figure 14B](#)), airway responsiveness to Mch challenge, or serum antibody responses; similar results were obtained from sham-locally sensitized mice (data not shown). Consequently, subsequent
statistical analyses examined effects of CAPs exposure, route of sensitization, and presence of mast cells in allergic groups only.

**Mast cells partially mediate allergic airway responsiveness.** When only allergic mice were examined by 3-way ANOVA, WT mice had significantly greater respiratory system resistance in response to i.v. Mch challenge than MCD mice (Figure 15). These results indicate that mast cell mediators enhance central airway responses to nonspecific contractile agonists in allergic mice. At the third dose of Mch (316 µg/kg), a significant interaction of genotype and systemic sensitization increasing resistance was observed, and a separate interaction of genotype, sensitization route, and exposure to CAPs was observed to increase respiratory system elastance, indicative of peripheral airway responses.

**Mast cells contribute to enhanced inflammation following CAPs exposure.** Serum IgG_{2a} was not detected in any group, but all samples from allergic mice had detectable levels of total IgE and OVA-specific IgG_{1} following antigen challenge. On average, IgE was 4-fold greater in systemically sensitized mice compared to locally sensitized mice (Table 6, \( P < 0.001 \)). Relative to optical densities (O.D.), a 10-fold increase in OVA-specific IgG_{1} was observed in systemically sensitized mice compared to locally sensitized mice (data not shown). Wild type mice had significantly higher levels of IgE than MCD mice \( (P < 0.05) \), while exposure to CAPs had no significant effect on antibody levels. There was no detectable BAL fluid IL-4 in any group, and BAL fluid IL-5 levels were low with no difference between any allergic groups.

CAPs exposure caused a significant increase in total BAL cell numbers in allergic mice (Table 7, \( P < 0.0001 \)), which was independent of genotype and the route of sensitization. A major portion of this inflammatory response can be attributed to
macrophages which increased about 40% relative to air-exposed mice (+CAPs: $27 \times 10^4$, -CAPs: $19 \times 10^4$, $P = 0.0045$). BAL eosinophils were increased more than 100% in CAPs-exposed mice relative to air-exposed mice (Table 7, $P = 0.0032$; Figure 16). A similar increase was observed for WT mice compared with MCD mice (Table 7, $P = 0.0018$; Figure 16). Eosinophils were increased about 125% in systemically-sensitized allergic mice compared with locally-sensitized mice (Table 7, $P < 0.0001$; Figure 16). Interestingly, local sensitization increased BAL lymphocyte numbers more than 200% relative to systemic sensitization (Table 7, $P < 0.0001$; Figure 16C and 16D). There was no effect of any factor on BAL neutrophil numbers, which were minimal (<2%).

*Mast cell numbers are not affected by CAPs exposure.* The inflammation observed in the BAL fluid of allergic mice was confirmed by histological analysis. There were numerous inflammatory cells surrounding the bronchioles of both WT and MCD allergic air-exposed mice, including eosinophils, lymphocytes, and monocytes (Figure 17B and 17C). CAPs exposure did not significantly alter lung pathology in either WT or MCD allergic mice (Figure 17E and 17F). Mast cells were observed exclusively in the tracheobronchial region of WT mice (Figure 17A and 17D), and were never found in MCD mice. Neither exposure to CAPs nor sensitization protocol caused any changes in mast cell numbers counted in tracheobronchial regions of WT mice. Nonallergic mice were lacking any evidence of inflammatory cells in the tissues, but leukocytes were observed in blood vessels surrounding bronchioles in both air- and CAPs-exposed mice (data not shown).
DISCUSSION

In this study, CAPs exposure did not cause overt toxicity in nonallergic or allergic mice, or significantly alter airway reactivity to methacholine. However, CAPs exposure significantly increased lavage macrophage numbers and potentiated antigen-induced infiltration of eosinophils into lungs of allergic mice. Mice lacking mast cells did not develop eosinophilia to the same degree as wild type mice. The enhancement of eosinophil infiltration by CAPs was independent of the route of antigen sensitization, although allergic systemically-sensitized mice had greater total BAL cell numbers and greater airway hyperresponsiveness compared to locally-sensitized mice. These findings demonstrate that exposure to concentrated fine ambient particles can potentiate allergic inflammation in mice and that mast cells are significant contributors to this response.

Several studies have supported the hypothesis that soluble transition metals or organic compounds can promote oxidative stress and the production of pro-inflammatory cytokines by alveolar macrophages (AM) and airway epithelial cells (AEC) [560, 574, 575, 584]. While organic carbon and sulfates were detected on the CAPs filters, there were few detectable transition metals, making it less likely that metals contributed to the observed effects. Given that carbon compounds comprised ~20% of the particle mass, and that significant inflammation was evident in CAPs-exposed allergic mice, organic compounds may have participated in the observed enhancement of pulmonary inflammation [585]. Regardless of which components are responsible for the observed effects, this study demonstrates that ambient PM in the form of CAPs is rather innocuous in nonallergic mice, but can enhance inflammation if accompanied by a pre-existing,
an antigen-specific inflammatory response. Furthermore, these data are reflective of studies in which healthy humans are not affected by acute PM exposures, but asthmatics may suffer more severe symptoms, potentially attributable to enhanced inflammation [567].

Exposure of allergic asthmatics to aeroallergens leads to the potential development of both the mast cell-mediated early asthmatic response and subsequent eosinophil recruitment in the late asthmatic response [568]. In mouse models, eosinophils are variably correlated with Mch responsiveness in the late response [152, 493, 586]. Mast cells are critical in the early response in asthma, but a role in the late response has not been precisely defined [493, 568]. The current study showed that the presence of mast cells was associated with hyperresponsiveness to Mch in the late response. Although CAPs exposure increased numbers of BAL eosinophils, CAPs did not independently enhance Mch responsiveness. However, exposure to CAPs did significantly increase respiratory system elastance in systemically sensitized WT mice, and a trend for increased resistance was also observed. Taken together, these data indicate that airway hyperresponsiveness to Mch in the late response is dependent on the presence of mast cells, and that systemic sensitization and exposure to CAPs can enhance this responsiveness.

Mast cells are involved in CAPs-induced eosinophil recruitment into the airways, but the mechanism by which inflammatory cell recruitment is up-regulated by environmental pollutants is unclear. A communication between resident airway cells and mast cells via soluble mediators or direct cellular contact has been suggested [587]. The current study supports such a communication link since there was no significant increase in AEC shedding or LDH in BAL fluid following CAPs exposure, yet there was still an enhancement of eosinophilic inflammation. Mast cell numbers were not increased in
allergic wild type mice following CAPs exposure, indicating that individual cells were stimulated to promote eosinophil recruitment by an undefined mediator. An attractive candidate is stem cell factor, secreted by resident lung cells such as AECs and AMs after uptake of particles, which is capable of promoting mast cell activation [566]. In support of this hypothesis, the inflammatory response to ozone is attenuated in MCD mice at time points preceding significant AEC shedding [537].

In contrast to our findings, it has been reported that systemically sensitized juvenile OVA-allergic BALB/cJ mice do not display enhanced eosinophil infiltration or AHR following CAPs exposures [588]. This study used a different strain and age of mice, and examined particles concentrated from the Boston airshed, which could have contributed to the observed differences between the studies. In contrast, residual oil fly ash (ROFA), which has a very high content of soluble transition metals including vanadium, nickel, and iron, significantly increases AHR [561, 588] and BAL eosinophils [561]. The bioavailable metal content of ROFA, especially vanadium, appears to be essential for ROFA-induced effects on airway epithelial gene expression, cytokine production, and cytotoxicity through a reactive oxygen species-dependent mechanism [584]. ROFA acts as an adjuvant to enhance allergen sensitization to soluble protein antigens such as ovalbumin in the normally non-responsive neonatal immune system [589]. The conflicting results observed in neonatal mice between CAPs and ROFA exposures [588] illustrate the importance of relevant exposures and animal models in determining the risks associated with PM.

Systemically-sensitized OVA-allergic mice had clearly greater eosinophil and antibody responses than locally-sensitized mice. Regardless of the route of sensitization,
the effects of CAPs exposure on the eosinophilic response to antigen challenge were relatively similar (~3-fold increase). However, in evaluating the adjuvancy of PM, the route of sensitization must be recognized, since PM may shift the balance of local Th1/Th2 cytokine production by specific subpopulations of mucosal cells, including B cells, γδ T cells, NK cells, and mast cells [590].

In conclusion, our data demonstrate that a non-cytotoxic exposure to CAPs potentiates mast cell-mediated antigen-induced airway eosinophilia in the mouse. Exposure to CAPs had no significant effect on physiologic endpoints, but airway responsiveness in the late response is clearly dependent on the presence of mast cells in systemically sensitized allergic mice.
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**EXPERIMENT 1**

**Conclusion for Experiment 1:**

Mast cells are involved in PM-induced pulmonary eosinophilia in the allergic mouse model of asthma. By contrast, PM exposure had no effect on physiologic endpoints for this study, but mast cells were found to partially mediate airway reactivity.
Hypothesis for Experiment 2:

CAPs exposure enhances pulmonary eosinophilia in the late phase of the allergic response by promoting mast cell activation in the early phase of the allergic response.
THE ROLE OF MAST CELLS IN LEUKOCYTE RECRUITMENT FOLLOWING EXPOSURE TO CONCENTRATED AMBIENT PARTICLES

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INTRODUCTION

The immune response is a dynamic process involving sequential communication between the innate and the adaptive immune systems. Mast cells are an important link between these two systems since they can be activated innate-mediated processes by toxic insults, such as ozone exposure, or by antigen via IgE-mediated signaling to promote adaptive immunity. Mast cell participation in the inflammatory response is most evident during the immediate phase of allergic reactions, however they can also continue to secrete a variety of mediators throughout the late phase of the inflammatory processes.

The previous study indicated that mast cells are pivotal to CAPs-induced enhancement of allergic inflammation, and specifically for eosinophil recruitment at 48-hours post-OVA challenge (day 2). However, since the observed time point was during the T cell-mediated, late phase of the inflammatory response it is difficult to fully appreciate the mast cell’s contribution to eosinophil recruitment. Therefore, the current study examined earlier time points in the inflammatory response at 8- (day 0) and 24-hours (day 1) post-OVA challenge, in order to more closely assess the contribution of mast cells to CAPs enhancement of pulmonary eosinophilia. This study also assessed the kinetic profiles of inflammatory cell recruitment for the different sensitization protocols in air exposed mice and the dose dependent changes in cytotoxic markers related to compounding CAPs exposures. Of note, the current study was designed to repeat the CAPs exposures achieved in the first study (C x T = ~12,000 µg·hr/m³), but due to adverse weather conditions (namely Hurricane Floyd) the CAPs exposures were ~4-fold lower than in the original study with day 0 mice receiving a C x T of 1500 µg·hr/m³ and
day 1 mice receiving a total C x T = 3000 µg·hr/m³. Protocol changes are detailed in the Materials and Methods.

The results of this study show that eosinophil recruitment is critically dependent on mast cells, but that this effect is not evident until 24-hours post-OVA challenge in systemically sensitized mice. CAPs significantly enhanced eosinophil recruitment in the BAL fluid of systemically, but not locally sensitized mice at day 0, without evidence of a concurrent increase in total cellularity normally comprised of cells of innate resistance including macrophages and neutrophils. The limited cellularity observed in the BAL fluid of CAPs exposed mice was not due to a cytotoxic response, since CAPs also significantly decreased LDH and NAG levels. From these data we conclude that CAPs has the potential to significantly alter the early events (8-24 hrs) of both innate resistance and IgE-mediated adaptive immune responses.
MATERIALS AND METHODS

Animals. Eight- to ten-week old male WBB6F1/J wild type (WT), and mast cell deficient (MCD) WBB6F1/J-Kit\textsuperscript{W}/Kit\textsuperscript{W-v} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in an AAALAC-approved facility and maintained on a 12-hour light/dark cycle with food and water \textit{ad libitum}. All animals were held for a minimum of 4 days before treatment. Protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were conducted using national guidelines for the care and protection of animals.

Ovalbumin Sensitization. In both local and systemic sensitization protocols, mice were sensitized by administration of a total of 30 µg of OVA. Local sensitizations were carried out by three separate intratracheal instillations (i.t.) of 10 µg of OVA prepared in 50 µl of sterile phosphate buffered saline (PBS) plus 0.05% Tween-80 (Pierce, Rockford, IL) and performed in three day intervals. Sensitizations were carried out at least 2 weeks before OVA challenge (Figure 18). The instillation technique has been described in detail elsewhere [578]. Systemic sensitizations were carried out by two separate i.p. injections of 15 µg OVA in 0.2 ml of aluminum hydroxide (Alhydrogel, Accurate Scientific, Westbury, NY) adjuvant (ADJ). Systemic sensitizations were performed at 1 week intervals with the final sensitization at least 2 weeks before OVA challenge (Figure 18). Since the previous study (Experiment 1) indicated that CAPs had no effect on sham-sensitized mice, nonallergic mice were not included as a control for the following experiment (Figure 14).

Ovalbumin Challenge and CAPs Exposure. All mice were challenged 2-4 weeks after the final sensitization as previously described [561]. In brief, mice were
challenged by whole body exposure to an aerosol of 1% OVA in saline for one hour (Figure 18). On day 0 beginning 1 hour after OVA challenge and on day 1, mice were exposed to concentrated ambient particles (CAPs, PM$_{2.5}$) in a whole body inhalation exposure system as described by Sioutas et al. with modifications (Figure 11) [579]. Control mice were exposed to unconcentrated air at the same temperature, pressure and length of time as mice exposed to CAPs. Since the concentration of particles in the CAPs exposure system is dependent on ambient PM levels and weather conditions, a protocol was devised to produce equivalent total exposure to fine PM despite fluctuating exposure concentrations. Equivalent exposures were achieved by exposing mice until a daily pre-determined concentration x time (C x T) constant of 1500 µg•hr/m$^3$ was attained (Figure 12). Real-time chamber concentrations were measured using a tapered element oscillating microbalance (TEOM; Rupprecht & Patashnick, Albany, NY) and recorded at 10-minute intervals. Final confirmation of the C x T product was determined following exposure by gravimetric analysis of the chamber filter weight and multiplying this concentration by the total time of exposure.

**BAL Fluid Cells, Proteins, and Cytokines.** Animals were sacrificed at 8- and 24-hours after OVA challenge and CAPs exposures. Those mice sacrificed at 8-hours (d0) received a CAPs exposure of 1500 µg•hr/m$^3$, while mice sacrificed at 24-hours (d1) received a CAPs exposure of 3000 µg•hr/m$^3$ (Figure 18). Mice were anesthetized with 1.5-2.0 g/kg urethane and tracheotomized with an 18G cannula and lavaged with two aliquots of Ca$^{2+}$, Mg$^{2+}$ and phenol red-free Hanks’ balanced salt solution (HBSS; Life Technologies, Bethesda, MD) using a machine designed to perform the technique at physiologically relevant pressures [591]. Approximately 2 ml of BAL fluid were
recovered from each animal resulting in an average lavage volume of 35 ml/kg. BAL fluid recovery was estimated by recording pre- and post-lavage body weights. The BAL fluid was maintained on ice and centrifuged at 360 x g for 12 minutes at 4°C. Supernatants were transferred to a separate tube in order to prepare aliquots for biochemical analyses. BAL cells were resuspended in 1 ml of HBSS and counted (Coulter Z1, Hialeah, FL). Cytospin preparations of BAL cells were made for each sample and stained with Wright’s Giemsa using an automated slide stainer (Hematek 2000, Elkhart, IN). Differentials were performed by counting 500-1000 cells per slide. Assays for total protein, albumin, lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAG) were carried out as previously described [557].

**Statistical Analysis.** Biochemical and inflammatory cell endpoints were analyzed by 4-way ANOVA (Figure 13) with tests for interactions for the following factors: time (day 0 vs. day 1), genotype (WT vs. MCD), route of sensitization (local vs. systemic) and CAPs exposure (air vs. CAPs). In instances where statistical interactions could be identified by 4-way ANOVA, data were further divided into all possible comparisons based on the factors involved and again analyzed by ANOVA.
RESULTS

Local and Systemic Sensitization Protocols are Temporally Different. There is a significant difference between the local and systemic sensitization protocols in response to OVA challenge at early time points (Table 8). Both locally and systemically sensitized mice rapidly recruit inflammatory cells to the lung following OVA challenge and air exposure (Figure 19A). Neutrophils account for a large proportion of the observed increase in total cellularity at day 0 (>50%, Figure 19C), however the neutrophilic response rapidly tapers off by day 1 in locally sensitized mice, particularly in locally sensitized WT mice, compared to systemically sensitized WT mice (Figure 19C). Mast cell-mediated recruitment of inflammatory cells was observed at day 0 in both locally and systemically sensitized mice exposed to air (Figure 19A and Figure 19C), however this effect was lost in locally sensitized mice by day 1 (Figure 19A and Figure 19C).

CAPs alters biochemical endpoints of lung injury. In comparison with air exposed mice, CAPs was shown to significantly limit cellular infiltration in locally sensitized mice at both day 0 (8 hrs) and day 1 (24 hrs), and in both WT and MCD mice (Figure 19B vs. 19A, and Table 9-10). Similar changes were not evident in systemically sensitized mice until day 1 (24 hrs), but both WT and MCD mice displayed limited inflammatory cell infiltration compared to the air controls (Figure 19B vs. 19A, and Table 9-10). Given what appears to be a depression of cellular infiltration in CAPs exposed mice, an increase in biochemical endpoints of cytotoxicity would suggest that the observed CAPs effects were due to cytotoxicity. However, CAPs exposed mice
tended to have lower levels of N-acetyl-b-D-glucosaminidase (NAG) and lactate dehydrogenase (LDH) in response to CAPs exposure as well (Table 11).

BAL fluid NAG was evident by 8-hours post-OVA challenge, tapering off by day 1 in locally sensitized mice (Figure 20C). Overall, locally sensitized mice had greater levels of NAG compared to systemically sensitized mice (Table 11). Mast cells appear to have a protective role against cytotoxic responses, since there were significantly greater levels of NAG in the BAL fluid of MCD mice compared to WT mice (Table 11, p=0.0018), particularly in air exposed mice (Figure 20C and 20D). Locally sensitized WT and MCD mice exposed to CAPs had significantly greater levels of BAL fluid NAG compared to systemically sensitized mice at day 0 (Table 12, WT: 1.34-fold, MCD: 1.45-fold). However, NAG level in the BAL fluid of CAPs exposed mice were still significantly lower than air exposed mice. Systemically sensitized air exposed mice displayed the highest LDH levels on day 1 (Table 11), while CAPs exposed mice at the same time point had much lower LDH levels (Figure 20B). Locally sensitized mice did not display large changes in LDH levels from day 0 to day 1 (Figure 20A), however despite the low LDH levels, a significant decrease in LDH was detected in locally sensitized mice following CAPs exposure (Table 13). Mast cells did not appear to influence LDH levels in any way (Table 13). Total protein levels were not affected by CAPs exposure, but albumin levels were significantly increased by CAPs exposure (Table 11).

**CAPs enhances eosinophil recruitment.** Despite the limited cellular infiltration following CAPs exposure, CAPs significantly enhanced eosinophil recruitment in systemically sensitized WT mice within 8-hours (day 0) after exposure (Figure 21A, Table 14, p = 0.0366). Since mast cell-mediated eosinophil recruitment was not evident
until day 1 (24 hrs) after OVA challenge in systemically sensitized mice (Figure 22B, Table 14, p = 0.0006), the enhanced eosinophil recruitment at day 0 (8 hrs) cannot be attributed to mast cell activation. Significantly greater eosinophil recruitment was observed in systemically sensitized mice compared to the locally sensitized mice (~5-fold) and there were no significant changes in eosinophil numbers following CAPs exposures in locally sensitized mice (Figure 22A). Furthermore, there was no evidence of mast cell-mediated eosinophil recruitment in locally sensitized mice (Table 14).

**CAPs and mast cells alter lymphocyte recruitment.** In general, BAL fluid lymphocyte numbers were depressed in CAPs exposed mice compared to air exposed mice (Table 8, p = 0.0146). However, a significant time dependent increase in lymphocyte numbers was observed in locally sensitized CAPs exposed mice by day 1 (4.5-fold) without significant changes in locally sensitized air exposed mice (Table 15). It is important to note that locally sensitized air exposed mice had 10-fold more lymphocytes than locally sensitized CAPs exposed mice (Figure 22C), but the relative increases following CAPs exposure at this early time point may be critically important to the ensuing enhancement of allergic inflammation (Figure 21B). Additionally, mast cells appear to mediate lymphocyte tissue infiltration at early time points since systemically sensitized air exposed WT mice had significantly fewer lymphocytes in their BAL fluid than MCD mice (~2-fold, Table 15, p = 0.0050, Figure 22D).
DISCUSSION

CAPs does alter the early events associated with the allergic response by enhancing eosinophil infiltration into the lung, however, this response does not appear to coincide with mast cell activation since no significant difference in eosinophil numbers recovered from the BAL fluid were ascertained between systemically sensitized, CAPs exposed WT and MCD, mice by 8 hours (day 0) post-OVA challenge. By contrast, the early CAPs effect was lost by 24 hours post-OVA challenge (day 1) where the effects of mast cell activation became evident (WT > MCD). This does not necessarily mean that CAPs is not altering the type I response, it simply indicates that in this study, the observable BAL fluid cell numbers could have been measured at their lowest limits of detection, and therefore, the ability to differentiate between mast cell-mediated eosinophil recruitment vs. alternative mechanisms of eosinophil recruitment, was not possible at the earliest time points. Limited BAL fluid recovery of inflammatory cells at the earliest time points could be due to differences in the total volume of BAL fluid recovery as a whole, since the residual volume of BAL instillate remaining in the lungs of MCD mice was far less than that of WT mice (Table 16). This could be an indication that the airways of WT mice were more constricted than that of the MCD mice, since it is well known that mast cells mediate bronchoconstriction during the EAR, as well as the LAR (Figure 15). The implications of this finding are that differential cell counts of BAL fluid cells may not be the ideal means by which to determine eosinophil infiltration as the final endpoint. A histological quantitation of eosinophil numbers may be more useful.
Since CAPs does promote inflammation in allergic mice, and since mast cells are known to be the gatekeepers for the entry of inflammatory cells into tissues, evidenced here by the fact that neutrophils recovered from the BAL fluid of MCD mice were significantly lower than that of WT mice, it is clear that there is some level of communication between the air to lung interface (alveolar macrophages and airway epithelial cells) and the mast cells in the submucosa which serves to modulate pulmonary inflammation.
ACKNOWLEDGEMENTS

The authors thank Hassel G. Hilliard, Edwin R. Lappi, Judy H. Richards, and Debora L. Andrews for their skillful technical assistance, and Najwa Haykal-Coates and Dennis E. House for statistical analyses. I would particularly like to thank Matt Campen, Julie Nolan, and Mette C. Jackson (Mette C.J. Schladweiler), my close friends, for helping me with this experiment when I needed them most.
Conclusion for Experiment 2:

CAPs-induced eosinophil recruitment and mast cell-mediated eosinophil recruitment do not coincide between 8 and 24 hours post-OVA challenge.
CONCLUSIONS
The results from this study support the epidemiologic findings that exposure to fine particulate matter (PM$_{2.5}$) may enhance asthmatic symptoms by increasing eosinophilic inflammation, and that mast cells do participate in this process.

One criticism of environmental PM exposure studies is that the particles obtained from filter samples do not reflect the ambient particles that humans are actually being exposed to, since the removal of the particles from the atmosphere may alter the particle’s physiochemical properties. This work has addressed this criticism by using unaltered, ambient particles for all exposures. These concentrated particles enhanced the number of eosinophils recovered from the BAL fluid of allergic WBB6F$_1$/J mice (day 0 and day 2), however the magnitude of this response was not overt. This fact was not alarming, because the air quality in North Carolina is very good (www.epa.gov). The state is compliant with the NAAQS, and there are very few transition metals associated with these particles as shown in Table 4.

Another reason for the low numbers of eosinophils recovered from the BAL fluid is that the WBB6F$_1$/J mouse originates from the C57BL/6 background, a strain well known for limited Th$_2$ responsiveness. In order to produce measurable results, we relied on a sensitization and challenge protocol (systemic) known to produce high IgE titers and robust eosinophilic responses in BALB/cJ mice, and for which PM with a high transition metal content (ROFA) has been shown to enhance Th$_2$ responsiveness by upregulating the production of IL-4 and IL-5 leading to enhanced eosinophil recruitment [1]. In the WBB6F$_1$/J mouse, allergic sensitization did occur with both the local and systemic sensitization protocols as evidenced by the resulting enhancement of IgG$_1$ production, and the production of IgE. However, in comparison with the BALB/cJ mouse, the
endpoint of interest, pulmonary eosinophilia, was rather limited in the WBB6F1/J strain. Given this limited eosinophilia, and the fact that the concentrated particles were shown to be virtually innocuous in normal animals, the finding that CAPs exposure can enhance pulmonary eosinophilia in WT mice is an important one, because it supports the results observed in BALB/cJ mice while simultaneously addressing the use of relevant, ambient PM exposure.

Mast cells were shown to participate in the recruitment of eosinophils following allergen challenge, and at some level, CAPs alter mast cell-mediated responses. However, a duplication of this study utilizing ROFA may help to elucidate some of the subtle differences observed in this study. While not addressed in the scope of this work, one hypothesis that could be tested with mast cell deficient mice and a more toxic PM exposure is that PM exposure in allergic mice increases the release of SCF by alveolar macrophages and airway epithelial cells, which in turn synergizes IgE-mediated mast cell activation to enhance eosinophil recruitment.

While the controversy still remains as to the true contribution of mast cells to eosinophil recruitment, I feel that greatest value of this study rests on the knowledge gained with respect to the use of relevant exposures for both the allergic sensitization and challenge protocols (local), the CAPs exposure system. This model system will likely play a prominent role in the risk assessment process as its results are applicable to personal exposure measures to relevant particles, which will assist in extrapolating animal data back to the human condition.
REFERENCES


390. Nakano, T., et al., *Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice.* Evidence


393. MacDonald, A.J., et al., Rat bone marrow-derived mast cells co-cultured with 3T3 fibroblasts in the absence of T-cell derived cytokines require stem cell factor for their survival and maintain their mucosal mast cell-like phenotype. Immunology, 1996. 88(3): p. 375-83.


TABLES AND FIGURES
Table 1. Notable air pollution episodes prior to 1965.

<table>
<thead>
<tr>
<th>Year of Episode</th>
<th>Location of Episode</th>
<th>PM Data Collected</th>
<th>Excess Deaths</th>
<th>% Increase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1930</td>
<td>Meuse Valley, Belgium</td>
<td>-</td>
<td>50</td>
<td>90%</td>
<td>Firket 1936</td>
</tr>
<tr>
<td>1948</td>
<td>Donora, PA</td>
<td>-</td>
<td>20</td>
<td>80%</td>
<td>Ciocco 1961</td>
</tr>
<tr>
<td>1952</td>
<td>London, England</td>
<td>Black Smoke</td>
<td>1540(^b)</td>
<td>160%</td>
<td>Logan 1953</td>
</tr>
</tbody>
</table>

\(^a\)All 3 locations listed above are topologically situated in a valley. The air pollution episodes resulted from a winter inversion whereby cloud cover trapped air pollutants resulting in their accumulation over the course of several days.

\(^b\)The 1952 London Fog caused an estimated 4,000 excess deaths in London and its suburbs. This number reflects metropolitan London.

\(^c\)Percent Increase = \(100 \times \frac{\text{(number of actual deaths – number of expected deaths)}}{\text{number of actual deaths}}\)
Table 2. NAAQS\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Legend ppm (µg/m\textsuperscript{3})</th>
<th>The Criteria Pollutants\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO</td>
</tr>
<tr>
<td>1-h Average</td>
<td>35</td>
</tr>
<tr>
<td>3-h Average</td>
<td></td>
</tr>
<tr>
<td>8-h Average</td>
<td>9</td>
</tr>
<tr>
<td>24-h Average</td>
<td></td>
</tr>
<tr>
<td>Quarterly Average</td>
<td></td>
</tr>
<tr>
<td>Annual Arithmetic Mean</td>
<td>0.053 (100)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The Clean Air Act federally mandates monitoring of the 6 criteria pollutants, carbon monoxide (CO), nitrogen dioxide (NO\textsubscript{2}), ozone (O\textsubscript{3}), lead (Pb), particulate matter (PM), and sulfur dioxide (SO\textsubscript{2}), in order to ensure the attainment of safe ambient levels as determined by the National Ambient Air Quality Standards (1997).

\textsuperscript{b}Values shown are the highest acceptable level for each pollutant within the designated time allotted in parts per million (ppm, top number) and the concentration in micrograms per cubic meter (µg/m\textsuperscript{3}, bottom number in parentheses, (see Legend).
Table 3. Particle concentration and concentration x time (C•T) values for concentrated ambient particles (CAPs) exposures, based on TEOM and gravimetric analysis.

<table>
<thead>
<tr>
<th>Expt Number</th>
<th>Sensitization Protocol</th>
<th>Date (M/D/Y)</th>
<th>Exposure Time (hr)</th>
<th>Concentration (µg/m³)</th>
<th>C•T * (µg•hr/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TEOM</td>
<td>Gravimetric</td>
</tr>
<tr>
<td>1</td>
<td>Local</td>
<td>7/28/98</td>
<td>1.58</td>
<td>4,310</td>
<td>4,528</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/29/98</td>
<td>2.53</td>
<td>2,508</td>
<td>2,799</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/30/98</td>
<td>2.77</td>
<td>2,175</td>
<td>2,299</td>
</tr>
<tr>
<td>2</td>
<td>Systemic</td>
<td>9/29/98</td>
<td>3.77</td>
<td>1,636</td>
<td>1,983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9/30/98</td>
<td>6.00</td>
<td>902</td>
<td>836</td>
</tr>
<tr>
<td>3</td>
<td>Local, Systemic</td>
<td>7/27/99</td>
<td>3.77</td>
<td>1,647</td>
<td>1,645</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/28/99</td>
<td>2.58</td>
<td>2,445</td>
<td>1,623</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Daily Mean and (SD) n = 7 days</td>
<td></td>
<td>3.29 (1.42)</td>
<td>2,232 (1,073)</td>
<td>2,245 (1,178)</td>
</tr>
<tr>
<td></td>
<td>Mean and (SD) n = 3 experiments</td>
<td></td>
<td>6.94 (2.58)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2 Day Sum † Mean and (SD) n = 3 experiments</td>
<td></td>
<td>12.287 (624)</td>
<td>-</td>
<td>12.241 (1,740)</td>
</tr>
</tbody>
</table>

* C•T values were calculated by multiplying exposure time by chamber concentration as determined by TEOM or gravimetric analysis.
† Values represent total time or C•T for the 2-day exposure protocol. For experiment 1, values for the two exposure combinations (7/28/98 + 7/29/98; 7/29/98 + 7/30/98) were averaged before determining mean and SD for all 3 experiments.
Table 4. Chemical analysis of filters from CAPs exposures. *

<table>
<thead>
<tr>
<th>Element</th>
<th>Mean (n = 7)</th>
<th>SD</th>
<th>Above DL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>8.73</td>
<td>2.02</td>
<td>7</td>
</tr>
<tr>
<td>Fe</td>
<td>0.22</td>
<td>0.09</td>
<td>6</td>
</tr>
<tr>
<td>K</td>
<td>0.20</td>
<td>0.13</td>
<td>6</td>
</tr>
<tr>
<td>Ca</td>
<td>0.13</td>
<td>0.07</td>
<td>5</td>
</tr>
<tr>
<td>Mn</td>
<td>0.03</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>Zn</td>
<td>0.04</td>
<td>0.04</td>
<td>2</td>
</tr>
<tr>
<td>S in SO₄ form</td>
<td>26.19</td>
<td>6.05</td>
<td></td>
</tr>
<tr>
<td>Other elements above</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic C</td>
<td>19.67</td>
<td>16.80</td>
<td></td>
</tr>
<tr>
<td>Elemental C</td>
<td>0.88</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>% Total weight on filter</td>
<td>47.35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Elements were analyzed by XRF and expressed as percent of total particle weight on filter. Mean and standard deviation of values from 7 exposure dates, and number of filters with levels of elements above detection limits (above DL) are shown. Organic and elemental carbon were analyzed by thermal optical analysis.
Table 5. Biochemical indicators of lung injury in BAL fluid. *

<table>
<thead>
<tr>
<th></th>
<th>Allergic Status</th>
<th>Mast Cells</th>
<th>Sensitization</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>n</td>
<td>40</td>
<td>106</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>74</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>25 (1.9)</td>
<td>30 (1.2)</td>
<td>32 (1.4)</td>
<td>26 (1.4)</td>
</tr>
<tr>
<td></td>
<td>28 (1.4)</td>
<td>30 (1.4)</td>
<td>27 (1.4)</td>
<td>30 (1.4)</td>
</tr>
<tr>
<td>Protein (µg/ml)</td>
<td>174 (11.2)</td>
<td>215 (6.89)</td>
<td>212 (8.30)</td>
<td>196 (8.30)</td>
</tr>
<tr>
<td></td>
<td>189 (8.36)</td>
<td>218 (8.25)</td>
<td>206 (8.36)</td>
<td>201 (8.25)</td>
</tr>
<tr>
<td>Albumin (µg/ml)</td>
<td>26 (1.5)</td>
<td>26 (0.93)</td>
<td>28 (1.1)</td>
<td>24 (1.1)</td>
</tr>
<tr>
<td></td>
<td>24 (1.1)</td>
<td>28 (1.1)</td>
<td>27 (1.1)</td>
<td>25 (1.1)</td>
</tr>
<tr>
<td>NAG (U/l)</td>
<td>3.4 (0.22)</td>
<td>3.7 (0.13)</td>
<td>4.0 (0.16)</td>
<td>3.3 (0.16)</td>
</tr>
<tr>
<td></td>
<td>3.2 (0.16)</td>
<td>4.0 (0.16)</td>
<td>3.5 (0.16)</td>
<td>3.8 (0.16)</td>
</tr>
</tbody>
</table>

* Values shown are means and (SEM).
† Significant effects as determined by 4-way ANOVA (p < 0.05).
Table 6. Serum IgE levels for WT and MCD allergic mice exposed to air or CAPs. *

<table>
<thead>
<tr>
<th></th>
<th>MCD</th>
<th>WT ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>CAPs</td>
</tr>
<tr>
<td>Local</td>
<td>268</td>
<td>334</td>
</tr>
<tr>
<td></td>
<td>(55)</td>
<td>(54)</td>
</tr>
<tr>
<td>n=</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Systemic †</td>
<td>2135</td>
<td>1935</td>
</tr>
<tr>
<td></td>
<td>(342)</td>
<td>(182)</td>
</tr>
<tr>
<td>n=</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

* Values represent means and (SEM) for total IgE as determined by ELISA
† P < 0.001 vs. locally-sensitized groups.
‡ P < 0.05 vs. MCD IgE levels.
Table 7. Bronchoalveolar lavage fluid cell numbers in allergic mice *.

<table>
<thead>
<tr>
<th>n</th>
<th>Mast Cells</th>
<th>Sensitization Route</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- (+)</td>
<td>+ (-) P†</td>
<td>- (+) P†</td>
</tr>
<tr>
<td>54</td>
<td>54</td>
<td>52 56 P†</td>
<td>54 54 P†</td>
</tr>
<tr>
<td>Total Cells</td>
<td>26 (1.8) 28 (1.8) -</td>
<td>27 (1.8) 28 (1.8) -</td>
<td>22 (1.8) 33 (1.8) 0.0001</td>
</tr>
<tr>
<td>Macrophages</td>
<td>23 (1.7) 23 (1.7) -</td>
<td>24 (1.7) -</td>
<td>19 (1.7) 27 (1.7) 0.0045</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1.7 (0.38) 3.6 (0.38) 0.0018</td>
<td>1.6 (0.38) 3.6 (0.37) 0.0001</td>
<td>1.7 (0.38) 3.6 (0.38) 0.0032</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.75 (0.11) 0.67 (0.11) -</td>
<td>1.1 (0.12) 0.35 (0.112) 0.0001</td>
<td>0.67 (0.114) 0.76 (0.114) -</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.35 (0.063) 0.38 (0.063) -</td>
<td>0.29 (0.065) (0.062) -</td>
<td>0.30 (0.06) 0.42 (0.06) -</td>
</tr>
</tbody>
</table>

* Data were analyzed by 3-way ANOVA for allergic mice only. Values shown are means and (SEM) in units of 10⁴ cells.
† Probability values for significant differences between factors as determined by 3-way ANOVA (shown for values < 0.05).
Table 8. Statistical analysis for the timecourse of inflammatory cell infiltration in allergic mice.

<table>
<thead>
<tr>
<th></th>
<th>Day</th>
<th>Sensitization Route</th>
<th>Mast Cells</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>L</td>
<td>1</td>
<td>Pb</td>
</tr>
<tr>
<td>Total Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.3</td>
<td>29.5</td>
<td>2.51</td>
<td>2.48</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.4</td>
<td>19.5</td>
<td>0.945</td>
<td>0.943</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.282</td>
<td>1.36</td>
<td>0.258</td>
<td>0.258</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.211</td>
<td>0.299</td>
<td>0.0388</td>
<td>0.0388</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.9</td>
<td>5.79</td>
<td>2.07</td>
<td>2.07</td>
</tr>
</tbody>
</table>

a4-way ANOVA was performed on transformed data (square root) and is presented in original form as the mean, SE, and n, respectively, in units of 1 x 10^6 cells/ml BAL fluid.

bProbability value for significant differences between factors as determined by 4-way ANOVA.
**Table 9. Statistical analysis of Day x Route interaction for total cells.**

<table>
<thead>
<tr>
<th>Interacting Factors</th>
<th>Independent Factors</th>
<th>P Values&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>Route</td>
<td>WT</td>
</tr>
<tr>
<td>0</td>
<td>Local</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>Systemic</td>
<td>49.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.67</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>Local</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
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<td>3.14</td>
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</tr>
<tr>
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</tbody>
</table>

<sup>a</sup>A significant statistical interaction between the day and route of sensitizations was observed for total cells by 4-way ANOVA (Table 8, p = 0.0323).

<sup>b</sup>Values shown for independent factors are presented as the mean, SE, and n, respectively.

<sup>c</sup>Probability value for statistically significant effects attributable to mast cells or CAPs exposure as determined by 2-way ANOVA.
Table 10. Statistical analysis of Route x Genotype interaction for total cells.

<table>
<thead>
<tr>
<th>Interacting Factors&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Independent Factors&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P Values&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route</td>
<td>Genotype</td>
<td>Day 0</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>-------</td>
</tr>
<tr>
<td>L</td>
<td>WT</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
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<td>10</td>
</tr>
<tr>
<td>L</td>
<td>MCD</td>
<td>36.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>S</td>
<td>WT</td>
<td>49.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>S</td>
<td>MCD</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.02</td>
</tr>
<tr>
<td></td>
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<td>10</td>
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</table>

<sup>a</sup>A significant statistical interaction between the route of sensitizations and the genotype was observed for total cells by 4-way ANOVA (Table 8, p = 0.0512).

<sup>b</sup>Values shown for independent factors are presented as the mean, SE, and n, respectively.

<sup>c</sup>Probability value for statistically significant effects attributable to time or to CAPs exposure.
Table 11. Statistical analysis for biochemical endpoints in allergic mice for Experiment 2a.

<table>
<thead>
<tr>
<th>Day</th>
<th>Sensitization Route</th>
<th>Mast Cells</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 Pb b</td>
<td>+ - Pb b</td>
<td></td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>8.54 15.5 0.844 0.934 0.0001</td>
<td>11.9 11.3 0.883 0.905 -</td>
<td>13.7 9.21 0.863 0.928 0.0037</td>
</tr>
<tr>
<td></td>
<td>3.50 3.04 0.125 0.139 0.0025</td>
<td>2.95 3.63 0.132 0.130 0.0018</td>
<td>3.66 2.88 0.127 0.135 0.0001</td>
</tr>
<tr>
<td>NAG (U/l)</td>
<td>8.50 15.5 0.844 0.934 0.0001</td>
<td>2.95 3.63 0.132 0.130 0.0018</td>
<td>3.66 2.88 0.127 0.135 0.0001</td>
</tr>
<tr>
<td>Total Protein (µg/ml)</td>
<td>8.07 15.5 0.844 0.934 0.0001</td>
<td>2.95 3.63 0.132 0.130 0.0018</td>
<td>3.66 2.88 0.127 0.135 0.0001</td>
</tr>
<tr>
<td>Microalbumin (µg/ml)</td>
<td>25.7 21.5 1.29 1.42 -</td>
<td>21.3 26.2 1.35 1.35 0.0130</td>
<td>19.8 28.1 1.32 1.38 0.0001</td>
</tr>
</tbody>
</table>

aData were analyzed by 4-way ANOVA and are presented as the mean, SE, and n, respectively.

bProbability value for significant differences between factors as determined by 4-way ANOVA.
Table 12  Statistical analysis of Day x CAPs x Genotype Interaction for NAGa.

<table>
<thead>
<tr>
<th>Interacting Factorsa</th>
<th>Independent Factorsb</th>
<th>Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>CAPs</td>
<td>Genotype</td>
</tr>
<tr>
<td>0</td>
<td>Air</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>Air</td>
<td>MCD</td>
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<tr>
<td>0</td>
<td>CAPs</td>
<td>WT</td>
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<tr>
<td></td>
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<td>6</td>
</tr>
<tr>
<td>0</td>
<td>CAPs</td>
<td>MCD</td>
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<td>6</td>
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<tr>
<td>1</td>
<td>Air</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>Air</td>
<td>MCD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
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<tr>
<td>1</td>
<td>CAPs</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>CAPs</td>
<td>MCD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

aA significant statistical interaction between time, CAPs exposure and genotype was observed for N-acetyl-β-D-glucosaminidase (NAG) by 4-way ANOVA (Table 11, p = 0.0097).
bValues shown for independent factors are presented as the mean, SE, and n, respectively.
cProbability value for statistically significant effects attributable to the route of sensitization as determined by ANOVA.
Table 13. Statistical analysis of Day x Route interaction for LDH.

<table>
<thead>
<tr>
<th>Interacting Factors</th>
<th>Independent Factors</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>Route</td>
<td>WT</td>
</tr>
<tr>
<td>0</td>
<td>Local</td>
<td>7.00</td>
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<tr>
<td></td>
<td></td>
<td>0.728</td>
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<tr>
<td></td>
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<td>12</td>
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<tr>
<td>0</td>
<td>Systemic</td>
<td>9.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>Local</td>
<td>8.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>Systemic</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\)A significant statistical interaction between the day and route of sensitizations was observed for lactate dehydrogenase (LDH) by 4-way ANOVA (Table 11, p = 0.0014).

\(^b\)Values shown for independent factors are presented as the mean, SE, and n, respectively.

\(^c\)Probability value for statistically significant effects attributable to time or the route of sensitization as determined by 2-way ANOVA.
<table>
<thead>
<tr>
<th>Day</th>
<th>Route</th>
<th>WT</th>
<th>MCD</th>
<th>Air</th>
<th>CAPs</th>
<th>Genotype</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>L</td>
<td>0.171</td>
<td>0.898</td>
<td>0.836</td>
<td>0.0738</td>
<td>0.0623</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0623</td>
<td>0.555</td>
<td>0.450</td>
<td>0.0291</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>S</td>
<td>0.433</td>
<td>0.502</td>
<td>0.299</td>
<td>0.719</td>
<td>0.161</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.161</td>
<td>0.119</td>
<td>0.087</td>
<td>0.179</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>L</td>
<td>0.384</td>
<td>0.643</td>
<td>0.614</td>
<td>0.360</td>
<td>0.122</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>0.122</td>
<td>0.325</td>
<td>0.278</td>
<td>0.0892</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>S</td>
<td>5.73</td>
<td>1.44</td>
<td>3.11</td>
<td>4.30</td>
<td>1.28</td>
<td>0.0006</td>
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<tr>
<td></td>
<td></td>
<td>1.28</td>
<td>0.256</td>
<td>0.632</td>
<td>1.82</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* A significant statistical interaction between the day and route of sensitizations was observed for eosinophils by 4-way ANOVA (*Table 8, p < 0.0001*).

*b* Values shown for independent factors are presented as the mean, SE, and n, respectively.

*c* Probability value for statistically significant effects attributable to mast cells or CAPs exposure as determined by 2-way ANOVA.
Table 15. Statistical analysis of Route x CAPs interaction for lymphocytes.

<table>
<thead>
<tr>
<th>Interacting Factorsa</th>
<th>Route</th>
<th>CAPs</th>
<th>Independent Factorsb</th>
<th>P Valuesc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td></td>
<td>0.350</td>
<td>0.314</td>
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<td>0.0814</td>
<td>0.102</td>
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<td>11</td>
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<td>0.0329</td>
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<td>0.384</td>
<td>0.423</td>
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<td>0.0815</td>
<td>0.0621</td>
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<td>12</td>
<td>12</td>
</tr>
<tr>
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<td>CAPs</td>
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<td>0.306</td>
<td>0.532</td>
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<td>8</td>
<td>8</td>
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</table>

aA significant statistical interaction between the route of sensitizations and CAPs exposure was observed for lymphocytes by 4-way ANOVA (Table 8, p = 0.0065).
bValues shown for independent factors are presented as the mean, SE, and n, respectively.
cProbability value for statistically significant effects attributable to time or to CAPs exposure as determined by 2-way ANOVA.
Table 16. Statistical analysis of pre- and post-lavage body weights and BAL fluid recovery.a

<table>
<thead>
<tr>
<th></th>
<th>Mast Cells</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pre-BW</td>
<td>29.62</td>
<td>26.19</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Post-BW</td>
<td>29.93</td>
<td>26.46</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Difference</td>
<td>0.33</td>
<td>0.28</td>
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<tr>
<td></td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Recovery Ratio&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pre- and Post-lavage body weights (BW) were analyzed by 4-way ANOVA. There was no significant effect due to the day of exposure or the route of sensitization, therefore this data is not presented. The difference in Pre- and Post-lavage body weights (Difference) was also analyzed as an indicator for lavage recovery. Data are presented as the mean, SE, and n, respectively.

<sup>b</sup>Probability value for significant differences between factors as determined by 4-way ANOVA.

<sup>c</sup>Since the BAL fluid remaining inside the lung following lavage is proportional to the recovery, the 'Recovery Ratio' was calculated by taking the inverse of the 'Difference' (1/Difference). The higher the ratio, the more BAL fluid recovered.
“It was one day, as I was Walking in Your MAJESTIES Palace, at WHITE-HALL (where I have sometimes the honour to refresh my self with the Sight of Your Illustrious Presence, which is the Joy of Your Peoples hearts) that a presumptuous Smoake issuing from one or two Tunnels neer Northumberland-House, and not far from Scotland-yard, did so invade the Court; that all the Rooms, Galleries, and Places about it were fill'd and infested with it; and that to such a degree, as Men could hardly discern one another for the Cloud, and none could support, without manifest Inconveniency. It was not this which did first suggest to me what I had long since conceived against this pernicious Accident, upon frequent observation; But it was this alone, and the trouble that it must needs procure to Your Sacred Majesty, as well as hazzard to Your Health, which kindled this indignation of mine, against it, and was the occasion of what it has produc'd in these Papers.”

Figure 1. *Fumifugium*. This excerpt from John Evelyn’s essay, *Fumifugium*, represents early evidence of poor air quality in the 17th century, even prior to the Industrial Revolution.
Figure 2. The NAAQS regulate PM based on the respirable fraction. Monitoring of ambient particles in the 1970s was based on the measurement of total suspended particulates (TSP) in the atmosphere. The new standards for the PM criteria pollutants are based on their size, which in turn relates to the distribution of particles in the lung to either the proximal regions (tracheobronchial) or the distal lung (respiratory bronchioles and alveoli) [12, 117].
Figure 3. Formation of PM$_{2.5}$. Fine mode particles are formed as secondary pollutants in the atmosphere due to the nucleation of ultrafine particles from combustion sources (1º particle) and water vapor in the atmosphere. The particle prototype consists of an insoluble core (>50% of particle mass), which is coated during the condensation process by sulfates, ammonium, soluble transition metals, and polycyclic aromatic hydrocarbons (PHAH). It is believed that < 5% of the particle’s mass confers toxicity since both transition metals (vanadium, nickel, and iron) and PHAHs have been associated with pulmonary injury [12, 117].
Figure 4. The prevalence of allergies and asthma. According to the 2000 Census, there are 281,421,906 Americans living in the United States (http://census.gov), ~30% of which (unconfirmed) suffer from allergies. Additionally, a study released by the Centers for Disease Control in 1997 indicates that nearly 15 million Americans have self-reported asthma. A large proportion of asthmatics are also allergic to aeroallergens [8], making allergic sensitization the primary risk factor for the development of asthma.
“Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli.”

Figure 5. Definition for asthma provided by the National Heart, Lung, and Blood Institute.
“Asthma is an ongoing inflammatory disease which occurs when eosinophils are allowed to differentiate in the bone marrow, selectively home to the lung, and become activated in the absence of an ongoing infection. In the lung, cellular activation causes the release of toxic eosinophil mediators and cytokines leading to a recurrent cycle of inflammatory cell recruitment, cellular damage and repair. The consequences associated with recurrent inflammation are increased airway hyperresponsiveness and mucous secretions both of which contribute to a decrease in pulmonary function.”

Figure 6. Definition for asthma provided by the author, Sharon L. Madison.
“The very first experiment in immunology, Jenner’s successful vaccination against smallpox, is still the model for assessing the presence of such protective immunity. The assessment of protective immunity conferred by vaccination has three essential steps. First, an immune response is elicited by immunization with a candidate vaccine. Second, the immunized individuals, along with unimmunized controls, are challenged with the infectious agent. Finally, the prevalence and severity of infection in the immunized individual is compared with the course of the disease in the unimmunized controls.”

Figure 7. Description of a typical vaccination protocol.
Figure 8. Genetics partially determine the efficiency of OVA-sensitization. In addition to the route of sensitization, the outcome of OVA-sensitization is dependent upon the genetic predisposition to produce Th$_2$ cytokines involved in the isotype switch from IgG to IgE (namely IL-4). Two strains of mice commonly used in the laboratory are the BALB/c and the C57BL/6. In general, the BALB/c is considered to be a good model for the Th$_2$ phenotype due to the abundant production of IgE following sensitization and challenge with an allergen. By contrast, the C57BL/6 produces substantial quantities of IgG$_1$ in response to sensitization and challenge, rather than IgE, indicating a lower efficiency of the “T cell help” necessary for IgE production. Similarly, the C57BL/6 strain may be generalized as a Th$_1$-type responder. It is possible to achieve comparable endpoints for these genetically distinct strains, with respect to the cellular infiltrate and airway hyperresponsiveness, but this outcome is dependent upon variations in the challenge protocol.
Figure 9. Mast cells are part of an integrated communication network in tissues. The bi-directional communication between mast cells and the surrounding tissue make them uniquely suited to serve as a link between the immune system and the neuroendocrine system.
Figure 10. Experimental protocol. Wild Type (WT) and mast cell deficient (MCD) mice were sensitized to ovalbumin (OVA) either locally or systemically. Locally sensitized mice were intratracheally instilled with 10 µg OVA 21, 18 and 15 days prior to OVA challenge, while systemically sensitized mice were injected i.p. with 15 µg OVA plus adjuvant 20 and 14 days prior to OVA challenge. Control WT and MCD mice were sham sensitized (sham-local or sham-systemic) with vehicle only. On day 0, all mice were challenged with a 1% solution of OVA for 60 minutes. Following OVA challenge, mice were sham-exposed to air or exposed to concentrated ambient particles (CAPs) on days 0 and 1 for a total C x T target of 12,000 µg•hr/m³. On day 2 (2 days post-OVA challenge and 1 day post-CAPs exposure) mice were tested for their reactivity to Mch challenge and lavaged to measure inflammatory endpoints.
Figure 11. Schematic diagram of particle concentrator. Fine mode particles are originally collected from the ambient air using a size selective inlet (1.0 - 2.5µ). Next, the particles are concentrated by passing through a series of stages which divert the majority of the airflow out of the system at a right angle. Due to the mass and velocity of the particles, they are separated from the diverted air (Major Airflow, comprised of gases and ultrafine particles <0.01µ). As the particles travel through each successive stage, the particles are concentrated in the remaining air volume (Minor Airflow). Concentrating efficiencies in these studies ranged from an 80 - 100 fold increase in [PM$_{2.5}$] relative to ambient levels.
\[ C \times T = (\Sigma \mu g/m^3) \times \text{(hours/day)} \times 2 \text{ days} \]

Daily Target 6,000 \( \mu g/hr/m^3 \times 2 \) exposures = Total exposure \( \mu g/hr/m^3 \)

Figure 12. Concentration x Time (C x T) exposure protocol for CAPs exposures (see Experiment 1, Materials and Methods).
### Variables

<table>
<thead>
<tr>
<th>Allergic</th>
<th>Route</th>
<th>Genotype</th>
<th>Day</th>
<th>CAPs</th>
</tr>
</thead>
</table>

### Experiment 1

<table>
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<th>Part B</th>
<th>Part C</th>
</tr>
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<tbody>
<tr>
<td>+/- L/S</td>
<td>+/- L/S</td>
<td>L/S</td>
</tr>
<tr>
<td>WT/MCD</td>
<td>WT/MCD</td>
<td>WT/MCD</td>
</tr>
<tr>
<td>Air/CAPs</td>
<td>Air/CAPs</td>
<td>Air/CAPs</td>
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### Experiment 2

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<td>Air/CAPs</td>
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</tbody>
</table>

**Figure 13. Variables examined for analysis of variance.** Due to practical limitations, Experiment 1 was performed in three parts (A, B, C) and statistical analyses were performed on all data given the following comparisons: Allergic – allergic, OVA-sensitized mice vs. sham-sensitized mice (+/-), Route - local vs. systemic sensitization (L/S), Genotype - WT vs MCD mice (WT/MCD), Day – day of endpoint collection for Day 2, or Day 0 vs. Day 1 (0/1), CAPs - Air vs CAPs exposure (Air/CAPs). In Experiment 1, the only time point examined was Day 2. Since nonallergic mice did not respond to CAPs exposure in parts A and B, we reasoned that the allergic mice should be considered as a distinctly separate groups due to the fact that allergic sensitization irreversibly alters their phenotypic expression of a variety of genes. Given that our goal is to observe the effects of PM in a susceptible subpopulation, we excluded nonallergic mice from further experiments (part C and Experiment 2, Timecourse), since the only real benefit of using this group is for observing the effects of PM on a normal population, which as established here is absent.
Figure 14. Total BAL fluid cells and protein in WT and MCD sham-systemically sensitized (i.p.) nonallergic mice. Mice were lavaged 2 days after OVA challenge and one day after final air or CAPs exposure. Values shown are means and SE for A) total BAL cells and B) BAL fluid protein concentration (n = 5 per group). Data were analyzed by ANOVA with no significant factors or interactions observed.
Figure 15. Airway responsiveness to intravenously administered methacholine (Mch) in locally sensitized and systemically sensitized, allergic WT and MCD mice. Mice were challenged with OVA and exposed to air or CAPs as described in Figure 1, and airway responsiveness in allergic mice was measured 2 days after OVA challenge. Results shown are time-integrated change over one minute for total respiratory system resistance ($R_T$) and elastance ($E_T$) ($n = 4$ / group). * Effect of genotype (WT > MCD) independent of sensitization route or exposure to CAPs ($P < 0.05$; $P = 0.058$ for 3162 µg/kg Mch dose). † Significant interaction of sensitization route and genotype ($P < 0.05$). ‡ Significant interaction of sensitization route, genotype and CAPs exposure ($P < 0.05$).
Figure 16. Total numbers of eosinophils and lymphocytes recovered from BAL fluid of allergic WT and MCD mice exposed to air or CAPs as described in Figure 10. Values shown are the means ± SEM for BAL fluid eosinophils (A and B) and lymphocytes (C and D) in locally (A and C) and systemically (B and D) sensitized mice (n = 13-14 mice per group). Statistical differences in eosinophil numbers as determined by 3-way ANOVA were found for all 3 factors and are shown in Table 7. Significantly greater numbers of lymphocytes were recovered from locally-sensitized mice compared with systemically-sensitized mice (Table 7).
Figure 17. Tracheobronchial sections from systemically sensitized OVA-allergic WT and MCD mice exposed to air or CAPs. Sections were stained with either toluidine blue (panels A, D; bar = 100 µm) or H&E (panels B, C, E, F; bar = 25 µm). Mast cells (red circles) were enumerated in all WT sections (A: air; D: CAPs). Interstitial inflammatory cells were present in H&E sections from WT (B, E) and MCD (C, F) mice exposed to either air (B, C) or CAPs (E, F).
Figure 18. Experimental 2 protocol. Wild type (WT) and mast cell deficient (MCD) mice were sensitized to ovalbumin (OVA) either locally or systemically. Locally sensitized mice were intratracheally instilled with 10 μg OVA 21, 18 and 15 days prior to OVA challenge, while systemically sensitized mice were injected i.p. with 15 μg OVA plus adjuvant 20 and 14 days prior to OVA challenge. All mice were challenged with a 1% solution of OVA for 60 minutes. Following OVA challenge, mice were sham-exposed to air or exposed to concentrated ambient particles (CAPs) on days 0 (d0) and day 1 (d1) with a C x T product of 1,500 and 3,000 μg•hr/m³, respectively. Eight hours after OVA challenge and immediately following CAPs exposures (C x T = 1,500) d0 mice were lavaged to assess inflammatory endpoints. The following day, d1 mice received a second exposure to CAPs (C x T = 3,000) and were sacrificed immediately after the CAPs exposure, but 32 hours after OVA challenge.
Figure 19. Route of sensitization and time dependent changes in the innate immune response following CAPs exposure. Total cells and neutrophils recovered from BAL fluid of allergic WT and MCD mice exposed to air or CAPs as described in Figure 10 on days 0, 1 and 2. Values shown are the means ± SE for BAL fluid total cells (A and B) and neutrophils (C and D) in air (A and C) and CAPs (B and D) exposed mice (n = 4-6 mice per group). Statistical differences as determined by 4-way ANOVA are shown in Table 8. Asterisks (*) in panel B indicates a significant decrease in total cellularity after CAPs exposure compared to air controls in panel A as determined by 2-way ANOVA (combined LWT and LMCD, d0, p = 0.0104, combined LWT and LMCD, d1, p = 0.0042, combined SWT and SMCD, d1, 0.0041, Table 9). Single cross (†) in panel B indicates a significant decrease in total cellularity compared to air controls in panel A and mast cell-mediated inflammatory cell recruitment mechanisms as determined by 2-way ANOVA (SMCD, d1 < SWT, d1, p = 0.0106, and LMCD, d1 < LWT, d1, p = 0.0213, Table 9). Shaded boxes indicate that day 2 is from a separate experiment with the same sensitization protocol, but different C x T for CAPs exposures, therefore, only OVA-challenge plus air exposure values are presented (panels A and C). Neutrophil recruitment was significantly decreased following CAPs exposure in all groups, however neutrophil recruitment was dependent upon mast cell activation at d0 and d1(Table 8).
Figure 20. Biochemical markers of cytotoxicity recovered in BAL fluid of allergic WT and MCD mice exposed to air or CAPs as described in Figure 10 on days 0, 1 and 2. Values shown are the means ± SE for BAL fluid lactate dehydrogenase (LDH, A and B) and N-acetyl-b-D-glucosaminidase (NAG, C and D) in local (A and C) and systemically (B and D) sensitized mice (n = 4-6 mice per group). Statistical differences as determined by 4-way ANOVA are shown in Table 11. Asterisks (*) in panel A and B indicates significantly lower levels of LDH in CAPs exposed mice compared to air controls as determined by 2-way ANOVA (combined LWT and LMCD, d1 p = 0.0394, and combined SWT and SMCD, d0, p = 0.0396, Table 14). Panel C illustrates significantly greater NAG levels in locally sensitized d0 mice compared to systemically sensitized d0 mice as determined by ANOVA (LWT, CAPs, d0 > SWT, CAPs, d0, p = 0.0274, and LMCD, CAPs, d0 > SMCD, CAPs, d0, p = 0.0309, Table 12). Cross (†) in panels C and D indicate a significant decrease in NAG at d1 compared to d0 as determined by ANOVA (LMCD, Air, d0 > LMCD, Air, d1, LWT, CAPs, d0 > LWT, CAPs, d1, and SMCD, CAPs, d0 > SMCD, CAPs, d1, Table 13). Shaded boxes indicate that day 2 is from Experiment 1 with the same sensitization protocol, but different C x T for CAPs exposures, therefore only OVA-challenge plus air exposure values are presented.
Figure 21. CAPs effects on the adaptive immune response. Statistical analyses reported in Table 14 and 15 indicate a significant increase in eosinophils and lymphocytes following CAPs exposures for combined groups. Values shown in panel A are the mean ± SE for systemically sensitized WT and MCD mice exposed to air or CAPs on day 0. Significant increases in eosinophils were identified following CAPs exposures compared to air controls as determined by 2-way ANOVA (combined SWT and SMCD, CAPs, d0 > SWT and SMCD, Air, d0, Table 14, p = 0.0366). Values shown in panel B are the mean ± SE for locally sensitized mice exposed to CAPs on day 0 and day 1. Dose dependent increases in lymphocytes were identified following CAPs exposures for both WT and MCD mice as determined by 2-way ANOVA (combined LWT and LMCD, CAPs, d0 < LWT and LMCD, CAPs, d1, Table 15, p = 0.0083).
**Figure 22.** Route of sensitization and time dependent changes in the adaptive immune response following CAPs exposure. Eosinophils and lymphocytes were recovered from BAL fluid of allergic WT and MCD mice exposed to air or CAPs as described in Figure 18 on days 0, 1 and 2. Values shown are the means ± SE for BAL fluid eosinophils (A and B) and lymphocytes (C and D) in local (A and C) and systemically (B and D) sensitized mice (n = 4-6 mice per group). Statistical differences as determined by 4-way ANOVA are shown in Table 8. Asterisks (*) in Panel B indicate a significant greater number of eosinophils in WT mice compared to MCD mice (combined SWT, Air and CAPs, d1 > SMCD, Air and CAPs, d1, Table 14, p = 0.0006), panel D indicates a significantly greater number of lymphocytes in MCD mice compared to WT mice (combined SWT, Air, d0 and d1 < SMCD, Air, d0 and d1, Table 15, p = 0.0050). Shaded boxes indicate that day 2 is from a separate experiment with the same sensitization protocol, but different C x T for CAPs exposures, therefore only OVA-challenge plus air exposure values are presented.