4-27-2018

The Role of the Microbiome in Regulating House Dust Mite-Induced Asthma

Alexander J. Adami
University of Connecticut School of Medicine and Dentistry, alexander.adami@uconn.edu

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Asthma is a complex and highly heterogeneous disease characterized by inflammation of the airways, leading to a host of respiratory symptoms including wheeze and dyspnea. Asthma is the product of multiple interconnected immunological processes and represents a constellation of related, but distinct, disease phenotypes. Disturbingly, the prevalence of the disease has more than doubled since the 1980s, and efforts to understand this increase have led to the consideration of the microbiome, or the communities of microorganisms that dwell on and within us, as a key player in the pathology and regulation of this disease. While recent years have seen an explosion of new research in this area, we are only beginning to untangle to mechanisms by which the microbiome may influence asthma. Utilizing a house dust mite-induced model of experimental asthma, we explored the relationship between the microbiome and the development of both allergic airway disease and the development of tolerance and disease resolution. Herein, we report that HDM exposure has can alter the gut microbiome, producing distinct changes in the microbial community over the course of HDM exposure, but that these effects are not consistent between treatment groups. We demonstrate that oral tolerance is not inducible for HDM, in contrast to a large body of evidence in other experimental allergens such as ovalbumin, suggesting that the inhalational tolerance seen with this allergen forms by independent
mechanisms within the lungs. We describe a new, clinically-relevant model of early-life antibiotic exposure that produces heightened asthma severity, including the first evidence that brief exposure to antibiotics after weaning is sufficient to alter the pathophysiology of asthma later in life. Furthermore, we demonstrate that the enhanced disease seen in this model may arise from deficiencies in pulmonary regulatory T Cells driven by loss of diversity in the microbiome. Finally, we explore the ability of antibiotic treatment to influence the development of inhalational tolerance, reporting that lifelong exposure to antibiotics can impair tolerance development through mechanisms that may be mediated by a loss of regulatory T and B Cells.
The Role of the Microbiome in Regulating House Dust Mite-Induced Asthma

Alexander John Adami

BS, Rensselaer Polytechnic Institute, 2010

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy
at the University of Connecticut

2018
I extend my thanks to Dr. Roger Thrall for his support, patience, and guidance to me during the four years of my PhD studies. I would also like to thank the members of my dissertation committee, past and present: Dr. Joerg Graf, Dr. Kamal Khanna, Dr. Leo Lefrançois, Dr. Louise McCullough, and Dr. Craig Schramm. My thanks also go out to Dr. Julia Oh for serving as the external reader and reviewer of my dissertation.

The advice, camaraderie, and encouragement of members of the Thrall and Schramm Laboratory, particularly Ms. Linda Guernsey and Dr. Sonali Bracken, was essential to the successful completion of this project, and they have my enduring gratitude for their steadfast support. Many aspects of this project would also not have been possible without the help of colleagues in the Matson and Graf laboratories, for which I thank them. I would also like to thank the members of the Immunology Graduate Program and the MD/PhD Program for their helpful advice and suggestions and the staff of the Departments of Immunology and Pediatrics for their administrative support.

I am grateful to Dr. David Benson for graciously lending space in his laboratory while I worked at UConn Storrs and I am also grateful to Dr. Pramod Srivastava for supporting my studies during the initial years of my PhD training.

This project was funded by the National Institutes of Health (grant R01 AI-43573 to Roger S. Thrall and Craig M. Schramm and grant F30 HL-126324 to Alexander J. Adami) and the Neag Comprehensive Cancer Center (grant to Roger S. Thrall).
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Portions of the following chapter were adapted into a publication in September of 2016 in the Yale Journal of Biology and Medicine:


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CHAPTER 1
INTRODUCTION

What is Asthma?

The term "asthma" conjures many images. To the public, it represents inhalers, wheezing, and difficulty breathing. Physicians think of corticosteroids, bronchodilators, and the patients they care for in the throes of asthma attacks. An immunologist sees the immunopathology and immunological forces behind the disease, such as eosinophils or inflammatory cytokines. Asthma is all these things and many more besides, and untangling the complexity of the disease has challenged medical science since the dawn of the profession[1].

At its heart, asthma is a disease of dysregulated immune responses resulting in chronic inflammation of the airways[2]. This inflammation promotes thickening of the airway wall, primarily due to fibrotic remodeling and airway smooth muscle (ASM) hypertrophy and hyperplasia[3]. The thickened wall is augmented by goblet cell hyperplasia and increased production of mucus[4]. This increased mucus, and the need to remove it, is one factor behind the cough seen in many suffering from the disease[4], and together with thickening of the ASM it reduces the cross-sectional area of the airway lumen significantly, restricting airflow. Spasm and hyper-reactivity of the thickened ASM layer further constrict the lumen and impede airflow[2]. For asthmatics, this process is what produces the wheeze, chest tightness, and dyspnea that are responsible for much of the suffering of the condition. In the early stages of asthma, this constrictive, obstructive process is characteristically reversible with bronchodilators, but after many years of asthmatic inflammation and remodeling, the obstruction can become irreversible and constant[5].

Asthma may fundamentally be a disease of airway inflammation, but this inflammation can manifest itself in multiple ways. While commonly thought of as a disease of childhood, asthma may appear throughout life[6]. The severity of symptoms, frequency of severe asthma attacks (a
sudden worsening of asthma symptoms), and response to treatment vary between and within age groups[4]. Some children who acquire asthma grow out of it, while others seem unable to shake the condition[7]. Indeed, the heterogeneity of the disease may explain why the most effective therapies we have are broadly immunosuppressive (inhaled or systemic corticosteroids), act on ASM to dilate the airway and restore airflow (bronchodilators), or perform a combination of the two actions (leukotriene antagonists)[4].

The recognition of asthma’s heterogeneous presentation prompted a reconsideration of the very nature of the disease. Long deemed a (if not the) prototypical T Helper Type 2 (Th2)-mediated disease, subsets of asthma that display few or no characteristics of Th2 inflammation are today well-recognized[8]. The concept of asthma is now one of multiple phenotypes, each with distinct features and immunological processes[8–10]. This new understanding has led to some, such as the editors of *The Lancet*, to call for the abandonment of asthma as a single disease concept[1]. One asthma researcher, Fernando Martinez, has likened the modern concept of asthma to fever, which was considered a disease unto itself until the 1800s[11]. No modern physician would describe fever as a disease, for we understand that fever can arise from a host of distinct processes. In the coming years and decades, we may realize the same to be true for asthma.

**The Actors on Asthma’s Immunologic Stage**

As complex as asthma’s presentation can be, the cast of immunologic actors that put on the asthma “show” is more complex still[8; 9]. Consider the classic picture of allergic asthma, that of a Th2-driven response against an innocuous environmental antigen, characterized by eosinophilia, airway hyper-reactivity (AHR), and abundant production of IgE[2]. CD4+ T cells reside at the center of this process, directly or indirectly providing cytokines and chemokines to promote the development of eosinophils, expansion of goblet cells, and development of AHR[8; 9]. Despite this seemingly complete picture, new discoveries are constantly expanding on the details regarding the players participating in the Th2 response. To pick just one recent example,
a subset of interleukin (IL) 21-producing CD4+ T cells was shown to be capable of modulating Th2 responses in mice, and the loss of these cells led to reduced airway inflammation in an experimental asthma model[12].

Intriguingly, CD4+ T cells are not the only orchestrators of Th2 immunity, and a recently-described player of increasing importance is the innate lymphoid cell, type 2 (ILC2). ILC2s are members of a group of cells collectively dubbed innate lymphoid cells (ILCs) that straddle the fence between adaptive and innate immunity, exhibiting many of the activities of T cells without the accompanying T cell receptor or clonal expansion in response to antigen[13]. Multiple experimental models have described ILC2s, in particular, as critical mediators for the induction of Th2 responses. While some of the earliest evidence of ILC2 participation in Th2 responses was in the context of parasite expulsion[14] (n.b. “nuocytes” refers to ILC2s in this and other papers), a growing body of work links ILC2s to the mediation of Th2 responses in experimental asthma[15–18]. Their importance is such that loss of these cells provides resistance to asthma development in mice[16–18]. While many of the details of ILC2 mediation of Th2 responses remain to be determined, we know that they are capable of producing many of the same cytokines of a classic Th2 response, including IL-4, IL-5, and IL-13[16; 17], and that they promote the migration of dendritic cells (DCs), a class of professional antigen-presenting cells, to lung-draining lymph nodes to present allergen to T cells[16; 18]. The latter is of such importance that Th2 memory responses are impaired in the absence of ILC2 activity[18]. Perhaps most interestingly, ILC2s can induce robust Th2 responses even in the absence of the adaptive immune system (particularly CD4+ T cells)[15], suggesting that these cells alone may be capable of driving asthma pathogenesis. Unfortunately, while our knowledge of how ILC2s orchestrate asthma in the mouse is growing, the challenges of studying specific immune pathways in humans mean that relatively little is known about the role of ILC2s, or ILCs in general, in the development of human asthma[13; 19].
One particularly interesting development in asthma pathogenesis has been the realization that pathways outside the traditional Th2 axis can drive asthmatic responses, particularly in adult-onset disease[8; 9]. In these subjects, and experimental animal models mimicking their disease, neutrophils dominate the inflammatory infiltrate, with eosinophilia and other characteristics of Th2 immunity being seemingly absent. Experimental work in animal models of asthma has pointed to the involvement of Th17 cells, a subset of helper T cells known for production of IL-17. Both Th17 cells and the IL-17 they produce have been linked to the recruitment of neutrophils to airways[20], increased severity of airway inflammation[21], and the promotion of AHR[22]. In humans, airway neutrophilia negatively correlates with airway function[23], and asthmatic airways on the whole tended to have more Th17 cells than controls[24]. Intriguingly, Th17 cells in animal models have been shown to be resistant to steroid therapy[21], and human Th17 cells seem to share this property[25], potentially explaining why human adults with severe asthma are often resistant to steroid treatment[26]. However, work in this area is far from complete, and many questions remain. For example, one early study of IL-17 in allergic asthma showed a protective effect against allergic inflammation[27], likely due to replacement of Th2 activity (and corresponding allergic disease parameters like eosinophilia) with Th17-driven activity. As suggested in this study, the effect of Th17 immunity may depend on when in the disease process it is roused. Given the difficulty of treating individuals with the most severe forms of asthma, further study of the role of Th17 immunity in asthma is desperately needed.

Thus far, the discussion has focused on pro-inflammatory immune mechanisms, but regulation of immunity is of equal, and if not greater, importance to asthma and to efforts aimed at treating the disease. Asthma can be thought of as an imbalance between regulation and inflammation, with too much inflammation and too little regulation. One of the most studied regulatory cell types is the regulatory T cell (Treg), a class of T lymphocytes first identified as being protective against autoimmune inflammation[28]. Since the initial identification of these cells, the role of Tregs in
regulating inflammation, including that seen in asthma, has only grown[29]. Treg expression of transforming growth factor β (TGF-β) has been shown to mediate tolerance, or lack of immune response, to inhaled allergens[30], and several studies have shown that the suppression of experimental asthma is accompanied by accumulation of Tregs in the lung and lung-draining lymph node[31; 32]. Tregs have been shown to protect against experimental asthma by reducing the activity of DCs[33] as well as suppressing Th17 cells and production of IL-17[34]. Finally, inducible Tregs (iTregs), or Tregs which form in peripheral tissues rather than in the thymus, protect against Th2 inflammation in both the gut and lungs[35].

Importantly, there is strong evidence to suggest that Tregs play an important role in the regulation of human asthma. Human Tregs treated with steroids are able to upregulate production of the anti-inflammatory cytokine IL-10[36], hinting at one potential mechanism for the efficacy of this class of drugs. Tregs have been shown to be reduced in the blood of asthmatic individuals relative to control subjects and also possessed diminished suppressive capability[37]. Interestingly, comparisons of Treg numbers in the lungs of asthmatic and non-asthmatic individuals (using broncho-alveolar lavage, or BAL, to assess airway cell populations) have been mixed. One study demonstrated that airway Tregs are diminished in asthmatic adults[24], and a pediatric study reported these Tregs to be functionally impaired in asthmatic children [38]. However, earlier work in adult asthmatics reported increased numbers of Tregs in asthmatic airways[39], although they did not examine Treg function. One possible explanation is that these studies assessed patients broadly classified as having “asthma” but who may have exhibited subtly different asthma phenotypes. More study, particularly comparing human Treg populations between asthma classifications, is required.

Tregs are not the only regulators of asthma pathology, a perhaps unsurprising revelation given the numerous effector cell types seen in asthma. Among these additional regulators are a sub-population of B cells, the precursors of the antibody-producing plasma cells, known as regulatory
B cells (Bregs)[40]. Bregs have been linked to suppression of inflammation in a number of disease states, often through interaction with Tregs[41], and evidence for their suppressive ability in asthma is increasing. Bregs have been found to suppress symptoms of experimental asthma[42] and later work linked this suppression to induction of Tregs[43]. Bregs may exert their regulatory powers in the lung-draining lymph node, where they have been shown to accumulate during the stage of tolerance development[41]. Unfortunately, evidence of a role for Bregs in human asthma is relatively limited. One recent investigation reported that B cells from asthmatic patients produced less IL-10 than those from controls, suggesting that a qualitative difference in Bregs may exist in human asthma[44].

A further cell type with regulatory activity is the DC. A subset of DCs has been shown to be protective in animal models of asthma[45]. Subsequent studies have extended this finding and reported that disease protection is mediated through DC induction of Tregs[46], that proteolytic modification of DC surface markers can reduce asthma pathology[47], and that one subset of regulatory DCs critical for asthma protection is characterized by surface expression of CD103[48]. Interestingly, at least one human study of asthmatics has reported CD103+ cells in the vicinity of sites of inflammation in the lung[39], although they appeared to be T cells rather than DCs and their exact function was not assessed. However, the DC-asthma story is complex, and there is equally good evidence that subsets of DCs help promote Th2 inflammation[49]. Which role DCs play may depend on their interactions with other immune cell types, including ILCs[13] and Tregs[33].

**Asthma: A Growing Epidemic**

Asthma has been a scourge of humanity since ancient times, but in the latter half of the 20th century asthma prevalence took a sharp turn upwards. Representing what one group dubbed the “asthma epidemic”[50], rates of childhood asthma more than doubled, rising from under 4% prior to the 1980s to a peak of nearly 10% by 2009[50–54]. While there is some recent evidence that
overall prevalence may be stabilizing, certain populations, including poor children, adolescents, and those dwelling in the Southern United States, continue to experience rising rates of asthma[54]. The numbers of affected children continues to rise, with a 20% increase in prevalence over the span of a decade[53].

However, one important point to consider with respect to asthma prevalence studies is that the heterogeneity of asthma presentation and lack of a gold-standard diagnostic test for the disease have long made estimates of asthma prevalence challenging for epidemiologists[10; 50]. To what degree prevalence numbers are an overestimate (or an underestimate) remains uncertain, but that they are imprecise is a certainty. For example, some types of asthma that are not accompanied by a Th2 response may be missed by surveys that utilize IgE and pinprick testing as key components of diagnosis.

Regardless of whether prevalence continues to rise or has stabilized, asthma continues to produce millions of missed school and work days[52], representing a significant drain on the national economy and a hindrance to learning. Even more troubling, asthma remains the third leading cause of hospitalization in children, behind only accidents and pneumonia[55], a costly burden both to children and the larger healthcare system. From a health disparities perspective, asthma is challenging disease for many minority populations in the United States. African American children have higher prevalence of asthma, approaching 15% compared to under 9% for other groups, a disparity that has worsened significantly since the 1980s[54]. US children of Puerto Rican descent fare event worse; for them, asthma prevalence has risen every year since 2001, and in 2015 asthma prevalence lay between 15 and 20%, the highest of any group[54]. Unfortunately, efforts to staunch the rising tide of asthma are hampered by an understanding of how to prevent asthma that remains minimal at best[52]. Until we can uncover the etiology (or, more likely, etiologies) of asthma, the best we may hope for is stabilization and not increase.
Cleaner Is Not Always Better: the Hygiene Hypothesis

In the decades since the upward trend in asthma prevalence first became apparent, many theories have been advanced to explain the rise. Among the most compelling, and the most supported by epidemiological evidence, is the hygiene hypothesis. Originally raised in a study of hay fever by Strachan[56], the hygiene hypothesis states that the increasingly clean and sterile environment of modern man has promoted the development of many diseases, including asthma. In the quarter-century since Strachan’s proposal, evidence in support of his hypothesis has gone from a few observations to a host of epidemiological studies[57] and, in some instances, interventions.

What, though, is the common thread? Interestingly the answer may have been proposed over 20 years prior to Strachan’s hygiene hypothesis paper. In the 1970s, a Canadian team comparing native communities with white communities made the observation that native communities suffered far less from asthma and allergic disease than their white counterparts. Far ahead of their time, the researchers opined that the higher frequency of asthma and allergy was merely “the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths”[58]. Microbes, or lack thereof, could be the source of the asthma epidemic[59].

Bring on the Bugs: the Microbiome

The term “microbiome” was popularized by Joshua Lederberg in the beginning of this century[60], refers to the microorganisms that inhabit our bodies and their interactions with the environment in which they live, namely us (for a discussion of the term microbiome and its conflicting definition, refer to Box 1). While the science of the microbiome has soared primarily in the last two decades, the idea that we possess microbiomes and that they can impact our health is far from new. Escherich’s description of bacteria from the infant digestive tract is perhaps the earliest mention
of microorganisms living within humans[61], and his observations were soon followed by those of Döderlein[62] for the vaginal microbiome. A more remarkable concept is the idea that we may be able to harness our knowledge of the microbiome in order to promote healthier and longer lives, a concept Metchnikoff first proposed over a century ago[63].

However, while recognition of the existence and some of the effects of the microbiome on human health is not new, it took the advent of next-generation sequencing (NGS) techniques to meaningfully advance the study of the specific mechanisms of human-microbiome interactions. Methods to culture some organisms (E. coli, K. pneumoniae, S. aureus, etc) have long been known, but as many as a quarter of organisms present in the human gut may be unculturable[64]. This may be an underestimation; for soil, it is estimated that over 99% of all bacterial species are not able to be cultured with modern techniques[65]. NGS changed the equation, removing the need to culture and allowing a broader group of scientists, even those without expertise in microbial culture and identification, to analyze the entire microbiome and its constituents[66].

Today, efforts to uncover the secrets of the microbiome are influencing every field of medical science, from neuroscience[67] to metabolism[68–70] and beyond. Broad, interdisciplinary studies led by the governments of the United States[71; 72] and Europe[73] go further still, and seek to not simply determine which microorganisms are part of the microbiome but what they are doing and what they are making.

The explosion in science and knowledge related to the microbiome has revealed many exciting new possibilities for advancing human health, but it has also revealed a disturbing trend. Humans have likely coexisted with our microbiome since before we were a species. However, as we have shaped our environment into one that is increasingly clean and sterile, we have fewer opportunities to acquire or be exposed to microorganisms. Put another way, our increasingly hygienic world has led to the loss of many so-called microbial friends[74]. Dubbed the “old friends” hypothesis[75], this addition to the hygiene hypothesis predicts that it is the loss of exposure to
microorganisms, many of whom we co-evolved with, that lies behind increases of many diseases of the modern age, from diabetes and obesity to allergy and asthma.

**The Benefits of Dirty Living**

The idea that microbial exposure can protect against asthma has a long history of clinical evidence to support it. In addition to the aforementioned comparison of native and white communities in Canada[58], two large studies took advantage of natural human experiments in Germany and Karelia. Prior to reunification, East and West Germans shared common heritage and culture but had decidedly different living situations. Examining the risk of asthma between the two states, a pioneering team reported that the West Germans, despite their “advanced” economy and better living conditions, suffered higher rates of asthma than the East[76]. The group hypothesized that this difference may have arisen from the East German tendency to place their children in day care from an early age while mothers went to work[76], a hypothesis which has been validated since[77]. Karelia, a region of southeastern Finland and northwestern Russia, has a common culture but has long been divided by the Finnish-Russian (formerly Finnish-Soviet) border. In Russian Karelia, the population is poorer, lives in less clean environments, and has lower rates of asthma compared to Finnish Karelians[78]. Intriguingly, those in Finnish Karelia who lived in a more biodiverse environment (a farm, for example) had lower rates of asthma than their peers[78].

Such dramatic natural human experiments are few in number, but one human difference which is very common is that between the children of farmers and nonfarmers. The idea that residing on a farm, where exposure to microorganisms is commonplace, could protect against asthma and many other diseases has a long history[79]. A host of studies have found farm living in general[80–82] and exposure to animals and dirty work on farms in particular[83; 84], as protective against the development of wheeze and asthma. One intriguing element of these studies, and one certainly most suggestive of a microbial factor in the differences found, is that exposure to endotoxin, a component of gram negative bacterial cell walls, negatively correlated
with the development of asthma in children on farms[85]. Perhaps most striking was that the inverse association between endotoxin and asthma was most prominent in nonfarmers. In other words, endotoxin exposure has an outsized influence on asthma development if one does not grow up on an already-dirty farm[85].

The inverse correlation between endotoxin and asthma outside the farm has been found repeatedly and worldwide[86–88]. More recently, the association has been extended beyond bacterial endotoxin to encompass a number of bacterial and even fungal products, with the combined effect of microbial product exposure providing protection from asthma development[89]. Interestingly, this study found the greatest protection came in the middle of the range of exposure levels, with those exposed to the highest levels of microbial products actually at higher risk of developing asthma. Too much of a good thing, even when it comes to the microbiome, can be a problem.

However, assessing exposure to endotoxin and other microbial products is relatively nonspecific. It may be able to tell you which microorganisms are behind the exposure in a general sense (e.g. gram negative bacteria producing endotoxin), but it cannot give specifics, such as genus or even class or phylum, of which organisms are behind the exposure. A growing number of studies have sought to change that, and today we know of multiple distinct microbial populations that may protect from or predispose to asthma[90–100]. While many differences exist between studies, two commonalities that have arisen relate to Firmicutes and Clostridium difficile. C difficile, an enteric microorganism most known for its pathogenic properties, was found to predispose to asthma[100], an important reminder that just as too much overall microbial exposure may be a bad thing, not all individual microorganisms are protective. Contrastingly, increased contact with organisms of the gram-positive phylum Firmicutes conveys asthma protection[92; 94; 97; 98; 101]. As gram positive organisms, the influence of Firmicutes would have escaped notice in
earlier studies of endotoxin levels, further underscoring the need to assess the specific members of the microbial community if we are to identify targets for asthma therapy.

Unfortunately, but perhaps not surprisingly, there appears to be no single microorganism or group of microorganisms common amongst all studies, suggesting that it may be broader exposure to microbes (as hinted by earlier work studying farms and endotoxin) that is protective. Indeed, many of the aforementioned microbiome assessments have found that the greater the diversity of microorganisms that one encounters as a child, the lower the lifetime risk of asthma[92; 95]. However, other studies have shown no link between overall microbial diversity and asthma development[93; 96–98] or even a tendency toward asthma development with increased microbial diversity[99]. Our understanding of how microbial diversity can impact asthma is clearly incomplete, and whether more or less is protective remains to be determined.

Among existing studies are several which point to fungi, rather than bacteria, as protective against asthma. Both individual fungal genuses, such as Eurotium[92] and Cryptococcus[96], and overall fungal diversity[96] have been linked to protection from asthma. There is a relative paucity of information available on the fungal microbiome, particularly in its relationship to asthma, and more study in this area is needed to determine which host-fungal relationships drive asthma protection.

**The Dangers of Very Clean Living**

Dirty living may be beneficial, but isolating which factor or factors is responsible for the increase in asthma seen in “cleaner” populations is challenging given the number of variables involved. Two “very clean” factors that have shown particular association with asthma are delivery by cesarean section (CS) and exposure to antibiotics, particularly early in life.

**Cesarean Section**

One of the earliest (although perhaps not the earliest given recent reports of the placental microbiome[102]) exposures to microorganisms occurs during vaginal delivery, when the infant
encounters the diverse flora of the vaginal tract. However, delivery by CS bypasses the vagina, sparing the infant contact with vaginal flora. Put another way, all children begin life with a clean slate, but a CS-delivered infant may start life with an even cleaner slate. The impact of this missed contact is not simply theoretical. Evaluating the microbiome of infants delivered by CS or vaginally demonstrated that both infant groups had relatively homogeneous microbial communities across their body sites, but those infants delivered by CS had microbiomes similar to that of the mother’s skin, while infants delivered vaginally had microbiomes similar to that of the mother’s vagina[103]. Such differences may have lasting consequences. A meta-analysis of 23 clinical trials comparing vaginal birth to CS found higher odds of developing asthma when the delivery mode was CS[104]. Subsequently, large studies evaluating hundreds of thousands to millions of children, in some cases over many decades, in Norway[105], Scotland[106], and Denmark[107] have also shown CS to be a risk factor for asthma development, among other diseases. The precise microorganisms responsible, and the mechanisms by which they shape host physiology, are not yet completely understood, but it is clear that missing early exposure to the diverse microbiome of the vagina may have lasting consequences for human health.

**Early-Life Antibiotics**

The introduction of antibiotics into clinical practice has saved countless lives, and one need only look to growing panic over rising antibiotic resistance to understand the necessity of antibiotic use[108]. Unfortunately, we are discovering that antibiotics may affect the development of asthma even as they protect us from truly deadly pathogenic microorganisms. We know that the microbiome, or at least the gut microbiome, rapidly changes in early life[109; 110], and that antibiotic treatment can have lasting effects on both the microbiome and the host[110; 111]. Many studies have linked early-life antibiotic exposure, particularly in the first year of life, to the development of asthma, and the effect has been reported in populations from Seoul to the United States[112–115]. Indeed, a meta-analysis of 20 studies of antibiotics in the first year of life found
that it was associated with greater than 50% higher odds of asthma development[116]. This is not a phenomenon isolated to humans. Antibiotic administration can lead to allergic asthma development in mice[117], and at least one study has reported that neonatal, but not adult, antibiotic exposure produces more severe allergic airway inflammation [118].

The Microbiome Governs the Immune System

Thus far, our discussion of links between the environmental exposures and human health has been on associations, not mechanisms. Knowing that farm living is protective from asthma is certainly important, but it provides little in the way of directly-targetable options for asthma treatments, save perhaps that a prescription option for the pediatrician should be “farm work”. Asthma is an immune-mediated disorder, and the next important consideration is how the microbiome affects the immune system, and how some of these effects have been tied to asthma.

The relationship between the microbiome and the host immune system is one of the most important concepts to have arisen from the field of microbiome research. Today, we know that the microbiome affects all aspects of immunity, from the development of specific immune cell types to the formation of immune organs and tissues[119–123]. The following section will explore some of the most important microbiome-immune associations with respect to asthma pathogenesis and pathophysiology. Importantly, most of what we know about microbiome-immune interactions comes from work in mouse models of asthma, and unless otherwise indicated, all reported relationships are from experimental asthma in laboratory mice.

Immune Regulation: Tregs and DCs

The ability of the microbiome to influence regulatory arms of the immune system, particularly Tregs, has fundamentally changed our view of what the microbiome means for host immunity and for asthma control. One of the earliest specific links was made to Bacteroides fragilis, which is able to induce Tregs in the mouse gut[124]. We later learned that this is mediated through
sensing of the *B. fragilis* polysaccharide A (PSA) by toll-like receptor 2 (TLR2)*[125]*, and that loss of PSA leads to a Th17 rather than a Treg response to *B. fragilis*[126]. This balance between Treg and Th17 immunity could have implications for severe, neutrophilic asthma, where perhaps a deficiency of Tregs leads to Th17 dominance.

*B. fragilis* is not the only microorganism capable of inducing Tregs. Just a few years after the discovery of PSA’s role, another group reported that a mixture of organisms in the class Clostridia could induce Tregs not only in the gut but also systemically*[127]. Soon thereafter, the mechanism became clear: Clostridial metabolism of dietary fiber to produce short chain fatty acids (SCFAs), particularly butyrate but also propionate, which then act through the receptor GPR43 to induce Tregs*[128–130]. Further work narrowed down the responsible Clostridia to a specific group of 17 organisms within clusters IV, XIVa, and XVIII*[131]. Interestingly, this is one instance where mechanism preceded organism, as the role of SCFAs and GPR43 in Treg induction was identified four years prior, with a report demonstrating that a lack of GPR43 increased the severity of experimental asthma in mice*[132].

The importance of SCFA production to asthma has become increasingly evident. Multiple groups have shown that putting mice on a high fiber diet*[133; 134]* or directly feeding them SCFAs*[134]* is protective from experimental asthma. One of these studies provided important insight into how gut microbial metabolism can impact the lung. Those investigators found that SCFAs travel to the bone marrow and promote the development of DCs that are highly phagocytic but poorly able to induce Th2 responses*[133]. This DC phenotype is similar to that of tolerogenic DCs, known Tregs inducers*[46].

Beyond these organisms, other bacteria linked to Tregs include *Bifidobacterium infantis*[135], *Helicobacter pylori*[136–138], and *Lactobacillus johnsonii*[139]. Interestingly, the protective mechanism of both *H. pylori* and *L. johnsonii* appears to mirror SCFAs; namely, induction of a similar population of Treg-promoting regulatory DCs*[136–139]. *L. johnsonii* was found in
abundance in the house dust of homes with dogs, further evidence for the role of a “dirty” environment in providing protection from asthma[139].

One Treg-microbiome relationship whose prominence has greatly increased over the past decade has been the notion that parasitic infection can influence Treg development[140]. Parasites including *Heligmosomoides polygyrus*[141–145], *Schistosoma mansoni*[146], and *Strongyloides ratti*[147] have all been found to be inducers of Tregs. *H. polygyrus* infection has also been shown to protect against experimental asthma, either by transfer of parasite-induced regulatory cells or by direct host infection[141; 142; 145]. The mechanism by which *H. polygyrus* induces Tregs appears to be indirect, with the parasite seemingly influencing the composition of the overall gut microbiome, including through promotion of *Lactobacillus* species[144] and SCFA production by other gut microbiome community members[145], to promote Treg induction. The reasons for parasite Treg promotion may be entirely selfish: without Tregs, at least one of the parasites (*S. ratti*) is quickly killed by the host immune system[147].

Unfortunately, the available human data on microbiome Treg induction are mixed. A study over 10 years ago lends support to the regulatory DC axis, as human blood DCs cultured with two *Lactobacillus* species switch phenotype to one that drives development of a population of Tregs which are particularly potent suppressors of effector T cell proliferation[148]. However, *B. fragilis* and Clostridial species in cluster XIVa represent a more complicated story. One available clinical study links abundance of these microorganisms at infancy to increased asthma at age three[101]. However, these data may not be as discordant as they first appear. In the study of the effect of day care on asthma development, children in day care had more severe wheeze as toddlers but less asthma by age six[77], suggesting that early promotion of asthma may belie protection from disease later in life. For SCFAs, human clinical data are supportive but mixed. Maternal intake of acetate but not butyrate or propionate was associated with less wheeze and physician office visits in the first 12 months of infant life[134]. While this study may also need to look further
forward in time for a clear answer, it is perhaps just as possible that those SCFAs that drive mouse Treg development are not the same SCFAs which drive human Treg development.

The Balance Between Th2 and Th17 Responses

The microbiome may also play a role in driving asthma phenotype polarization. Antibiotic treatment in early life, in addition to predisposing to asthma in general, is capable of driving the systemic immune system in a Th2 direction[101], potentially promoting the development of allergic, eosinophilic asthma. In germ-free (GF) mice, who are born and live without a microbiome, the immune system is polarized in to produce Th2 responses that are incapable of being suppressed[149]. Together, these two studies suggest that the rise in asthma seen with a poorly-diverse microbiome may arise from polarization of the human immune system in a Th2 direction.

One interesting element of the Th2 polarization process which could have microbial involvement relates to pulmonary uric acid production. In 2011, one group reported that induction of experimental asthma in mice was accompanied by uric acid production in the airways, and that this uric acid production induced a Th2 response that was diminished by the addition of uricase[150]. Most interestingly, this seemed to apply to human asthmatics. Assessment of the BAL fluid of asthmatics revealed that they had higher uric acid levels than controls, and that those individuals with higher uric acid levels tended to produce stronger Th2 responses when challenged with allergen[150]. Where, then, is the microbial connection? At least one recent study has shown that one disease state characterized by heightened uric acid, end stage renal disease, was associated with an increase in microorganisms capable of metabolizing it (e.g. uricase-expressing)[151]. Perhaps one therapeutic for asthma could include drugs designed to increase the proportion of uricase-producing microorganisms, reducing the ability of pulmonary uric acid to promote Th2 immune responses.
As previously noted, removing PSA from *B. fragilis* turns a Treg inducer into a Th17 driver[126], and similar species to which lack PSA may be drivers of Th17 themselves. Indeed, the link between Th17 immunity and the microbiome is a strong one. In 2008, it was reported that certain microbial organisms were required for induction of Th17 cells in the mouse gut, but they were unable to identify the specific organisms[152]. Less than a year later, the culprit was revealed to be segmented filamentous bacteria (SFB), an organism within the class Clostridia, family Clostridiaceae[153].

While the evidence for SFB influencing asthma phenotype remains minimal, there is evidence for the influence of other organisms. *Haemophilus influenzae*, a common respiratory pathogen, has been found in the lungs of patients with severe neutrophilic asthma[154]. Infecting mice with *H. influenzae* during the induction of experimental asthma shifts the phenotype of disease, reducing markers of Th2 immunity, including eosinophils, and driving accumulation of neutrophils and production of IL-17[155]. If this relationship proves to be a key factor behind human Th17-driven asthma, it may be all the more reason to be sure that children receive the *Haemophilus influenzae* vaccine. Finally, one enteric pathogen, an *Enterococcus faecalis* subspecies, can suppress Th17 immunity and symptoms of allergic airway disease together[156], although specifically Th2-driven features of asthma (eosinophils, IgE) were not explored in that study. *E. faecalis* and similar organisms may represent targets worth evaluating to develop a therapeutic that moderates asthma and Th17 immunity simultaneously.

**B Cells and Immunoglobulin**

Unlike for Tregs, the connection between B cells, particularly Bregs, and the microbiome remains very poorly understood. We have some evidence that B cells need the microbiome and microbiome-associated lymphoid tissue, particularly that of the gut, to properly develop[157; 158]. One area in which there is a body of evidence linking regulatory B cells to the microbiome actually has its origin over a decade before the first reports linking Tregs to the same. In 1996, a
pioneering study reported that B10 cells, a population of Bregs characterized by production of the anti-inflammatory cytokine IL-10, are induced by the parasite *Schistosoma mansoni*, although they did not examine the phenotype of those Bregs much beyond IL-10 production[159]. Later work confirmed this link in patients suffering from the autoimmune disease multiple sclerosis, showing that B10 cell induction accompanied either *Trypanosoma cruzi* or *Paracoccidioides brasiliensis* infection[160]. More recently, two studies have shown that recognition of microbial antigens through TLRs[161] and the TLR signal mediator MyD88[162] produce B cells which can suppress inflammation during microbial infection. One recent experimental study demonstrated that *S. mansoni* can induce B10 cells (together with Tregs) and that transfer of the B10 cells to a naïve, non-infected animal is protective against experimental asthma[146]. While more work remains before this parasite-B10 axis can be targeted for asthma therapies, it is an exciting area of research and one further potential explanation for why a dirty childhood (including, perhaps, parasitic infection) can protect against asthma.

**Innate Lymphoid Cells (ILCs)**

ILCs, previously discussed as being players in the Th2 response, have also been linked to the microbiome[163], but the evidence that a microbiome-ILC-asthma axis exists remains tentative at best. We know that ILCs can interact with the microbiome[163], although the dependency of ILCs on microorganisms remains very poorly understood. The relationship between the two may be indirect. Two recent studies have shown that ILCs, specifically ILC3s, can modify the expression of membrane-bound receptors and signaling molecules on epithelial cells and that this modification changes how the epithelium interacts with the microbiome, including host susceptibility to pathogenic microbial invasion[164; 165]. With respect to asthma, one study has shown that antibiotics promote the allergic response to mold spores in mice, and that this response depends on IL-13 production[117]. Given the growing recognition that ILC2-produced IL-13 is a factor behind Th2-driven asthma (as previously discussed earlier in the chapter), this
disease exacerbation could be a result of antibiotic alteration of the microbiome subsequently impacting ILC development or function. Many more questions than answers remain in this area, and it is likely that we will learn much more about the relationship between the microbiome and ILCs in the coming years.

**What About the Locals? Enter the Lung Microbiome**

Prior discussion has centered on the how the microbiome of the gut may influence immunity in the lung but has not touched on the influence of the local lung microbiome. Despite the obvious potential relevance of the lung microbiome to asthma, this relationship, as with so many things related to the lung microbiome, remains poorly understood. This deficit is, to a large extent, an accident of history. While we have known for well over a century of the existence of gut microorganisms[61], the lung microbiome was long considered to be sterile in the absence of active disease[166–170] and was correspondingly excluded from the sites explored in the first Human Microbiome Project[71]. Today, we know that even healthy lungs harbor a microbiome, although precisely how it interacts with the pulmonary immune system and what constitutes a true “healthy” microbiome remain matters of great debate[171; 172].

In asthma, we know precious little, although some patterns are beginning to emerge. One common finding has been the association of Proteobacteria species with increased severity of asthma in BAL fluid from human asthmatics[173–176]. The explanation may return to Th17 induction, as one of these studies reported that genes related to Th17 immunity were upregulated in those individuals harboring abundant Proteobacteria[176]. It may also return to *H. influenzae*, as that microorganism happens to be a member of phylum Proteobacteria. When it comes to diversity of the lung microbiome, limited evidence suggests that the same patterns that hold true in the gut (i.e. higher diversity tends to be better for health) may not apply, as at least one large clinical study found higher diversity in asthmatics and bacterial burden in asthmatic lungs when compared to controls[174].
Just as early life gut microbiome exposures and perturbations appear to affect lifelong asthma risk, so too goes the lung. Two cohort studies of the human infant respiratory microbiome reported that the infant lung microbiome, like the gut, changes rapidly[177] but that colonization with Streptococcal organisms was a strong predictor of wheeze or asthma[177; 178]. One of these studies also mirrored results from adult asthmatics, reporting an association between two Proteobacteria, *Moraxella catarrhalis* and *H. influenzae*, and development of wheeze and asthma at 5 years of age[178].

The lung microbiome is one area where clinical evidence may outnumber experimental work, which remains exceedingly limited. One recent study may provide experimental support for the negative influence of Proteobacteria. In this report, newborn mice tended to produce exaggerated Th2 responses in an experimental asthma model, even in the face of high numbers of thymically-derived Tregs in the lungs[179]. However, as the lung microbiome bacterial load increased, Gammaproteobacteria were replaced by Bacteroidetes with a concomitant increase in iTregs, the very Treg population also generated by gut Treg inducers. This increase was evidently driven by interactions involving the cell surface receptor PD-L1. When challenged with allergen after this shift, mice were more resistant and developed less disease, and the exaggerated Th2 response disappeared[179]. Asthmatics, at least of the Th2-driven variety, may have seen the shift from Proteobacteria to Bacteroidetes either not occur or occur incompletely, imparting a correspondingly heightened risk of developing asthma on encountering an allergen.

In the past year, two elegant studies have shown that we may be able to manipulate the lung immune system through application of inhaled bacterial products. In one, endotoxin or farm dust (which contained endotoxin as well as a host of other microbial products) was administered intranasally to mice followed by the induction of experimental asthma[180]. Either endotoxin or farm dust suppressed the development of allergic disease through a mechanism mediated by induction of an immunosuppressive protein known as A20. Interestingly, this result conflicts with
at least one published clinical study, where higher levels of endotoxin were noted in the BAL fluid of asthmatics[175]. However, this could be another case of too much of a good thing, and perhaps the endotoxin burden in the human asthmatic is beyond that level at which it is capable of providing benefit. In the second study, a different bacterial product, flagellin, was administered simultaneously with the experimental allergen ovalbumin (OVA) or the common human allergen house dust mite (HDM). Whether administered with HDM or OVA, flagellin was found to protect from allergic airway inflammation[181]. The mechanisms of action, while not fully defined, may rely on regulatory DCs and Tregs, as administration of flagellin to human DCs polarizes them toward a regulatory, Treg-inducing phenotype[181].

**Therapeutic Targets and Interventions for the Microbiome**

We have recognized a relationship between the microbiome and asthma for many years, and it is perhaps surprising that we have no effective microbiome-based therapies to treat or prevent asthma. This paucity of therapies is not for lack of effort. Consider probiotics, or live microorganisms administered to provide a health benefit[182]. A number of experimental studies have fed mice probiotic microorganisms, or their products, particularly *Lactobacillus* spp., and found protection from experimental asthma[183–188]. This protection has been variously attributed to increased signaling through TLRs[183], increased numbers of systemic regulatory T cells[185; 187], and increased production of anti-inflammatory substances including IL-10 and TGFβ by macrophages[186]. However, a meta-analysis of 25 clinical trials of probiotics failed to show any beneficial effect of probiotics on asthma development[189]. While the reasons for the lack of translation from the mouse to the human remain unclear, one possibility is that adding a single microorganism or product has too small an effect to overcome any disease predisposition. It is worth noting that the only clinical microbiome therapy in widespread use today involves treatment with an entire microbial community, not just a few microorganisms. That treatment is fecal-microbiota transplantation (FMT) for antibiotic-resistant *C. difficile* colitis[190–192].
Successful asthma therapy using the microbiome may need to take a page from the \textit{C. difficile} playbook and look beyond the addition of just one or even a few organisms at a time.

That said, perhaps live organisms are not the answer. Consider the deep pool of evidence reviewed herein, both clinical and experimental, that endotoxin exposure prevents asthma development. Administration of endotoxin to children, or even adults, may provide replacement for our missing microbial friends and protection from asthma development. Indeed, perhaps such supplementation should go beyond endotoxin and administer a cocktail of microbial products, including endotoxin but also including fungal components and bacterial polysaccharides. Unfortunately, we may correct one problem and cause another, as at least one study shows that endotoxin and beta glucan shift the profile of asthma from Th2 to Th17\cite{193}.

Another potential option is the prebiotic, or a substance which is not alive but which modifies our microbiomes or is utilized by our microbiomes to produce a health effect\cite{194}. Dietary fiber is a potent modulator of both the microbiome and (through microbial metabolism) the immune system, and perhaps increased dietary fiber will prove effective in preventing asthma. At least one active clinical trial is assessing this very question\cite{195}. Several studies have looked at another prebiotic, oligosaccharides\cite{196; 197}, by administering them to human infants and assessing asthma symptoms later in life. Unfortunately, results are mixed, with one study reporting that wheeze was reduced later in life\cite{196} and others reporting no effect on wheeze\cite{197; 198}.

A final point to consider is the relationship between early-life interventions, particularly antibiotics and CS, and asthma. Avoiding all antibiotics in childhood would be foolish, and see many children suffer from diseases that are easily cured, just as avoiding CS entirely would see many children and mothers die during high-risk deliveries. However, both risk factors can be mitigated without unduly increasing danger to patients. CS should only be performed when medically warranted, not as an elective procedure, as recommended by the American College of Obstetricians and Gynecologists\cite{199} and the United Kingdom National Institute for Health and Clinical
Excellence[200]. For antibiotics, parents and physicians should carefully consider when to use antibiotics and when to not. If a child’s infection is deemed most likely to be viral, administering an antibiotic is not only costly but unnecessary and without benefit[108]. Indeed, there is evidence that providing a prescription for an antibiotic, but telling patients not to fill it unless they do not get better within a few days, produces better health outcomes than simply prescribing antibiotics for every upper respiratory infection that enters the clinic door[201]. For the cases where CS, antibiotics, and other hygienic interventions prove necessary, one means to potentially avoid ill effects could be FMT or other microbiome transplantation to correct the perturbation of the microbiome following treatment or intervention. Recently, evidence that such corrections could prove feasible was published in relation to CS. This report revealed that the microbiome can be “corrected” following CS[202], although no study has yet evaluated whether such interventions have any impact on the development of asthma or other disease.

Lost in Translation: Why Mice?

In the study of any human disease, asthma not excepted, one of the principle questions a researcher need ask is in what system they will undertake their work. Perhaps the most obvious choice would be to study human disease in the human system itself. Why, then, do so many studies, both in asthma and in other diseases, utilize animal models, particularly laboratory mice? One important consideration, particularly when it comes to an immunologic disease, is the ease with which the system lends itself to detailed mechanistic analysis. Isolating the immunologic pathways and mechanisms responsible for asthma pathophysiology requires that we are able to access tissues that, in a human, would be impossible or unethical to study absent extraordinary circumstances. For example, consider the study of Treg induction mediated by the lung microbiome and PD-L1[179]. Obtaining such access to lung tissue to determine this relationship would have been nearly impossible, if not altogether impossible, were humans the study subjects. Something as simple as access to the lymph nodes draining the lungs would be less challenging
but would still require invasive surgery that would be difficult to justify in a human as compared to a mouse.

However, the ability to precisely dissect immunologic pathways and the effects of experimental interventions may be meaningless if those dissected pathways, genes, and other players have no relevance for human disease. The dangers of this were illustrated in a 2013 report[203] that was widely commented on in the scientific and popular literature. In this study, the gene expression responses in the blood of mice and humans were compared in several inflammatory responses including trauma and sepsis. The investigators found little to no correlation between the species and concluded that the inflammatory response of the mouse differs too greatly from the human to be relevant for research[203]. This conclusion ignited a firestorm of controversy, with subsequent studies and commentaries[204] declaring that mice were alternatively useless and essential for biomedical research. In 2015, the waters were further muddied by a reanalysis of the original 2013 dataset, where a different team applied what they believed was a more appropriate analysis and found much similarity between the mouse and human inflammatory responses[205]. The debate has continued, with the authors of the original publication and others unrelated to either group declaring that one or the other assessments is not valid[206; 207]. While the exact relevance of the mouse to human research may remain unquantifiable for the immediate future, it is likely that the true answer falls somewhere between the extremes. Indeed, while one may question whether all mouse discoveries are directly applicable to humans, the available evidence suggests that at least some in the field of immunology and asthma (for example, that microorganisms can cause human dendritic cells to promote Treg development[148]) will prove to be shared between mice and men.

That said, the hopeful microbiome mouse researcher is confronted with an uncomfortable reality when it comes to improving human health: the veritable cordillera of published work has yet to translate into more than a handful of meaningful health advances, particularly with respect to
asthma. For many discoveries, such as the protective effect of prebiotic dietary fiber for murine asthma[133], the science is simply too new for an effect to have even been formally evaluated in humans. For others, particularly probiotics, we have excellent evidence of efficacy in mice[183–188] but poor evidence in human clinical trials[189]. This problem of poor translatability goes beyond asthma. Many fields have long bemoaned the failure of science to take findings in animal models and turn them into clinical advances, with many clinical trials based on promising preclinical animal data failing to show a benefit in man[208].

While one may be quick to blame the mouse, the true impediment to translation may be the human researcher. There is growing evidence that humans conducting less than rigorous animal studies are behind the failures of many promising preclinical results to show efficacy in humans. Consider the study of amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig’s disease. Efforts by ALS researchers to replicate the preclinical findings of dozens of mouse trials failed to replicate any when repeated in separate groups of mice[209; 210]. Many of these preclinical findings had failed to reproduce in humans, an unsurprising result when one considers that the original tests may have failed in mice in the first place. Indeed, efforts to blame the mice may also be obscuring significant concerns in human clinical studies. A recent analysis of 100 human psychology studies found that only 39% of reported effects were replicated, even with assistance from the authors of the original studies in designing the replications[211]. While some of the problem may lie in poor study design, part of the difficulty lies in the nature of the human animal. Unlike laboratory mice, humans are highly heterogeneous in behavior and lifestyle. Furthermore, while humans are far from genetically divergent from each other, even small genetic variations can produce enormous differences in responses to treatment[212]. Understanding these differences, and targeting therapies based on genetics, has blossomed into the distinct field of pharmacogenomics[212], and this may be another reason still for failures of animal studies (which benefit from highly
genetically homogeneous populations) to translate to human benefit. Mouse studies may have challenges and obstacles aplenty, but human studies carry just as many, if not more.

To abandon mice, however attractive it may seem, is to also abandon the rich legacy of discovery animal models have constructed. In immunology, many fundamental concepts have their roots in animal research. Consider the discovery of antibodies. Over a century ago, Behring and Kitasato described the protective effects of “antisera” against tetanus in rats and rabbits long before such concepts were extended to man[213; 214]. In the realm of asthma, similar animal discoveries abound. Cortisone, the first corticosteroid isolated, was found through the study of bovine adrenal glands nearly 90 years ago by Hench and Kendall[215], and any asthmatic who uses a steroid to control airway inflammation owes a debt of gratitude to those ruminants. The refinement of beta2-adrenergic agonists, the selective bronchodilators that save untold millions of asthma sufferers from serious morbidity and suffering, arose from detailed studies in animals[216]. Indeed, prior to the discovery of these specific receptor agonists, nonselective sympathomimetics such as epinephrine, which bronchodilate at the cost of a host of undesirable systemic effects, were the only means to provide relief to asthma sufferers[217]. Lest one think that only “old” medicine benefitted from animal work, consider omalizumab, the anti-IgE monoclonal antibody used today in the treatment of moderate to severe asthma[218]. Creating and refining this therapeutic so that it would provide specific IgE blockage without unwanted side effects (i.e. mast cell degranulation) took place in mice long before the drug was ever tested in humans[218].

Finally, there is cause for hope that mice may be an entirely appropriate platform for human microbiome studies. Mouse and human microbiomes, at least in the gut, do have similarities, although many of these are at higher taxonomic levels (i.e. phylum) rather than at the species level[219; 220]. One argument against reading too much into the dissimilarity of mouse guts from those of humans is that many studies only examine the guts of one strain of mice (often from one
supplier). We would do well to consider the microbiomes of all mouse strains together when comparing to humans. While variation would surely increase, variation is the currency of the human species, and we might find that the microbiomes of many different strains of mice together mimic the broader human microbiome. Perhaps future mouse studies of asthma will compare farm mice to conventional laboratory-raised animals, and the same patterns seen in human children may surface but now in a system amenable to manipulation and close, detailed study. Or, absent exact match between natural mouse microbiomes and human ones, we may be able to make mice more human. Transfer of human gut microbiomes to mice has shown that these microbiomes can implant stably, with relatively few differences from the original, donor human microbiome[70; 221]. Indeed, such transplants can even prompt physiologic changes, as we have previously noted with the gut microbiomes of obese and lean twins[70].

**Conclusion, Hypothesis, and Project Overview**

When it comes to the microbiome and asthma, the common rule of the microbiome applies: we recognize that there is an important relationship between the two, but we are only beginning to decipher precisely what that relationship is and which players direct it. Advances in the realm of microbiome-immune interactions have fundamentally changed our view of how microorganisms can regulate asthma, particularly when it comes to Tregs, but a host of questions remain to be answered. How can we harness microbial modulation of Tregs to control asthma? Is the microbiome responsible for the switch from Th2-driven, eosinophilic asthma to the severe, Th17-driven phenotype? Importantly from a translational research perspective, we recognize broad patterns in humans, such as early-life farm and endotoxin exposure and a lack of antibiotic exposure, in asthma development, but we have yet to understand how to harness these broad patterns to influence human disease.

The laboratory of Dr. Roger Thrall has made the study of regulation in asthma the subject of several decades of research. During that time, the lab utilized the experimental ovalbumin model
of allergic airway disease to first show that continuous, long-term inhalational exposure to ovalbumin induces a tolerant state, where further exposure to ovalbumin produces no appreciable disease[222]. Exploring this process of tolerance further, we demonstrated that Tregs accumulate as tolerance develops and experimental asthma is suppressed[31], that Bregs may interact with Tregs to promote this process[41; 42], and that other cells, including DCs[47] and natural killer cells[223], may have important roles to play in control of disease. Most recently, we have found that many of these principles extend beyond one asthma model and are, while not identical, very similar in a model utilizing the clinically-relevant human allergen house dust mite[32].

We are all exposed to allergens, the causative agents of Th2-driven asthma, but only a few of us ever develop asthma. It is possible that those of us who do not develop asthma actually experience disease but quickly develop tolerance to the offending allergen, potentially explaining the paradox of daycare children being at risk for early asthma but receiving protection later in life[77]. One potential driver of tolerance in some but not all people could be the microbiome. Our group has previously shown that regulatory elements of the immune system drive tolerance to inhaled allergens, and many microbiome researchers have shown that the microbiome drives regulatory elements of the immune system. However, efforts to link tolerance in asthma to the microbiome have been minimal at best. For my dissertation research, I took the expertise of the Thrall Laboratory in immune regulatory mechanisms in asthma and extended it to the microbiome, focusing on the following overarching hypothesis in the lab’s house dust mite (HDM) model of allergic airway disease:

*Exposure to antibiotics early in life increases the severity of asthma through microbiome-dependent reduction of regulatory T cells (Tregs).*

To evaluate my hypothesis, my dissertation traveled two parallel roads. On the first, I evaluated how the microbiome changes as experimental asthma progresses from disease to tolerance, linking these changes to shifting disease parameters. For the second, I disrupted the microbiome
using antibiotics and evaluated its impact on the development of disease and on the development of tolerance. The following chapters will detail the results of my endeavors in these areas as well as the future directions and potential clinical and scientific impact of my results on both microbiome research and asthma clinical care.
Box 1: Microbiome, Microbiota, or Metagenome?

Box 1. Microbiome, Microbiota, or Metagenome? The field of microbiome research utilizes many specific terms, and unfortunately some of these have no universally accepted definition, particularly microbiome[224]. Some researchers consider the word “microbiome” to be derived from a joining of the word “microbe” and suffix “ome”, implying all the genomes of all the microorganisms in a community. A second definition is broader, pairing the prefix “micro” with the word “biome” and implying the sum of all organisms, their activity, and their genes. As originally popularized by Joshua Lederberg, microbiome was closer to the latter, defined as “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space”[60]. Such a definition seemingly encompasses the organisms, their interactions with each other, and their interactions with the host (the entire biome, if you will). Furthermore, this definition fits with classic use of the term. In work dating to 1988[225], 1952[226], and even 1949[227], the microbiome referred to the entire ecological community of microorganisms and their interactions with their environment. I prefer to use microbiome in its classical sense, to refer to the sum of all the organisms, their genomes, and their environmental interactions. When discussing only the organisms, I shall use the term microbiota. To speak of the genomes of the microorganisms, I shall use the term metagenome.
CHAPTER 2
MATERIALS AND METHODS

Animal Subjects

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). For studies of allergen exposure and microbiome changes (no antibiotics included), female mice were purchased at 6-8 weeks of age. For studies of early-life antibiotic (ABX) exposure, nursing mothers with female pups were purchased at three weeks of age and upon receipt were immediately weaned and ABX treatment initiated. For studies of the effect of ABX throughout life on tolerance, pregnant mice were purchased and treatment initiated a few days prior to giving birth. Mice of both sexes were used for the latter studies.

Mice were housed conventionally in plastic cages with corncob bedding in the Center for Comparative Medicine at UCONN Health. Animal rooms were maintained at 22-24°C with a daily, 12-hour light/dark cycle. Chow and water were supplied ad libitum. Animal care and use was approved by the Institutional Animal Care and Use Committee at UConn Health, protocol #100983-0717.

House Dust Mite (HDM) Model

The HDM model of allergic airway disease (AAD) was performed as described in [32]. Briefly, mice were lightly anesthetized with vaporous isoflurane and intranasally (i.n.) instilled with droplets containing 25 μg lyophilized HDM extract (equal mixture of *Dermatophagoides pteronyssinus* and *D. farinae*, Greer Laboratories, Lenoir, NC) solubilized in 50 μl phosphate buffered saline (PBS). HDM was administered for five consecutive days, followed by two days of rest, for up to eleven consecutive weeks (Fig 1). Control groups received equal volumes of *i.n.* PBS in a time-matched manner. For studies assessing the active disease phase, mice were sacrificed after either short-term (two week) or intermediate-term (five week) HDM exposure time.
points. For studies of tolerance, mice were sacrificed at after long-term (eleven week) HDM exposures. All mice were sacrificed 72 hours post final HDM challenge.

**Broncho-Alveolar Lavage (BAL), Blood, and Tissue Collection**

To obtain BAL fluid and cells from mice, the trachea was exposed and cannulated at time of sacrifice. Lungs were lavaged *in situ* with 5 mL of sterile saline in 1 mL aliquots and BAL cells pelleted at 600xg for ten minutes at 4°C. Hilar (lung-draining) lymph nodes (HLN) were removed and mechanically disrupted into single-cell suspensions and filtered through a 100 µm Nytex screen (ELKO Filtering Co, Miami, FL). Lavaged half lungs (left lung) were digested with 150 U/mL collagenase (Gibco/Thermo-Fisher Scientific, Waltham, MA; Cat #17018-029) and mechanically disrupted into single-cell suspensions, after which erythrocytes were lysed using Tris-buffered ammonium chloride (TAC) solution (9 parts 0.83% w/v ammonium chloride; 1 part 2.57% w/v Tris; pH 7.0) at room temperature. For histopathologic assessment, lavaged half lungs (right lung) were removed and fixed with 4% buffered formalin for 72 hours.

For all tissue samples, manual total nucleated cell counts were obtained using a hemocytometer with nigrosin dye exclusion as a measure of viability. Cytospin preparations of BAL fluid were stained with May-Grünwald and Giemsa for differential cell analysis via light microscopy.

To isolate serum, blood was collected from mice through cardiac (right ventricular) puncture at the time of sacrifice and allowed to clot for 30 minutes. Clotted blood was spun down at 1600xg for ten minutes and serum removed and stored at -80°C until needed for analysis.

**Flow Cytometry**

Cells isolated from BAL fluid, HLN, and lung tissue were subject to flow cytometric analysis using the following monoclonal antibodies: CD3epsilon (1:200, Tonbo Biosciences, San Diego, CA; Clone 145-2C11; Cat #35-0031-U100); CD4 (1:200, eBioscience, San Diego, CA; Clone GK1.5; Cat #47-0041-82); CD5 (1:100, eBioscience; Clone 53-7.3; Cat #17-0051-81); CD8α (1:200,
Tonbo Biosciences; Clone 53-6.7; Cat #80-0081-U100); CD19 (1:200, Tonbo Biosciences; Clone 1D3; Cat #35-0193-U500); and CD45R/B220 (1:200, Tonbo Biosciences; Clone RA3-6B2; Cat #75-0452-U025). Samples were stained essentially as previously described[32; 41]. Cells were washed in PBS containing 0.2% bovine serum albumin and 0.1% sodium azide. Aliquots containing \(10^5-10^7\) cells were incubated with anti-mouse CD16/CD32 (1:200, Fc Shield, Tonbo Biosciences; Cat #70-0161-U500) for 15 minutes. Cells were then incubated with AlexaFluor350 as a viability marker (1:500, Invitrogen/Thermo-Fisher Scientific; Cat #A10168) and surface antibodies for 30 minutes at 4°C. For surface stains only, cells were then fixed with 4% paraformaldehyde for 10 minutes. For staining of Foxp3, cells stained with surface markers were fixed and permeabilized with Foxp3/Transcription Factor Staining Kit (Tonbo Biosciences; Cat #TNB-0607-KIT) according to the manufacturer’s instructions and stained with anti-Foxp3 (1:150, Tonbo Biosciences; Cat #20-5773-U100) for 30 minutes at 4°C. Samples were run with corresponding isotype controls on a BD LSR II (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo (Tree Star Software, Ashland, OR).

**Tissue Histology and Scoring**

Formalin-fixed lungs were placed in tissue cassettes and sent to the Connecticut Veterinary Medical Diagnostic Laboratory (CMVDL) in Storrs, CT for paraffin embedding, slide mounting, and both hematoxolyn and eosin (H&E) and periodic acid-Schiff with hematoxylin counterstain (PAS-H) staining. H&E-stained slides were used to assess gross pathologic changes, while PAS-H-stained sections were used to assess mucus production. Sections from all four lobes of the right lung were evaluated in their entirety and representative images taken using a BX53 research microscope with DP73 digital camera (Olympus, Waltham, MA). Pathologic scoring was performed essentially as described previously[32] with modifications made to the scoring rubric. Five blinded reviewers simultaneously graded the same visual fields on each slide using a multiheaded microscope. For inflammation scores, 0 corresponded to no detectable
inflammation; 1 to mild peribronchiolar/perivascular cuffing with inflammatory cells; 2 to significant peribronchiolar/perivascular clustering; and 3 to significant clustering and airway remodeling (e.g. smooth muscle hypertrophy and hyperplasia). For mucus scores, 0 corresponded to no visible mucus; 1 to occasional and punctate mucus staining in the airways; 2 to presence of ring-like mucus structures in less than 10% of airways; and 3 to presence of ring-like mucus structures in greater than 10% of airways. The scale was modified from [32] in that half scores (e.g. 0.5, 2.5) were permitted when graders determined a sample fell between two whole-number grades.

**Serum Immunoglobulin Assessment**

Isolated serum was assessed for HDM-specific IgE and IgG1 levels essentially as described previously[32]. Serum HDM-specific IgE levels was determined by ELISA. 96-well Nunc MaxiSorp flat-bottom plates (Thermo-Fisher Scientific) were coated with 10 ug/mL HDM extract in sodium bicarbonate buffer (pH 9.5) for 48 hours at 4°C. Following coating, plates came to room temperature for 15 minutes and were washed six times with 0.05% PBX/Tween 20 (PBST) and blocked for one hour at 37°C with BD OptEIA Assay Diluent (BD Biosciences, San Diego, CA; Cat #555213). After washing six times with PBST, serum samples were added in two-fold serial dilutions (range 1:20 to 1:2560) and incubated at room temperature for 1.5 hours. Plates were washed eight times with PBST and labeled with Biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech, Birmingham, AL; Cat #1110-08) diluted 1:5000 in blocking buffer for one hour at room temperature.

Serum HDM-specific IgG1 levels were determined by ELISA in a similar manner to detection of IgE. 96-well plates were coated with 2 ug/mL HDM extract in sodium bicarbonate buffer for 24 hours at 4°C. Plates were washed six times with PBST and blocked for one hour in BD OptEIA Assay Diluent. Plates were washed six times with PBST and incubated with 10-fold serial dilutions of serum (1:10 to 1:200,000,000) for 1.5 hours at room temperature. Plates were washed eight times with PBST and labeled with biotin-SP-conjugated AffiniPure goat anti-mouse IgG, Fcγ
subclass 1 (Jackson ImmunoResearch, West Grove, PA; Cat #115-065-205) diluted 1:100,000 in blocking buffer for one hour at room temperature.

For both assays, plates were then washed six times and incubated with streptavidin HRP (BD Biosciences, San Jose, CA; Cat #554066) diluted 1:1000 in blocking buffer at room temperature for thirty minutes. Plates were washed seven times with PBST and incubated with 3,3',5,5'-tetramethylbenzidine (TMB, KPL, Gaithersburg, MD; Cat #50-76-01) for 20 minutes in the dark at room temperature. The reaction was stopped using 1 M phosphoric acid at equal volume to TMB and the plates were read at dual 450nm-570nm wavelengths using an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

**Airway Hyper-Reactivity (AHR) Assessment**

AHR was assessed as previously described with modification[32]. Total respiratory system resistance (Rrs) responses to increasing doses (0-300 mg/mL) of acetyl-β-methacholine chloride (Sigma-Aldrich, St. Louis, MO; Cat #A2251) were measured by a flexiVent FX1 mechanical ventilator (SCIREQ Scientific Respiratory Equipment, Montreal, QC, Canada). Mice were anesthetized using urethane (Sigma-Aldrich; Cat #U2500) administered in saline by intraperitoneal (i.p.) injection at 0.8 g/kg - 5.0 g/kg body weight. Once insensitive to toe pinch, respiratory muscles were paralyzed using 500 ng/kg i.p. pancuronium bromide (Sigma-Aldrich; Cat #P1918) and mice were immediately placed under mechanical ventilation. Airway reactivity was determined by assessing forced oscillatory mechanics every ten seconds for four minutes following each methacholine challenge. Electrocardiogram measurements were monitored to ensure viability for the duration of mechanical ventilation.

**Antibiotic (ABX) Treatment**

ABX treatment consisted of one of three regimens. Mice were exposed to either tylosin (Sigma-Aldrich; Cat #T6271) and amoxicillin (Sigma-Aldrich; Cat #A8523), amoxicillin alone, or a
combination of ampicillin (Sigma-Aldrich; Cat #A0166), metronidazole (Sigma-Aldrich; Cat #M1547), neomycin (Sigma-Aldrich; Cat #N6386), and vancomycin (Sigma-Aldrich; Cat #V1130) in the drinking water. ABX-treated water was sterile filtered prior to placement in mouse cages and provided to mice ad libitum. Control water was treated identically to ABX-containing water (including sterile filtering) save for the lack of ABX.

For experiments assessing early life antibiotic exposure and AAD, the treatment schedule was adapted from the example of Nobel and colleagues[228]. Mice were exposed to amoxicillin, ABX mix, or control (untreated) drinking water for three four-day periods (Thursday to Sunday), with a three day rest between treatments where untreated water was supplied, beginning immediately after weaning (three weeks old). Following the third ABX treatment, mice were administered HDM for five weeks according to the model described earlier. Refer to Figure 2 for a schematic of the treatment strategy.

For experiments assessing the effect of antibiotics on tolerance development, three treatment schedules were utilized. The first was closely adapted from Nobel and colleagues[228]. Mice received two different ABX, tylosin and amoxicillin, in the drinking water in alternating four alternating, four-day periods (Thursday to Sunday), with a three day rest between treatments where untreated water was supplied, beginning immediately after weaning (three weeks old). The first treatment consisted of 0.4 g/L tylosin, the second 0.25 g/L amoxicillin, the third 0.4 g/L tylosin, and the fourth 0.25 g/L amoxicillin. HDM exposure began following the first tylosin exposure. Figure 3 details this treatment strategy. The second ABX exposure model followed the early life exposure model described previously but extended HDM exposure to eleven weeks (Figure 4). The third was adapted from the examples of reports studying perinatal antibiotic administration on asthma development in laboratory mice[118; 229; 230]. Here, mice were exposed to amoxicillin, ABX mix, or control (untreated) drinking water from approximately 2-3 days prior to birth until the conclusion of the experiment (i.e. throughout life). In both cases, mice were exposed
to eleven weeks of HDM according to the model described earlier. Refer to Figure 5 for a schematic of the treatment strategy.

Amoxicillin was selected because it is the most commonly prescribed ABX in childhood[231] and it can be dissolved in drinking water and administered to laboratory mice[232]. The cocktail of ampicillin, metronidazole, neomycin, and vancomycin (hereafter, “ABX mix”) was selected for its demonstrated effectiveness in broadly depleting the entire gut microbiome and its ability to be dissolved and administered in drinking water to laboratory mice[233; 234].

**Microbiome Sampling and DNA Extraction**

The mouse fecal microbiome was sampled by collecting fresh fecal pellets at the time of production. Mice were grasped by the scuff of the neck and restrained until defecation, whereupon sterile, RNA-/DNA-/RNAse-/DNAse-free microcentrifuge tubes were used to collect the pellet. Collected pellets were immediately placed at -80°C storage and kept frozen until DNA extraction.

DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA; Cat #12888-100) according to the manufacturer’s protocol with one modification. At step five (bacterial cell disruption), fecal samples were placed in PowerSoil bead tubes and samples were bead-beaten for five minutes using a single-speed (3450 oscillations/min) Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK). Extracted DNA was quantified using a Qubit dsDNA Broad Range Assay Kit (ThermoFisher Scientific; Cat #Q32853) and a Qubit 2.0 fluorometer (ThermoFisher Scientific Cat #Q32866).

**16S rRNA Amplification and Sequencing**

Amplicon library preparation, cleaning, and dilution were performed by the Microbial Analysis, Resources, and Services (MARS) Facility at the University of Connecticut, Storrs, CT. Prior to August, 2015, the V4 region of the 16s rRNA gene was amplified and sequenced using the
protocol and barcoded primers (515F and 806R, with 8 basepair Golay-barcode indices on the 3’ end) designed by Caporaso et al.[235]. Approximately 20 ng of extracted DNA was amplified in triplicate using Phusion High-Fidelity DNA Polymerase master mix (New England BioLabs, Ipswich, MA; Cat #M0530S). The PCR reaction was incubated at 94 ºC for three minutes followed by 30 cycles of 45 s at 94.0 ºC, 60 s at 50.0 ºC and 90 s at 72.0 ºC. Final extension took place at 72.0 ºC for 10 minutes.

From August 2015, the protocol was the same as above with three modifications. The V4 region of the 16s rRNA gene was amplified in triplicate using the dual barcoded primers (515F and 806R) of Caporaso et al (8 basepair Golay-barcode indices on the 3’ end[235]) and Kozich et al (8 basepair on the 5’ end[236]). Amplification was performed using 30 ng of DNA and AccuPrime Taq DNA Polymerase (Thermo-Fisher Scientific; Cat #12342010). The PCR reaction was incubated at 95 ºC for 3.5 minutes followed by 30 cycles of 30 s at 95.0 ºC, 30 s at 50.0 ºC and 90 s at 72.0 ºC. Final extension took place at 72.0 ºC for 10 minutes. Comparison of the same samples amplified by both polymerases and using both PCR reaction protocols revealed no significant differences between the two with respect to downstream diversity or community composition (genus-level and higher).

PCR products were pooled for quantification and visualization using the QIAxcel DNA Fast Analysis (Qiagen, Hilden, Germany). PCR products were normalized based on the concentration of DNA from 250-400 bp in length and pooled using the QIAgility liquid handling robot (Qiagen). The pooled PCR products were cleaned using the Gene Read Size Selection kit (Qiagen; Cat #180514) according to the manufacturer’s protocol. Samples were then denatured, diluted, and sequenced on an Illumina MiSeq using v2 or v3 chemistry according to the manufacturer’s instructions. PhiX (5-20%) was added to the run to increase sample complexity.
Analysis of 16S rRNA Sequence Data

Sequence data were analyzed using QIIME 1.9.1 software[237] in the High Performance Computing Facility of the Richard D. Berlin Center for Cell Analysis and Modeling. The analysis pipeline was derived from Nelson et al[238]. Briefly, samples were demultiplexed and paired-ends joined. A Phred quality score of 19 was used as the cut-off when splitting libraries. Joined reads were filtered so that only reads of between 245 and 260 base pairs (bp) were kept. Chimeric sequences were removed using UCHIME[239] within USEARCH6[240]. Operational taxonomic units (OTUs) were picked at 97% similarity using USEARCH6[240; 241] in an open-reference manner using the GreenGenes ribosomal database May 2013 release[242; 243] as a reference. Results were subsampled to 10,000 sequences, and any samples falling below this threshold were either excluded or re-sequenced until they exceeded the 10,000 sequences threshold. Alpha and beta diversity metrics were calculated using QIIME. PCoA of beta diversity metrics was performed using QIIME and included comparisons between groups using UniFrac distances[244]. For taxonomic tables, only organisms which appeared with an abundance of greater than 1% in more than one group or timepoint were represented as distinct bars. All organisms failing this cut-off were placed in the “Other” category. These organisms were included in all alpha and beta diversity analyses. Appendix A contains the analysis script used. Appendix B contains the settings specified in the QIIME parameters file referenced in the analysis script.

Fecal Microbiota Transplantation (FMT)

FMT was performed in a manner adapted from[245–248]. Fresh fecal pellets were collected in sterile saline at a ratio of approximately 1 mL of saline per 3.5 pellets. The pellet-saline mixture was vortexed at the highest speed for 3 minutes to disperse pellets. Tubes were then centrifuged at 300xg for one minute to pellet large particulate matter. The supernatant was drawn off, avoiding particulate matter, and immediately frozen at -80°C until use. Prior to use, frozen FMT solution was thawed and allowed to come to room temperature.
To perform FMT, 200 µL of FMT solution or saline control was lavaged into the stomach (gavaged) into mice. Gavage was performed by firmly grasping the mouse, without anesthesia, and advancing a 22 gauge, 1 inch stainless steel feeding needle with 1.25 mm ball time (Braintree Scientific, Braintree, MA) into the oral cavity, down the esophagus, and into the stomach. Contents of the syringe are then injected slowly into the stomach. Mice who has received early life antibiotic exposure as described above received three gavages of FMT solution (Monday, Wednesday, and Friday) in the first week following the final ABX treatment. Following the first week of gavages, mice began HDM i.n. exposure for two weeks. During those two weeks, mice received two FMT solution gavages (Tuesday and Thursday) per week for the subsequent two weeks. The ABX, FMT, and HDM treatment protocol is outlined in Figure 6.

**HDM Gavage**

To assess oral tolerance, mice were exposed to HDM by gastric lavage (gavage). Gavage was performed in the manner described for FMT. HDM-gavaged animals received an identical dose to that of mice exposed to intranasal HDM, 25 µg lyophilized HDM extract (equal mixture of *Dermatophagoides pteronyssinus* and *D. farinae*, Greer Laboratories, Lenoir, NC) solubilized in 50 µl phosphate buffered saline (PBS). HDM gavage was administered for five consecutive days, followed by two days of rest, for eleven consecutive weeks (Figure 7A). Control groups received equal volumes of gavaged PBS in a time-matched manner. In a second group of animals, mice received eleven weeks of HDM or PBS gavage followed by two weeks of i.n. HDM (Figure 7B) in the manner of Figure 1. All mice were sacrificed 72 hours post final HDM challenge.

**Statistical Analysis**

Statistical comparisons between two groups were made using the non-parametric Mann-Whitney U test. Comparisons between three or more groups were made using the non-parametric, one-way Kruskal-Wallis analysis of variance (ANOVA). Multiple-comparisons between individual
groups were made using Dunn’s multiple comparisons test or Sidak’s multiple comparisons test, as appropriate. Comparisons of serum immunoglobulin levels and AHR were made by computing the area under the curve (AUC) of all samples and comparing the AUCs using one-way ANOVA. These tests were performed using Prism 6 (GraphPad Software, La Jolla, CA). Measures of within-sample (alpha) and between-sample (beta) diversity were made using QIIME software. Biomarker and effect size assessment for taxa were made using LDA Effect Size (LEfSe)[249] on the Huttenhower Lab Galaxy Server[250; 251]. For all tests, a threshold of p < 0.05 was used to determine statistical significance.
Figures

Figure 1: House Dust Mite (HDM) model of allergic airway disease (AAD) and tolerance

**Figure 1.** House Dust Mite (HDM) model of allergic airway disease (AAD) and tolerance. HDM extract was administered intranasally to mice five days per week, Monday through Friday, for up to eleven weeks as described in the *Materials and Methods*. AAD was generated by two weeks of exposure and persisted at least through five weeks of exposure. By eleven weeks of exposure, tolerance developed and disease was suppressed. Mice were sacrificed 72 hours following the final HDM exposure for each timepoint (two, five, and eleven weeks).
Figure 2. HDM and Antibiotics Exposure Model, 5 Weeks. Mice received ABX exposure in an analogous manner to that described in Figure 2. ABX consisted of either amoxicillin or a combination of ampicillin, metronidazole, neomycin, and vancomycin (ABX mix). Untreated water served as a control. Following the first two ABX exposure periods, ABX-treated water was replaced with untreated water from Monday to Thursday. HDM exposure for five weeks (five days per week) began immediately following the final ABX exposure. Mice were sacrificed 72 hours following the final HDM exposure.
Figure 3: Early-Life Antibiotics and Tolerance Exposure Model: Amoxicillin and Tylosin

Mice received ABX exposure consisting of tylosin (ABX exposures at weeks 0 and 2) or amoxicillin (ABX exposures at weeks 2 and 4). Untreated water served as a control. Following the first three ABX exposure periods, ABX-treated water was replaced with untreated water from Monday to Thursday. HDM exposure for eleven weeks (five days per week) began immediately following the first ABX exposure. Mice were sacrificed 72 hours following the final HDM exposure.

Figure 4: Early-Life Antibiotics and Tolerance Exposure Model: Amoxicillin and Antibiotic Mix
**Figure 4.** Early-Life Antibiotics and Tolerance Exposure Model: Amoxicillin and Antibiotic Mix. Mice received ABX exposure in an analogous manner to that described in Figure 2. ABX consisted of either amoxicillin or a combination of ampicillin, metronidazole, neomycin, and vancomycin (ABX mix). Untreated water served as a control. Following the first two ABX exposure periods, ABX-treated water was replaced with untreated water from Monday to Thursday. HDM exposure for eleven weeks (five days per week) began immediately following the final ABX exposure. Mice were sacrificed 72 hours following the final HDM exposure.

**Figure 5: Continuous Antibiotics and Tolerance Exposure Model**

![Diagram](image)

**Figure 5.** Continuous Antibiotics and Tolerance Exposure Model. To ensure sustained disruption of the developing microbiome, mice were exposed to ABX continuously throughout life, including during the prenatal period. ABX consisted of either amoxicillin or a combination of ampicillin, metronidazole, neomycin, and vancomycin (ABX mix). Untreated water served as a control. HDM exposure began at six weeks of age and continued for eleven weeks (five days per week). Mice were sacrificed 72 hours following the final HDM exposure.
Figure 6. Fecal Microbiota Transplantation Model. Mice received ABX exposure in an analogous manner to that described in Figure 2. During the week following the third antibiotic exposure period (week 0), mice received 200 µL gavage of saline-suspended fecal contents three times: Monday, Wednesday, and Friday. HDM exposure began in the following week (week 1) and continued for two weeks. During the two weeks of HDM exposure, mice received an additional two gavages per week (Tuesday and Thursday) of 200 µL of saline-suspended fecal contents. Mice were sacrificed 72 hours after the final HDM exposure.
Figure 7: HDM Gavage Model

HDM Gavage (5 days/week)

0 1 2 3 4 5 6 7 8 9 10 11

Weeks

Figure 7. HDM Gavage Model. HDM extract was administered by oral gastric lavage (gavage) to mice five days per week, Monday through Friday, for eleven weeks as described in the Materials and Methods (A). Control animals received gavage of PBS alone. The dose administered by gavage was identical to that administered by intranasal instillation as described in Figure 1. After eleven weeks of gavage, half the HDM-gavaged and half the PBS-gavaged mice were sacrificed and assessed using the cellular and immunologic parameters described in the Materials and Methods. The remaining half of the HDM- and PBS-gavaged mice were
CHAPTER 3
THE IMPACT OF INHALED AND ORAL HDM EXPOSURE ON THE GUT MICROBIOME

Introduction

Immunologic tolerance (henceforth, “tolerance”) is the process by which the immune system does not react to an encountered substance, cell, or other recognizable antigen. Perhaps the most classic example of tolerance is the inability of the immune system to attack self tissue absent pathologic autoimmunity[252]. However, tolerance extends beyond self tissue and includes many non-self substances. One obvious example is food: we take in large quantities of food daily, all of which is decidedly foreign, but our body is normally able to tolerate the presence of food and not react to it[253]. Indeed, failure of the body to tolerate the presence of food is the source of many devastating food allergies, where the body reacts to entirely-harmless foods such as peanut[254] or the wheat protein gliadin[255], the latter reaction being the source of celiac disease.

Just as food allergy is a failure of immunologic tolerance, so too is allergic asthma (hereafter, “asthma”). The airway inflammation that is so characteristic of the disease is a result of that failure of tolerance against otherwise harmless inhaled allergens[256]. Work from our group[32; 222; 257] as well as several others[258–261] has shown that tolerance and suppression of asthma can be generated through prolonged inhalational exposure to the offending allergen. However, these experimental systems have important differences from human asthma. In mice, exposure to OVA or HDM always consistently produces the development of tolerance, whereas a minority (albeit a significant one, with prevalence approaching 15% of the pediatric population for some groups[54]) of humans fail to develop that tolerance despite exposure to the same allergens that others are tolerant to. Elucidation of the mechanisms that drive tolerance in animal models of asthma is
critically important to identify targetable mechanisms that could promote tolerance in human asthma sufferers. Our work[31; 32; 41] as well as that of others[30] has suggested that regulatory T cells (Tregs) are critically important for the generation and maintenance of tolerance to inhaled allergens. In humans, Tregs have been shown to be functionally impaired in a number of allergic diseases[262], and a growing body of evidence has revealed that many human asthmatics have decreased numbers of Tregs, diminished suppressive capacity in those Tregs that are present, or a combination thereof[24; 37; 38; 263].

One of the forces driving tolerance-promoting Tregs is the host microbiome. Multiple microbial populations have been shown to drive the formation of inducible Tregs (iTregs), particularly in gut-associated tissues[124; 125; 127; 131]. Despite the fact that these Tregs are generated in the gut, they can have protective affects in a number of tissues, and some have reported that it is these iTregs, rather than thymically-derived Tregs, that are most important for suppression of allergic inflammation in the lung[35]. The microbiome is shaped by its exposures, and these exposures influence its ability to regulate the immune system, including through Tregs. Several studies have shown that products produced by gut microorganisms as they ingest the food that we eat, particularly production of short-chain fatty acids (SCFAs) from dietary fiber, can resolve experimental asthma in the lung through induction of Tregs[133; 134].

Furthermore, allergens may themselves have a role to play. House dust mite (HDM), one of the most common human allergens, is highly complex, consisting of products from the mite itself as well as associated bacterial and fungal components[32]. At least one study has shown that certain kinds of HDM and the house dust they dwell in can contain protective microorganisms that colonize the gut of exposed animals and protect against experimental asthma[139]. However, the HDM commonly utilized in our laboratory and others is a standardized extract from Greer Laboratories, not a sample obtained from the complex environment of a home. Almost no information exists on the impact of this standardized HDM extract on the microbiome despite its
wide use. A single recent report described changes induced by HDM exposure that depended on the age of the animal[264]. However, this report evaluated changes in the microbiome after just three intranasal sensitizations and subsequent challenge with HDM 21 days later. Evidence for the effect of prolonged HDM exposure on the microbiome remains to be determined. For this investigation, we hypothesized that exposure to HDM would promote the development of a regulatory microbiome, and that this regulatory microbiome would promote the suppression of AAD through accumulation of organisms known to induce Tregs. In the present chapter, we explore this hypothesis through assessments of the fecal microbiome across our model of HDM-induced AAD.

Results

Sample Numbers and Groups

The fecal microbiome was assessed in three independent groups of animals subjected to our HDM model of asthma, one each for the years 2013-2015. One group (35) was assessed at each stage of the model (pre-intranasals, or naïve; 2; 5; and 11 weeks) for HDM as well as PBS-exposed mice. A second group (27) was assessed at each stage of the model in HDM-exposed animals alone. The final group was assessed at the naïve and 11 week HDM exposure timepoints. At least twenty HDM-exposed animals were assessed at each timepoint of the model. Details of sample numbers by group and timepoint may be found in Table 1. These numbers represent the samples analyzed using QIIME in Figures 8 and 9.

HDM Exposure Does Not Consistently Alter the Composition of the Fecal Microbiome

Analysis of the composition of the microbiome using QIIME revealed that the three groups of HDM-exposed mice experience changes in the fecal microbiome that differ between groups but tended to be similar to time-matched PBS controls (Figure 8). All naïve samples began with a
similar microbial community dominated by a group of organisms only classifiable as order Clostridales and the family S24-7. Family S24-7 have been previously shown to represent a large portion of the mouse fecal microbiome and represent an as-yet uncultured and poorly-described group of Bacteroidetes[265; 266]. Over time, all samples saw the proportion of genus Akkermansia increase from nearly undetectable in naïve animals (with the sole exception of group 27) to upwards of 20-40% of all organisms by eleven weeks of either PBS or HDM exposure. The proportion unclassifiable order Clostridales tended to decrease with time across all groups, as did the proportion of organisms within the family S24-7, although the latter organisms continued to make up the single most abundant group of organisms for all timepoints and exposures. In all groups and treatments, the proportion of genus Lactobacillus tended to decreases and the proportion of family Rickenellaceae tended to increase, although the abundance of the latter remained below 10% of all organisms across groups and treatments.

Those differences between PBS- and HDM-exposed animals which did exist were not consistent between experimental groups. In group 35, HDM-exposure was associated with increases in organisms of genus Blautia, genus Parabacteroides, order RF32 (a Proteobacteria), and genus Bacteroides as compared to PBS-exposed animals. Indeed, Blautia and RF32 were essentially present only in HDM-exposed animals. Contrastingly, an unidentified group of organisms within the family Ruminococcaceae were only present in PBS-exposed animals after eleven weeks of exposure. However, both group 6 and group 27 exhibited no appreciable population of Blautia, RF32, Parabacteroides, or Bacteroides. Furthermore, these two groups had members of the unidentified Ruminococcaceae in both HDM- and PBS-exposed populations. For group 6, HDM exposure was associated with no distinct populations of organisms when compared to PBS controls, although the proportions of the unidentified group of order Clostridales and unidentified organisms of family Lachnospiraceae were different between the HDM and PBS, the former being more abundant and the latter less abundant in HDM-exposed animals as compared to PBS.
Group 27 has no time-matched PBS control, but its final composition was similar to that of group 6’s HDM-exposed animals save for a higher proportion of genus Allobaculum.

**HDM-Induced Fecal Microbiome Changes Are Inconsistent and Often Similar to PBS-Induced Changes**

Principle coordinate analysis (PCoA) of the unweighted UniFrac distances between the groups confirmed the qualitative taxonomic comparisons seen in Figure 8. Comparisons of groups 6 (Figure 9, lower left), 27 (Figure 9, lower right), and 35 (Figure 9, upper right) revealed that all naïve samples tended to be similar at the start of the experiments, residing in the lower right of the coordinates plot. Additionally, all groups shifted in a similar manner over time, moving from the lower right of the coordinate plot up and to the left, with the majority of the change seen along principle coordinate 2. Group 35 differed the most from the other two, with eleven week and some five week HDM samples occupying a distinct position in the lower left from all other samples. Eleven week samples from groups 6 and 27 changed in a similar manner to each other but distinct from group 35. PBS-exposed animals in groups 35 and 6 shifted in a similar manner to time-matched HDM-exposed animals. Only animals in group 35 differed appreciably along both principle coordinates after eleven weeks of PBS or HDM.

**Inter-Group Variability Drives Fecal Microbiome Changes Rather Than HDM Exposure**

To assess the importance of individual taxa, including those whose abundance was below the 1% threshold for graphical depiction described in the Materials and Methods, LEfSe was utilized to identify taxa which differed between treatment groups (Figure 10A). Initial LEfSe analysis evaluating differences between treatment groups (the class, e.g. 11 week HDM vs 11 week PBS) initially identified several dozen taxa, including several known to have immunomodulatory effects (Figure 10A, e.g. genus *Lactobacillus* and genus *Clostridium*). However, secondary analysis with groups (the subclass, e.g. Group 35 vs Group 27) revealed only a single taxa with differential
representation between treatment groups, family Clostridaceae, genus Clostridium which was overrepresented in naïve animals (Figure 10B). This taxa is present in most naive animals (Figure 11) but not present in all but a handful of animals by 11 weeks, suggesting that its disappearance is a result of normal changes with aging rather than treatment effect.

**HDM Exposure Does Not Significantly Affect Microbial Diversity**

Diversity of the microbiome is an additional metric which may influence host health by itself. Analysis of Simpson’s Diversity Index across the 11 week experiment (a metric where higher numbers correspond to higher diversity) shows that diversity tends to increase as time passes (Figure 12A) but that there is no significant difference between HDM and PBS treatment by 11 weeks of exposure (Figure 12B).

**Discussion**

The present investigations suggest that intranasal exposure to HDM extract does not have a consistently differential impact on the composition of the fecal microbiome. These data have important implications related to tolerance research as well as the results of several other seminal asthma microbiome studies.

One potential contributing factor to the differences we noted between the groups exposed to HDM concerns the housing facility. The UConn Health Center for Comparative Medicine (CCM) underwent extensive renovations in 2014, with all animals moved from the old facility, which had not been significantly altered in the 37 years since its construction, to a new, cleaner space in the basement. This move occurred in January of 2014. Animals remained in the basement facility until February of 2015, when they were moved back to the newly-renovated original facility. Thus, the 2013 group experienced the original, dirtier facility; the 2014 group experienced the clean, basement facility; and the 2015 group experienced the clean, renovated facility, with no overlap in facilities for any group. This raises the possibility that the differences seen in 2013 were a
consequence of the facility, and that both cleaner environments in 2014 and 2015 did not harbor or promote the microorganisms that defined HDM exposure in 2013 (e.g. genus Blautia). This would also suggest a reason why the 2014 and 2015 groups were similar in composition, as both experienced new, clean facilities.

This facility effect is not unprecedented. One group at Emory University has undertaken several studies of TLR5 knockout mice[267; 268]. In the first instance, they noted that most mice exhibited a phenotype consisting of severe intestinal inflammation and colitis[267]. In the second, they noted that only some animals exhibited colitis, while the majority had mild inflammation but pronounced obesity. Through careful re-derivation in mothers from the source facility (the Jackson Laboratory), they discovered that the microbiome of their facility tended to promote colitis in the absence of TLR5, while the microbiome of the Jackson Laboratory tended to promote milder inflammation and obesity[268]. Clearly, the environment to which laboratory animals are exposed can have an enormous impact on the results of microbiome studies, and it is critical for important findings to be replicated in multiple facilities. Indeed, if a result does not replicate, this should be cause for excitement, as the differences in the microbiome between facilities could hold clues to organisms that could aggravate or alleviate the biological process under study.

A second possibility concerns the effect of cages. Mice housed in separate cages, even when they originated in the same facility, may diverge with time. This concern is one which has become increasingly recognized by microbiome researchers[269], particularly after reassessment of a series of studies in knockout mice. Initial reports of mice deficient in the immune signaling adaptor MyD88[270] as well as various TLRs[268] reported that these genotype alterations alone could drive changes in the microbiome. However, subsequent assessment by an independent group revealed that these effects were likely due more to the influence of the maternal microbiome and cage divergence following long periods of isolation from the original source environment[271]. Such cage effects, particularly over the long timecourse of the present study, could be the driving
One argument against this, particularly for the differences seen in 2013, is that multiple cages were utilized (four which received HDM and two which received PBS) and the effect of HDM exposure did not depend on source cage.

That changes did not depend on cage, and that changes were similar between groups, particularly in 2014 and 2015, suggests a simpler reason for the shifts observed: normal evolution of the microbiome with age. We know that the microbiome can change with age, particularly early in life. Assessment of the developing human microbiome revealed that its composition changes rapidly in the first few years of life[109]. In mice, analysis of older animals has shown that significant shifts continue even up to 800 days of age[272]. In fact, the sole existing study assessing the impact of HDM exposure on the microbiome found many changes, but most of these could be attributed to age or to HDM and age rather than to HDM alone[264]. An additional view on the matter comes from an unlikely source: the control animals in a study of early-life antibiotic (ABS) use by Martin Blaser’s group[273]. The ABX-exposed group shifted much in the first 30 weeks of life, as expected, but so too did the control group. Interestingly, some of the changes seen in the control mice were analogous to those in our groups, including the decline of Lactobacillus, a shift perhaps driven by the cessation of nursing after weaning. The fact that the sole taxa found to differ by LEfSe was found in naïve animals and not in animals after 11 weeks supports this theory.

One study for which the present work has great relevance is the investigation by Fujimura and colleagues that demonstrated that a particular bacteria in the house dust of homes with dogs, *Lactobacillus johnsonii*, was protective against experimental asthma in laboratory mice[139]. In the study, they describe obtaining house dust from the homes of dog owners and homes without dogs, and that the aforementioned bacteria was only found in homes with dogs. However, no mention is made of the diversity of the homes (or even the number) from which the house dust was obtained. Suppose that replication of this study across the homes of dozens or hundreds of
dog owners in different regions reported that only some homes with dogs harbored the protective
*L. johnsonii*? Suppose, too, that the homes which provided protection harbored house dust with
different microbial communities? Rather than being a disappointment, this could explain a
puzzling conflict in the literature concerning pet ownership and asthma. A number of studies have
reported that growing up in a home with a dog is protective against the development of
asthma[274; 275], while others have found no significant protective effect[276; 277] or even a
tendency to increase risk of developing asthma[278]. Similarly, studies of cat ownership have
reported asthma protection[276], asthma promotion[279], or no effect at all[277]. Studies of pet
ownership in general have been no less definitive, with some reporting increased risk of
asthma[280] and others no association at all[281]. What if these discrepancies were driven by the
environmental microbiome of the pets in question? Perhaps some breeds carry unique
microorganisms that can aggravate or alleviate asthma symptoms. The present work would
certainly seem to suggest that relying on only one or a few tests of HDM exposure is insufficient
to provide definitive evidence for protection or lack thereof.

A final important consideration concerning HDM exposure is one of microbiome location; namely,
have we selected the most appropriate one to analyze? Given the intranasal route of
administration, the lung microbiome might have been a more appropriate site to study. It is
possible that the lack of consistent, HDM-specific microbiome changes in the gut belies changes
in the lung that can be attributed to HDM and HDM alone. Some recent work has suggested that
inhalational tolerance (and pulmonary immune responses in general) requires a healthy
microbiome to develop[179]. However, this and other work are severely limited by a general lack
of knowledge concerning what a health or normal lung microbiome even is[171; 172]. Moreover,
many specific immune-microbiome relationships remain unclear within the lungs. Do Tregs
generated in the mucosa of the lung have the same relationship to the microbiome as those in
the gut, where much is known? Do dendritic cells in the lung sense the microbiome in the same way as those in the gut? Many of these questions remain to be answered.

The present study has uncovered a number of important questions but has provided strong support for the answer to another, namely that inhalational HDM exposure does not produce consistent changes in the gut microbiome, including no consistent induction of a organisms known to promote immune regulation. This suggests that a simple, organismal answer to the question of how (or if) the gut microbiome influences development of inhalational tolerance to HDM is not likely forthcoming, but it does not rule out other regulatory gut microbiome functions changing with HDM exposure (e.g. production of pro-regulatory products to differing degrees despite inconsistent community composition changes) or changes in the microbiome of an entirely distinct compartment (e.g. the lung). A final question concerns the impact of oral (swallowed) exposure to HDM in the process of intranasal administration. This will be addressed in Appendix A of this dissertation.
Table 1: Sample Numbers by Timepoint and Group

<table>
<thead>
<tr>
<th>Group</th>
<th>NAIVE</th>
<th>2 WEEK HDM</th>
<th>2 WEEK PBS</th>
<th>5 WEEK HDM</th>
<th>5 WEEK PBS</th>
<th>11 WEEK HDM</th>
<th>11 WEEK PBS</th>
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<td>32</td>
<td>6</td>
<td>32</td>
<td>6</td>
<td>24</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1. Sample Numbers by Timepoint and Group. Sample numbers represent samples analyzed using QIIME after rarefaction to 10,000 reads. Samples with fewer than 10,000 reads after quality filtering were not included in analyses. Figures 8-12 represent analyses of the samples in this table.
Figure 8: HDM Exposure Does Not Consistently Alter the Fecal Microbiome

QIIME was used to pick OTUs and analyze samples from mice that had received PBS or HDM gavage. OTUs picked at the 97% similarity level are represented as bars in (A). Bars represent the average abundance of each OTU. In (A), o__ refers to Order, f__ refers to Family, and g__ refers to Genus. OTUs lacking a lower classification (e.g. an organism identified at the Order level but not the Family or Genus level) refers to an unidentified OTU within that level, not all organisms at that level. For example, the OTU identified as Order Clostridiales does not represent all Clostridiales, merely one Clostridiales at the 97% similarity level that is not classified in GreenGenes below the Order level.
Not all groups contain samples from all timepoints. Refer to Table 1 for sample sizes by group and timepoint.
Figure 9: HDM-Induced Fecal Microbiome Changes Are Inconsistent & Similar to PBS
**Figure 9.** HDM-Induced Fecal Microbiome Changes Are Inconsistent & Similar to PBS. PCoA was performed on the distance matrix of the unweighted UniFrac distances and the two largest principal coordinates represented. Samples from each group were separated out from the same PCoA. Black arrows represent the direction of change from Naive samples to 11 Week HDM samples. Not all groups contain samples from all timepoints. Refer to Table 1 for sample sizes by group and timepoint.
Figure 10: Inter-Group Variability Drives Fecal Microbiome Changes Rather Than HDM Exposure
Figure 10. Inter-Group Variability Drives Fecal Microbiome Changes Rather Than HDM Exposure. Linear Discriminant Analysis with Effect Size (LEfSe) was performed on (A) the taxonomy table of abundances for all taxa based on treatment types as the class – naïve, 11 week HDM, or 11 week PBX – or (B) based on treatment types as the class and groups as the subclass – Group 35, Group 27, and Group 6. Blue bars represent taxa which are associated with naïve animals, green bars represent taxa associated with 11 weeks of PBS treatment, and red bars represent taxa associated with 11 weeks of HDM treatment. Refer to Table 1 for sample sizes by group and timepoint.
Figure 11: Family Clostridaceae, Genus *Clostridia* Variates with Age Rather than Treatment.

Percent of all organisms represented by Clostridaceae *Clostridia* in all samples by timepoint. Numbers represent percents (e.g. 0.1 is 0.1%, not 10%). Symbols represent individual animals at the specified timepoints. Data represent the mean ± the SEM. Black lines separate naïve timepoints (leftmost five columns), 11 week PBS timepoints (middle two columns), and 11 week...
HDM timepoints (rightmost three columns). Refer to Table 1 for sample sizes by group and timepoint.
Figure 12: HDM Exposure Does Not Produce Significantly Different Gut Microbiome Diversity

A

**Simpson's Diversity Index**

*Over 11 Weeks of Treatment*

B

**Simpson's Diversity Index**

*After 11 Weeks of Treatment*

- Group 35 (2013) HDM
- Group 35 (2013) PBS
- Group 27 (2014) HDM
- Group 6 (2015) HDM
- Group 6 (2015) PBS

ns
**Figure 12.** HDM Exposure Does Not Produce Significantly Different Gut Microbiome Diversity. Diversity of the gut microbiome represented by Simpson’s Diversity Index plotted across the 11 week experiment (A) and at 11 weeks (B). In (A), not all experimental groups have samples at all timepoints. In (B), data points represent individual animals in all three experimental groups, n=23 for HDM and 9 for PBS. Data represent the mean ± the SEM. Analyzed using non-parametric Mann-Whitney Test. Refer to Table 1 for sample sizes by group and timepoint. ns, not significant.
CHAPTER 4
EARLY-LIFE ANTIBIOTIC EXPOSURE INCREASES THE SEVERITY OF HDM-INDUCED AAD

Introduction

Asthma is an inflammatory disease of the airways that arises from an inappropriate immune response to innocuous environmental allergens[2; 4]. The prevalence of the disease has nearly doubled since the 1980s and today stands at nearly 10% of children[10; 50; 282]. In some populations, such as African Americans, prevalence is higher still, approaching nearly 15% of children[54]. The sharp increase in prevalence has been termed the “asthma epidemic”[50], and while the most recent data suggest that overall asthma prevalence may be stabilizing, it continues to rise in some populations, including adolescents and the poor[54]. Whether prevalence is stabilizing or still rising, this epidemic is a costly one, for asthma carries a high morbidity burden for patients. Indeed, the disease has long been the third leading cause of hospitalization in children[55].

One of the most important questions concerning the asthma epidemic has been determining what factors may underlie rising prevalence. One important complicating factor is that the lack of a gold-standard test for asthma[9] or even one single definition of asthma makes interpretation of asthma prevalence challenging[10]. Despite this, the evidence is overwhelming that prevalence has increased, even if the magnitude of the increase is not precisely measurable[50; 54; 282]. One factor which has been extensively investigated is the role of early childhood exposure to antibiotics (ABX) and the effect of ABX on the host microbiome. Over 20 clinical studies have shown that ABX, particularly in the first year of life, significantly increases the odds of developing asthma later in childhood[112–116].
Despite this clear association it remains just that: an association. Understanding the mechanisms behind the association is critical to the development of therapies that could halt the development of asthma or, perhaps, provide a cure for this as-yet incurable condition. Dissecting such mechanisms, particularly when they are related to the microbiome and the immune system, is complex, if not impossible, in human subjects. Recognizing this, multiple groups have explored the role of antibiotics on development of asthma using laboratory mice, and these studies have been unified in their message: ABX worsen subsequent experimental asthma and alter the immune response to asthma-inducing allergens[117; 118; 145; 229; 283–286].

However, these studies have important limitations when it comes to comparisons with human asthma and ABX. Several of these studies utilized germ free (GF) mice, where no microbial exposure occurs[284; 286], an extreme version of early-life ABX exposure. While GF mice are undeniably a useful model to assess how the microbiome affects the host immune system in the most extreme manner, no human child grows up in a germ free state, and the clinical relevance of the significant immune disturbances recognized in GF animals[287] remains unclear. Similarly, several investigations have focused on antibiotic exposure from prior to birth until later in life, which may be more akin to the GF mouse than human childhood ABX treatment[118; 229]. Finally, two reports exposed mice to ABX for prolonged periods of at least four weeks[145; 285], much longer than a typical childhood antibiotic course[288].

These studies have provided important insights into how antibiotic exposure may influence the developing immune system and susceptibility to asthma, but development of a more clinically-relevant animal model of early childhood antibiotic exposure would be of great benefit in determining which factors are behind the increased susceptibility to asthma seen in human children. In the present study, we hypothesized that exposing laboratory mice to ABX in a manner similar to that of human children (i.e. brief, but repeated, exposures) would increase the severity of house dust mite (HDM)-induced allergic airway disease (AAD) later in life.
Results

**Antibiotic exposure increased the severity of HDM-induced AAD**

Mice developed profound airway leukocytosis (Figure 13A) following HDM exposure, in agreement with our previously published results for the model[32]. Exposure to HDM and the cocktail of four ABX (HDM + Antibiotic Mix) resulted in three-fold higher BAL leukocytosis (Figure 13A) as compared to HDM alone. By contrast, leukocyte counts in the BAL of mice exposed to HDM and amoxicillin (HDM + Amoxicillin) were not significantly elevated over HDM or HDM + Antibiotic Mix animals. All leukocyte counts were many-fold higher than the level seen in naïve mice not exposed to HDM or ABX, represented by a dotted line in Figure 13A.

As expected based on our previous results[32], exposure to HDM resulted in airway eosinophilia (Figure 13B), with over 20% of BAL leukocytes being eosinophils in HDM-exposed animals. HDM + Antibiotic Mix animals had two-fold higher relative eosinophilia as compared to HDM animals (43% vs 20%, p < 0.01) and HDM + Amoxicillin animals (43% vs 28%, p < 0.05). HDM + Amoxicillin animals did not significantly differ in eosinophil proportion from HDM animals. Similarly, total eosinophil numbers (Figure 13C) were nearly five-fold higher in HDM + Antibiotic Mix animals as compared to HDM exposed animals (2.3 x 10^6 vs 4.6 x 10^5 eosinophils, p < 0.05). HDM + Amoxicillin mice fell between HDM and HDM + Antibiotic Mix mice and did not significantly differ in eosinophil numbers from either group. In all cases, the relative increase in eosinophils was at the expense of the relative proportion of macrophages, which make up more than 90% of the cells in a naïve mouse airway[32].

**Antibiotic exposure increased the severity of tissue inflammation in the lungs**

HDM exposure led to evidence of perivascular and peribronchial inflammation in the lungs (Figure 14A-C), particularly clusters of inflammatory cells surrounding the airways and vasculature and airway smooth muscle (ASM) hyperplasia and hypertrophy around the small airways. Blinded
comparison of the inflammation and ASM remodeling revealed that HDM + Antibiotic Mix mice harbored more severe histopathologic changes than either HDM or HDM + Amoxicillin mice, both of whom were not significantly different from each other (Figure 14G). Pathology scores for HDM mice were similar to those noted in our previous description of the model[32]. By contrast, evaluation of mucus production (Figure 14D-F) showed no significant differences in mucus scores between any two groups (Figure 14H).

**Serum HDM-specific IgE is elevated in mice treated with antibiotics early in life**

A hallmark of allergic asthma and AAD is the appearance of allergen-specific IgE in the serum. This has been demonstrated in multiple models of allergic asthma in mice, including ovalbumin[222] and HDM[32]. In agreement with our previous model data, serum HDM-specific IgE appeared following HDM exposure (in naïve animals, it is virtually undetectable[32]). However, HDM + Antibiotic Mix animals had significantly higher levels of HDM-specific IgE than HDM animals (Figure 15). HDM + Amoxicillin animals did not significantly differ from either group, although there was a trend toward higher levels of HDM-specific IgE as compared to HDM animals (p = 0.078).

**Airway hyper-reactivity (AHR) was significantly elevated in antibiotic-treated mice**

Exposure to HDM induces ASM remodeling that results in AHR and bronchoconstriction in response to allergens and agents such as the non-selective muscarinic receptor agonist methacholine[32]. This bronchoconstriction is a factor in the characteristic chest tightness and wheeze seen in asthmatics[4]. Experimental assessment of respiratory function using the flexiVent system revealed that HDM + Antibiotic Mix animals had significantly higher increases in total respiratory system resistance (Rrs) in response to methacholine challenge as compared to HDM animals (significant effect of the interaction of methacholine dose with treatment, 2-way ANOVA, p < 0.05, Figure 16A). These differences were most pronounced at higher doses of
aerosolized methacholine, particularly 300 mg/mL (p < 0.01, HDM vs HDM + Antibiotic Mix, Figure 16A). Furthermore, comparison of the area under the curve (AUC) of the Rrs for HDM vs HDM + Antibiotic Mix animals showed significantly higher AUC for those animals exposed to both HDM and Antibiotic Mix as compared to HDM alone (3.84 vs 5.168, p < 0.05, HDM vs HDM + Antibiotic Mix, Figure 16B).

**Antibiotic exposure reduced the proportion of regulatory T cells in the lung-draining (hilar) lymph node (HLN)**

Our laboratory[31; 32; 41] and others[263; 289] have shown that Foxp3+ regulatory T Cells (Tregs) play a critical role in the control of asthma and allergic disease. Furthermore, previous work from our lab has shown that Tregs increase and orchestrate the development of tolerance and disease suppression to both inhaled ovalbumin[31; 41] and HDM[32] from within the in the lung-draining, or hilar, lymph node (HLN). Assessing the frequency of Foxp3+ Tregs in the HLN revealed that HDM + Antibiotic Mix mice had significantly lower proportions of Tregs as a percent of total CD4+ T Cells than HDM mice (8.93% vs 11.1%, p < 0.05, Figure 17). HDM + Amoxicillin mice fell between the two groups and did not significantly differ from either HDM or HDM + Antibiotic Mix.

**Antibiotic exposure reduced the diversity of the fecal microbiome**

We have known for over half a century that antibiotics rapidly alter the composition of the gut microbiome[290], and more recent studies have shown the specific microbial community changes that result from antibiotic administration in both humans[109] and laboratory animals[111], including a loss of many bacterial genuses. A similar pattern was seen between in the present study. Antibiotic Mix treatment resulted in a sharp reduction in phylum Bacteroidetes, particularly order Bacteroidales, which was nearly absent in Antibiotic Mix-treated animals as compared to No Treatment animals (Figure 18A). Phylum Firmicutes remained present after Antibiotic Mix treatment but with a very different composition. Class Clostridia, particularly organisms of order
Clostridiales such as family Lachnospiraceae, are much reduced when compared to No Treatment animals, and organisms of the Firmicutes order Turicibacterales and Lactobacillales (genus Turcibacter and Enterococcus, respectively) dominate. The latter two genuses are nearly absent in No Treatment animals.

Following HDM exposure, animals not treated with antibiotics (denoted HDM in Figure 18) have similar community composition to No Treatment animals, dominated by Bacteroidales and Clostridiales, although organisms of the clostridial family Lachnospiraceae are reduced and organisms of the Firmicutes genus Allobactum appear. However, in Antibiotic Mix mice, five weeks of HDM treatment and no antibiotic exposure (HDM + Antibiotic Mix) witnessed some recovery of the microbial community, but not to the level of either the No Treatment or HDM animals. Phylum Bacteroidetes reappears, but its lower-level composition differs. Some order Bacteroidales organisms returned, but nearly half of Bacteroidetes consisted of organisms of the genus Parabacteroides, an organism almost absent from either No Treatment or HDM animals. Nearly half of the organisms present were of the phylum Verrucomicrobia, genus Akkermansia, an organism making up less than 6% of the microbiome in both No Treatment and HDM animals.

Principle coordinate analysis (PCoA) of the unweighted UniFrac distances confirmed these changes (Figure 18B). No Treatment and HDM animals were virtually indistinguishable by analysis of PC1 and PC2 (which together represented over 50% of the variance within the samples). Both differed considerably from either Antibiotic Mix or HDM + Antibiotic Mix animals along PC1, which accounted for most of the variance between the samples (nearly 43%), and also differed considerably along PC2, which accounted for just over 19% of the variance amongst samples. However, this latter difference was not the same between Antibiotic Mix and HDM + Antibiotic Mix, as the two differed from each other along PC2.

In addition to assessment of microbiome diversity by examining community composition (Figure 18A), it is possible to calculate the diversity of the community using one of many indices, such as
the Shannon diversity index[291]. Assessment of the alpha diversity (within-sample diversity) revealed that the Shannon diversity index significantly differed between HDM and HDM + Antibiotic Mix animals at the time of sacrifice (Figure 18C). The mean diversity of the fecal microbiome of HDM mice was a third higher than HDM + Antibiotic Mix (0.954 vs 0.747, p < 0.01).

To separate the effects of antibiotic exposure from HDM exposure on the composition of the microbiome, LEfSe was utilized to identify taxonomic “biomarkers” linked to antibiotic treatment and not confounded by HDM exposure (Figure 19A). The identified taxa lost with antibiotic treatment match those described in Figure 18 and include many, such as Lachnospiraceae and Clostridium, with known immunologic activity. Those taxa which increase in proportion with antibiotic treatment include organisms, such as genus Enterococcus, with known pathogenic properties. Further analysis to isolate those organisms whose effects are due to HDM and not ABX (Figure 19B) shows several organisms, including those with pro-regulatory properties such as genus Blautia, more represented in HDM-treated animals. However, the absence or near-absence of these organisms in the ABX treated and HDM exposed animals (Figure 18A) suggests this effect may be driven solely by the non-ABX treated, HDM-exposed animals.

**Decreased diversity of the fecal microbiome directly correlates with the proportion of Tregs in the HLN**

The diversity of the fecal microbiome directly correlated with elements of immune regulation. Plotting the Shannon diversity index against the proportion of Foxp3+ Tregs in the HLN (Figure 20) for both HDM and HDM + Antibiotic Mix mice revealed that there was a positive correlation between microbiome diversity and the proportion of Tregs (Spearman r=0.7109, 0 < 0.01). As diversity increased, so too did Treg proportions.
Discussion

We are not the first to explore alternatives to commonly-used ABX treatment regimens in laboratory mice. Martin Blaser and his group have shown that brief, repeated administration of ABX early in life can induce dramatic changes in metabolic activity that persist to a minimum of six months of age[228]. However, while this report contained many details on metabolic changes wrought by ABX exposure, it did not assess how such ABX may affect immune function. The present study demonstrates that brief, repeated ABX exposures can worsen subsequent experimental asthma, confirming the efforts of others who have administered long-term ABX regimens[145; 285] or evaluated ABX treatment from before birth until later in development[118; 229] and found the same.

The present study also adds to two reports that paired brief ABX treatment with secondary fungal spore inoculation[117; 283]. In both, the combination of spores and ABX significantly enhanced the severity of subsequent experimental asthma. This, paired with data that an environment with significant fungal exposure is related to the development of asthma[292], would seem to indicate that brief ABX exposure and fundal inoculation is a good model of pediatric ABX exposure and asthma. However, the same report linking environmental fungal exposure to asthma also found less than 12% of asthmatics were sensitized to the fungi in question[292], suggesting that fungal exposure may promote the development of asthma in conjunction with early-life ABX exposure but that it does not account for all the childhood asthma risk ABX carries.

The link between early life microbiome diversity and asthma that we report, and the association of said diversity with Treg proportions, add mechanistic explanation to reports linking low early life diversity of the gut microbiome[95] and the environmental microbiome[96] to subsequent asthma and allergy development. However, not all such reports are in agreement. One recent analysis of an ongoing study of children in Canada found no link between diversity and the development of asthma, although they did report that the loss of a number of genera was
associated with asthma severity[97]. There is one important difference between the studies which may complicate the comparison. This report examined children at ages two or three[97], while the other two studies evaluated asthma at age seven[95; 96]. In an early study of children in day care, it was noted that day care was associated with increased wheeze in the toddler years but reduced asthma by age six[77]. We may need to await further results from the Canadian cohort before final judgement on the relevance of microbiome diversity to asthma development.

Interestingly, one group of organisms whose reduction was association with asthma development in the Canadian study were those of the genus Lachnospira[97], falling within the family Lachnospiraceae, one of the families that was nearly eliminated by ABX treatment in the present study, although its proportions subsequently recovered somewhat. Members of this genus could have a common effect between humans and mice, and are worth exploring for potential probiotic use. Indeed, these organisms have been linked to Treg induction in mice, as members of the family Lachnospiraceae fall within the clostridial clusters known to induce Tregs through fermentation of dietary fiber and production of short-chain fatty acids[131]. Their loss in this study may be one of the factors behind the decrease in Tregs seen, even five weeks after the cessation of ABX therapy.

Considering probiotics, the present study may provide some insight into the failure of many probiotics to translate from benefits in mice to protection in humans. Multiple clinical trials have failed to demonstrate any benefit from the administration of probiotics[189] despite abundant evidence of their benefit in laboratory mice[183–188]. It may be that the addition of one or even a few organisms is insufficient to correct a profound loss of microbiome diversity in humans, and that only therapies which meaningfully impact diversity will have an effect on asthma susceptibility. Fecal microbiome transplantation (FMT), which provides an entire, diverse microbiome to the recipient, may be the means to overcome the loss of diversity seen with ABX administration. FMT is already available in capsule form for the treatment of *Clostridium difficile*
infection[192], and a future course of ABX for a childhood infection could be accompanied by an FMT pill to correct the disruption of the microbiome once the ABX had eradicated the pathogenic organism.

However, despite the evidence of the dangers of ABX use in childhood with respect to asthma development, it is important to remember the critical benefits of the drug class. Diseases from acute rheumatic fever to bacterial meningitis killed millions of children prior to the introduction of ABX[293], and to reduce ABX use indiscriminately in an effort to reduce asthma prevalence would be foolish and ethically unsound. However, there are many steps that can be taken to reduce unnecessary ABX use without endangering children. Consider the common childhood ailment otitis media, otherwise known as an ear infection. ABX are commonly administered to treat otitis media, but multiple analyses (summarized in a meta-analysis of 63 such studies[294]) found that the majority of cases spontaneously resolved without the need for any intervention. Better targeting of ABX to only those children at greatest need of pharmacological support could reduce the risk of developing asthma that unnecessary ABX use carries.

An important obstacle to such efforts is the perceived need to prescribe ABX seen by many physicians. Confronted with limited time to see each patient and the demand to increase patient satisfaction, many physicians simply prescribe ABX even when they suspect it is unwarranted, assuming that little harm will come of the misdeed[295]. Fortunately, recent data suggest that physicians can both rationally prescribe ABX and increase patient satisfaction and outcomes in the same blow. In one recent randomized controlled trial evaluating uncomplicated respiratory infections, simply providing an ABX prescription immediately (but instructing patients not to fill it unless symptoms persisted) or telling patients to return to the clinic for an ABX prescription if they continued to feel unwell or worsen was sufficient to reduce the duration of symptoms when compared to patients not given a prescription at all[201]. In this way, ABX prescription was reduced without adversely affecting clinical outcomes. A true “win-win” scenario for any clinician.
The case of amoxicillin in the present study is an intriguing one. For nearly all parameters, including HLN Treg proportions, amoxicillin-treated mice fell somewhere between those mice exposed to HDM alone and those mice exposed to HDM and the mix of four ABX. While there was no significant increase in disease severity, this broad tendency to higher disease severity and lower Tregs could combine with other factors to influence disease. Asthma arises from a fundamental imbalance between regulatory and inflammatory responses of the immune system[296]. While amoxicillin exposure may not provide sufficient impetus to shift the balance on its own, it could remove just enough from the regulatory side to increase susceptibility to or increase severity of asthma in the presence of other contributing factors, such as a lack of exposure to dirty environments when young (e.g. a child in a clean, suburban house as compared to one living on a farm[83]) or one of the known genetic risk factors for asthma such as variations in the gene encoding thymic stromal lymphopoietin[297]. Early-life amoxicillin use could be one factor that initiates the so-called “atopic march” that characterizes so many children with asthma, allergic rhinitis, and other childhood allergic diseases[298], but it may require help to meaningfully increase the risk of developing the disease.

One point of concern with the choice of the cocktail of four ABX is the lack of clinical relevance of that particular combination. Some severe diseases can warrant such broad-spectrum ABX cocktails, such as necrotizing enterocolitis and other complicated intra-abdominal infections[299], but most children will not be routinely exposed to such treatment early in life (if ever). While the use of this regimen is far from ideal, the use of a series of three short doses rather than weeks or more of similar cocktails is closer to the clinical reality than previous studies have been. An important avenue for future investigation would be to determine if a subset of this cocktail was sufficient to increase disease severity (e.g. vancomycin alone or neomycin and metronidazole together). An important consideration is that whether an antibiotic increases the severity of
disease can be dependent on the disease as much as on the antibiotic[118; 230], so results found for asthma may have only partial relevance for other diseases.

To conclude, the present study describes a clinically-relevant model of the effects of early-life ABX exposure on the subsequent severity of experimental asthma later in life. Exposure to a cocktail of four ABX increased the severity of all parameters of experimental asthma, including BAL eosinophilia, AHR, and serum allergen-specific IgE levels. Increased disease severity was accompanied by a loss of Tregs, known moderators of the allergic inflammatory response. ABX reduced the diversity of the fecal microbiome in a sustained manner, and this reduction in diversity correlated with the loss of Tregs seen with ABX administration, providing a mechanism behind clinical studies that have linked early-life microbiome diversity with subsequent asthma development in human children.
Figure 13. Early-Life Antibiotic Exposure Increases the Severity of Experimental Asthma. At sacrifice, broncho-alveolar lavage (BAL) was performed and total BAL leukocytes counted (A) and BAL cellular differentials determined using May-Grunwald Giemsa stain (B). Typical BAL cell count in a naïve animal, not exposed to HDM, is represented by a dotted line at 150,000 in (A). Total BAL eosinophils were then determined from these two assessments. Values in A and C represent mean ± the SEM. n=8 per group. * p < 0.05, ** p < 0.01; a: p < 0.01, eosinophil percent
in HDM vs HDM + Antibiotic Mix; b: p < 0.05, eosinophil percent in HDM + Amoxicillin vs HDM + Antibiotic Mix.

**Figure 14: Antibiotic Exposure Is Associated with Increased Disease Pathology**

**Figure 14.** Antibiotic Exposure Is Associated with Increased Disease Pathology. Formalin-fixed slides were processed in a standard manner and stained with hematoxylin and eosin (top row, A-C, 10X) or periodic acid-Schiff with hematoxylin counterstain (bottom row, D-F, 4X). Inflammation
scores (G) and mucus scores (H) were determined in a blinded manner on a severity scale of 0-3. Data represent mean ± interquartile ranges. n=3 slides per group for HDM and HDM + Antibiotic Mix, n=2 for HDM + Amoxicillin; five independent graders per slide. * p < 0.05, ** p < 0.01.

**Figure 15: Antibiotic Exposure Is Associated with Heightened Serum HDM-Specific IgE**

**Figure 15.** Antibiotic Exposure Increases Serum HDM-Specific IgE Levels. At sacrifice, serum was collected from animals exposed to HDM, HDM + Amoxicillin, and HDM + Antibiotic Mix. HDM-specific IgE levels were determined by ELISA. Dual absorbance was calculated at 450 nm and 570 nm over a range of serial dilutions. Data represent mean ± SEM values. Group comparisons were made by Kruskal-Wallis test with Dunn’s multiple comparisons test on the area
under the curves (AUC) of the samples. n=8 per group. a: p < 0.05, HDM + Antibiotic Mix vs HDM.
Figure 16: Antibiotic Exposure Significantly Increase Airway Hyper-reactivity

A
Respiratory System Resistance (Rrs)

B
Respiratory System Resistance (Rrs) AUC

Area Under the Curve (AUC) of Rrs

HDM
HDM + Antibiotic Mix
Figure 16. Antibiotic Exposure Significantly Increase Airway Hyper-Reactivity. Mice underwent tracheostomy and mechanical ventilation with the flexiVent system. Total respiratory system resistance (Rrs) was measured following increasing doses of aerosolized methacholine (A). Rrs was compared using Area Under the Curve (AUC) (B). Data represent mean ± SEM. n=4 for HDM, n=9 for HDM + Antibiotic Mix. a: p < 0.05 for the interaction of antibiotic treatment status and methacholine dose. * p < 0.05, HDM vs HDM + Antibiotic Mix AUC; ** p < 0.01, HDM vs HDM + Antibiotic Mix for 300 mg/mL methacholine.

Figure 17: Antibiotic Exposure Reduces Regulatory Immune Cells in the Lung-Draining (Hilar) Lymph Node (HLN)

![HLN Regulatory T Cells](image-url)

- HDM
- HDM + Amoxicillin
- HDM + Antibiotic Mix

Foxp3+ Tregs as % of CD4+ T Cells
Figure 17. Antibiotic Exposure Reduces Regulatory Immune Cells in the Lung-Draining (Hilar) Lymph Node (HLN). At sacrifice, HLNs were removed and cells isolated. These cells were stained to identify CD3+CD4+Foxp3+ regulatory T cells (Tregs) and assessed by flow cytometry. Error bars represent mean ± SEM. n=7 for HDM, n=8 for HDM + Amoxicillin and HDM + Antibiotic Mix. * p < 0.05.

Figure 18: Antibiotic Exposure Reduces the Diversity of the Fecal Microbiome

Figure 18. Antibiotic Exposure Reduces the Diversity of the Fecal Microbiome. Following 16S rRNA sequencing, QIIME was used to analyze the sequence data and pick operational taxonomic units (OTUs) against the GreenGenes database. OTUs present in the samples are represented as bar graphs of the average percent of all organisms represented by each OTU in each group.
(A). In (A), o__ refers to Order, f__ refers to Family, and g__ refers to Genus. OTUs lacking a lower classification (e.g. an organism identified at the Order level but not the Family or Genus level) refers to an unidentified OTU within that level, not all organisms at that level. For example, the OTU identified as Order Clostridales does not represent all Clostridales, merely one Clostridales at the 97% similarity level that is not classified in GreenGenes below the Order level. Simpson’s Diversity Index for the fecal microbiome following HDM exposure was calculated (B) and principal coordinates analysis (PCoA) of the unweighted UniFrac distances was performed on samples before and after HDM exposure (C). n=8 for No Treatment and HDM + Antibiotic Mix, n=5 for HDM, n=6 for Antibiotic Mix. ** p < 0.01.
Figure 19: Antibiotic Exposure Results in Proportional Changes in Multiple Taxa
Figure 19. Antibiotic Exposure Results in Proportional Changes in Multiple Taxa. Linear Discriminant Analysis with Effect Size (LEfSe) was performed on the taxonomy table of abundances for all taxa based on (A) antibiotic treatment as the class (either treated, ABX_Mix, or not treated, No_Tx) and HDM exposure as the subclass (pre-exposure or post-exposure) or (B) HDM exposure as the class (either exposed, HDM, or pre-exposure, PreHDM) and antibiotic exposure as the subclass (treated or not treated). In (A) green bars represent taxa associated with no antibiotic treatment (and not due to the effects of HDM exposure), and red bars represent taxa associated with antibiotic treatment (and not due to the effects of HDM exposure). In (B) blue bars represent taxa associated with HDM exposure (and not due to the effects of antibiotic treatment). n=8 for No Treatment and HDM + Antibiotic Mix, n=5 for HDM, n=6 for Antibiotic Mix.
Figure 20: Fecal Microbiome Diversity Directly Correlates with Regulatory T Cell Proportions

Simpson’s Diversity Index plotted against the proportion of all CD4+ T Cells in the HLN that are Foxp3+ Tregs. The Spearman r coefficient of correlation was calculated. The solid line is the linear regression line plotted on the data, with the equation shown below the graph. The curved, dotted lines represent the 95% confidence band for the linear regression line. n=5 for HDM, n=8 for HDM + Antibiotic Mix.
CHAPTER 5
ANTIBIOTIC EXPOSURE DISRUPTS THE DEVELOPMENT OF INHALATIONAL TOLERANCE

Introduction

A key objective of research endeavors aired at curing allergic asthma is restoration of immunological tolerance to the offending allergen. Current asthma therapies that target the immune system, such as corticosteroids and leukotriene inhibitors, broadly suppress immunity but do not promote tolerance\[4\], and they carry a host of side effects ranging from infection risk to reduced efficacy following repeated usage. Tolerance-promoting therapies would harness the immune system to control itself, suppressing inappropriate inflammation while allowing beneficial inflammatory responses, such as those to an invading pathogen, to proceed unimpeded\[256\].

Medical science has recognized that tolerance can be induced to foreign substances since the early part of the 20th century. Work by Wells and Osborne in 1911 described oral tolerance, or the process by which oral consumption of an allergen (for Wells and Osborne, ovalbumin consumption by guinea pigs) produced a lasting and broad tolerance to that allergen, even if exposed in an area other than the gut (such as application to the skin)\[300; 301\]. Others have shown that this extends beyond ovalbumin (OVA) and that oral tolerance can be induced to various pharmacologic compounds\[302\] as well as poison ivy extract\[303\]. The phenomenon has long been recognized in human subjects, from an early case report at a Middle Eastern military hospital of sulphanilamide desensitization in 1944\[304\] to modern efforts to promote tolerance to food allergens such as peanut by carefully exposing allergic subjects to the offending protein or protein(s)\[253; 305\].

As our understanding of oral tolerance grew, it became apparent that the microbiome was critical for tolerance development. Experiments in the 1980s demonstrated that germ free (GF) mice
either failed to develop oral tolerance or saw incomplete tolerance development the absence of a host microbiome[306; 307]. Later studies have shown that the lack of a microbiome produces a seemingly never-ending Th2 response, with no counteracting regulatory presence to reduce the allergic reaction[149], and that this imbalance is at least in part due to a lack of T cells within the gut-associated lymphoid tissue (GALT)[308]. This discovery reconnected us with work done nearly seventy years prior, when Glimstedt’s 1936 assessments of GF guinea pigs revealed that that Peyer’s patches, an important component of GALT, are fewer in number and that gut-associated lymph nodes have disorganized architecture[309], findings confirmed and expanded upon in laboratory mice decades later[310]. Since then, a host of research has demonstrated that a healthy microbiome is absolutely required for proper immune development and function, and that disruption of the microbiome can produce lasting immunological consequences, including for tolerance development[123].

While we know much of the interactions between the microbiome and the host which power oral tolerance, much less is known about the importance of the microbiome for the development of inhalational tolerance. A growing body of work has shown that disruption of the microbiome, particularly following antibiotic (ABX) exposure, increases the severity of or susceptibility to AAD in animal models[117; 118] and asthma in human subjects[112–116]. These studies have yielded important insights into disease pathogenesis, but revealed little about the effect of the microbiome on inhalational tolerance development. One recent study delved a little deeper into this area, exploring the relationship of the lung microbiome to the development of tolerance to HDM. This report suggested that lung microbiota, acting through the receptor PD-L1, provide protection against allergic inflammation, but it did not evaluate true tolerance, as the exposure period for HDM was only three weeks and the group did not see eosinophilia resolve even in control animals[133]. As we have shown, tolerance to inhaled allergens including OVA[257] and HDM[32] requires many weeks of continuous exposure (six and eleven, respectively).
In the present chapter, we undertook investigation of the effect of ABX administration on the development of inhalational tolerance. Based on prior results concerning AAD severity and the existing body of oral tolerance literature, we hypothesized that disruption of the microbiome using ABX would impair the development of inhalational tolerance and lead failure to suppress symptoms of AAD. The following experiments will explore this hypothesis using three ABX exposure models.

**Results**

**Tylosin and Amoxicillin Do Not Impair the Development of Inhalational Tolerance**

Animals were first exposed to a combination of amoxicillin and tylosin and HDM (HDM + Antibiotic Pair) or HDM alone in the manner of Nobel and colleagues[228], receiving exposure to ABX immediately after weaning. This ABX exposure protocol did not disrupt tolerance development from the perspective of leukocytosis and eosinophilia (Figure 21). The relative eosinophilia in the broncho-alveolar lavage (BAL) was not significantly different between HDM and HDM + Antibiotic Pair mice (Figure 21A-B). Both HDM and HDM + Antibiotic Pair mice had similar eosinophilia at eleven weeks (approximately 5% of BAL leukocytes or less) as has been previously reported for eleven weeks of HDM exposure by our group[32]. BAL eosinophil numbers (Figure 21C) also did not significantly differ between HDM and HDM + Antibiotic Pair mice. Overall leukocyte numbers in the BAL (Figure 21D), lungs (Figure 21E), and lung-draining (hilar) lymph node (HLN, Figure 21F) also did not differ significantly between the two groups.

**Tylosin and Amoxicillin Do Not Alter the Proportions of Foxp3+ Tregs in the HLN or Lung**

We[31; 32; 41] and others[30] have previously shown that regulatory T cells (Tregs) are key mediators of the development of inhalational tolerance, particularly in the lung and lung-
associated lymphoid tissues. Examination of the HLN and lung of these mice for CD4+Foxp3+
Tregs did not show a difference between HDM and HDM + Antibiotic Pair mice (Figure 22A-B).
Both had percentages of Tregs in the HLN and lung that were approximately the same as those
seen in our previous study of the model[32].

**Early-Life Exposure to Amoxicillin or a Mixture of Four Antibiotics Does Not**

**Impair the Development of Inhalational Tolerance**

Mice were next exposed to a different ABX treatment protocol, the same one which significantly
increased the severity of AAD after five weeks of HDM exposure (refer to Chapter 3). These mice
received either HDM, HDM and amoxicillin (HDM + Amoxicillin), or HDM and a mixture of four
antibiotics (HDM + Antibiotic Mix). There were no significant differences in the relative (Figure
23A-B) or absolute (Figure 23C) numbers of eosinophils in the BAL between HDM, HDM +
Amoxicillin, and HDM + Antibiotic Mix animals. Furthermore, there were no significant differences
in the number of leukocytes in the BAL between the groups. Data for all groups were consistent
with previously reported results following eleven weeks of HDM exposure[32].

**Lifelong Exposure to a Mixture of Four Antibiotics Impairs the Resolution of**

**Eosinophilia and the Development of Inhalational Tolerance to HDM**

Mice were next subjected to an ABX exposure protocol adapted from the prior experiment as well
as the work of Russell and colleagues[229; 230]. Amoxicillin or Antibiotic Mix was administered
to mice beginning prior to birth (i.e. to pregnant mothers) and continued throughout life, including
during HDM exposure. Control mice were raised in an identical manner but did not receive ABX
of any kind. HDM + Antibiotic Mix resulted in sustained relative eosinophilia (Figure 24A-B) that
was significantly elevated above both HDM and HDM + Amoxicillin animals (with a mean
proportion of eosinophils of approximately 10% of all BAL leukocytes vs below 5% for HDM and
HDM + Amoxicillin). The absolute BAL eosinophilia was also significantly elevated in HDM +
Antibiotic Mix mice, where the number of eosinophils was over ten-fold higher than in either HDM or HDM + Amoxicillin mice (Figure 24C).

Furthermore, HDM + Antibiotic Mix animals had significantly higher total leukocyte counts in the BAL and the lung as compared to both HDM and HDM + Amoxicillin animals (Figure 24D-E). Indeed, leukocyte counts for HDM + Antibiotic Mix animals in both compartments were approximately twice that seen for HDM animals. However, cell counts in the HLN did not differ significantly between any of the three groups of animals.

**Lifelong Exposure to a Mixture of Four Antibiotics Reduces the Proportions of Tregs and Bregs in the HLN and Lung**

In contrast to early-life exposure to tylosin and amoxicillin, HDM + Antibiotic Mix mice had significantly lower proportions of CD4+Foxp3+ Tregs in the HLN (Figure 25A) as compared to wither HDM or HDM + Amoxicillin mice. HDM + Antibiotic Mix mice had lower, although not significantly so, proportions of Tregs in the lung (Figure 25C). As for Tregs after five weeks of HDM (see Chapter 4), HDM + Amoxicillin animals fell between HDM and HDM + Antibiotic Mix animals and did not significantly differ from either.

In addition to Tregs, our lab has previously shown that regulatory B Cells (Bregs), which reside in the CD5+ B Cell population, play an important role in the development of tolerance in experimental asthma[41; 42]. HDM + Antibiotic Mix reduced the proportion of CD5+ B Cells in both the HLN (Figure 25B) and the lung (Figure 25D) by approximately a quarter. HDM + Amoxicillin animals again fell between HDM and HDM + Antibiotic Mix animals and did not significantly differ from either.

**Discussion**

The present study provides the first evidence that disruption of the microbiome using ABX can impair the development of inhalational tolerance. It was well-recognized that ABX could impair
the development of oral tolerance[311; 312], and long recognized that the microbiome had a
general role in oral tolerance development[306; 307], but little was known of the effect of
microbiome disruption on tolerance in the lungs.

We note that different antibiotic regimens can have differing impacts on lung immunity. This idea
is perhaps unsurprising given current literature. Consider the case of Russell and colleagues and
their studies of neonatal antibiotic exposure. In one landmark study of neonatal ABX exposure,
they reported that vancomycin, but not streptomycin, increased the severity of experimental
asthma later in life[118]. Three years later, they reported that an identical treatment scheme had
the opposite effect in a different disease model, hypersensitivity pneumonitis, where streptomycin
produced worse disease while vancomycin had no effect on disease severity[230].

Absent evidence from Chapter 3 that oral tolerance does not develop to HDM, one might conclude
that lifelong ABX treatment impairs tolerance merely by disrupting oral tolerance, but that is clearly
not the case. Instead, these results lend support to the idea that tolerance to HDM is orchestrated
within the lung itself. The recent study linking the respiratory microbiome to PD-L1-mediated
tolerance in the lung may provide the most mechanistic clue[179]. While that study only examined
a short window of allergen administration (a maximum of three weeks), the present results
suggest that the lung microbiome-PD-L1 axis may be relevant over the longer term. One
interesting avenue to explore in future studies would be the evaluation of the PD-L1 axis with
lifelong ABX treatment. Some of the same deficiencies seen early in life in mice lacking a
microbiome could conceivably persist throughout life so long as ABX are being administered.
Assessment of the lung microbiome over this period would also be illuminating, although
limitations of assessment (i.e. animals must be sacrificed to sample the lung microbiome)
necessarily mean that it could not be monitored over time in the same animals as is the case for
the fecal microbiome.
An important follow-up to this study is the assessment of the composition of the microbiome following sustained ABX treatment. As we saw in Chapter 4, diversity of the fecal microbiome directly correlates with the proportion of Tregs in the HLN. Given the similar relative lack of Tregs seen here in mice exposed to ABX for their entire lives, a similar diversity-Treg relationship may exist. Furthermore, it would also be interesting to determine if the organisms lost due to ABX treatment are members of the groups known to induce Tregs, such as Clostridial fermenters and \textit{B. fragilis}. Their absence, would explain the dearth of Tregs seen with sustained ABX treatment, particularly in light of evidence that iTregs induced in the gut can migrate to the lungs and moderate allergic inflammation\cite{35}. Evidence that gut Treg inducing microorganisms are down would open the door to possible therapeutic interventions. While dietary fiber supplementation might fail given ABX-depletion of the fermenters, perhaps direct supplementation with short-chain fatty acids (SCFAs) would correct the deficit of Tregs seen with ABX therapy and resolve the associated eosinophilia. A further possibility, albeit one with less clinical relevance, would be to transfer iTregs into ABX-treated animals to see if supplementation with the missing cell population could restrain allergic inflammation. Such studies have been performed in the absence of ABX therapy\cite{313; 314}, but whether it can correct for changes induced by extended ABX therapy remains to be determined. Similar experiments, perhaps to include experiments with concurrent Treg transfer, could also be performed for CD5+ Bregs, as we have shown that transfer of such cells is protective against allergic inflammation in an OVA mouse model of asthma\cite{41}. If transfer of either or both populations alleviated disease, it would strongly suggest that a population lost to ABX treatment was responsible for the Breg/Treg induction, maintenance, or a combination thereof.

A close examination of eosinophilia in mice exposed to lifelong ABX (Figure 24A-B) reveals a bimodal distribution of samples, with three tightly clustered at around 12% of all BAL leukocytes and two at approximately 5% or below. This distribution happened to mirror sex: the three animals
with high eosinophilia were female (as were all samples in all other groups), while the two lower samples were male. We had not previously noted sex differences in our HDM model[32], but there is a growing recognition that sex can interact with the microbiome. Indeed, the sex-microbiome axis has been termed by some the “microgenderome”[315]. Studies over the past three years have suggested that the microbiome can impact autoimmunity[316; 317]; microbiome metabolism and microbiome-diet interactions[318; 319]; and gastrointestinal immunity and cytokine production[319]. With respect to the microbiome, sex may have an impact on how the microbiome changes with time, including changes associated with circadian rhythm[320]. While data are limited in human subjects, early reports suggest that there may be sex-specific differences in the composition of the microbiome (including the vial microbiome) in humans, although the clinical significance of differences reported remains almost completely unknown[321–323]. Perhaps the microbiome response to long-term ABX therapy differs by sex, and the males maintain a more robust and ABX-resistance microbiome when compared to the females. Perhaps males retain a protective organism that is lacking in females. Or, perhaps males lack a pro-inflammatory organism such as Proteobacteria[173] that female animals possess. It is known that females experience more severe asthma than males[324], and it is interesting to consider that this could arise from a difference in the underlying host microgenderome.

A simple but important addition to this study is functional assessment. Eosinophilia is a key definition of active disease in our models[32; 257], but few asthma sufferers worry about how many eosinophils are patrolling their airways. Instead, the concern is with function, such as wheeze and airway hyper-reactivity (AHR). While obtaining an accurate description of current wheeze from a mouse remains a challenge as yet unsolved by neuroscientists, we do have means to evaluate AHR[32]. It will be important to assess AHR after continuous ABX therapy and HDM exposure to see if the persistent eosinophilia is coupled with decreased pulmonary function. If it
is, that would also suggest that airway smooth muscle hypertrophy and hyperplasia is enhanced or at the least made persistent by ABX therapy, a finding which could explain associations between wheeze and ABX therapy in human subjects.

An important consideration concerning the ABX protocol utilized is one of clinical relevance. Lifelong ABX therapy is not common in humans, nor is multi-ABX cocktail treatment of newborns common. However, there is an exception to the latter that may have relevance for the present study. Premature babies who experience sepsis, such as from necrotizing enterocolitis, require extensive antibiotic therapy to survive. These children, when followed through development, tend to have higher rates of asthma development[112]. Thus, while this model may be less relevant for the typical childhood ABX exposure and subsequent asthma risk, it could have much relevance for the septic neonate. An interesting test would be to only expose mice to ABX in the perinatal period, perhaps ceasing at weaning, and assessing if tolerance development was disrupted. This would be consistent with other groups who have also reported that neonatal and perinatal ABX exposure, but not exposure later in life, increases the severity of AAD[118; 229].

To conclude, the present study demonstrates that development of inhalational tolerance is dependent on an intact microbiome, and that disruption of the microbiome with ABX can impair tolerance development. Furthermore, disruption of inhalational tolerance using ABX requires exposure greater than simply at weaning, as was found to increase the severity of AAD in Chapter 4. However, just as ABX exposure early in life led to decreased lung-associated Tregs, so too did continuous ABX exposure produce a relative dearth of Tregs that was associated with failure to fully resolve eosinophilia. This loss of Tregs was accompanied by a relative deficit in CD5+ B Cells, a population known to contain Bregs. Further study, including assessment of AHR and the specific changes seen in the gut microbiome, will be required to begin to elucidate the mechanisms connecting ABX exposure with sustained eosinophilia in the BAL and diminished Tregs and Bregs in the HLN and lung.
Figures

Figure 21: Early-life Amoxicillin and Tylosin Treatment Does Not Impair the Development of Inhalational Tolerance
**Figure 21.** Early-life Amoxicillin and Tylosin Treatment Does Not Impair the Development of Inhalational Tolerance. BAL fluid was assessed for cellular populations using May-Grunwald stain (A) and counted. From that, the proportion of eosinophils (B) was determined. The total number of eosinophils (C), number of leukocytes in the BAL (D), number of leukocytes in the lung (E), and number of leukocytes in the HLN (F) were also determined. Data in B-F represent the mean ± the SEM. n=8 except for HDM HLN, where n=7. NS, not significant.

**Figure 22: Amoxicillin and Tylosin Treatment Has No Effect on Treg Populations**

![Graphs showing Treg populations](image)

**Figure 22.** Amoxicillin and Tylosin Treatment Has No Effect on Treg Populations. Single-cell suspensions of the HLN and Lung were stained with antibodies targeting CD3, CD4, and Foxp3 and assessed using flow cytometry. Regulatory T Cells (CD3+CD4+Foxp3+) were identified in the HLN (A) and lung (B). Data represent the mean ± the SEM. n=8 HDM Lung Tregs, HDM + Antibiotic Pair Tregs; n=7 HDM HLN Tregs, HDM + Antibiotic Pair Lung Tregs.
Figure 23: Pulsed Amoxicillin or Antibiotic Mix Treatment Does Not Impair the Development of Tolerance

**Figure 23.** Pulsed Amoxicillin Or Antibiotic Mix Treatment Does Not Impair the Development of Tolerance. BAL fluid was assessed for cellular populations using May-Grunwald stain (A) and counted. From that, the proportion of eosinophils (B) was determined. The total number of eosinophils (C) and number of leukocytes in the BAL (D) were counted. Data in B-D represent the mean ± the SEM. n=4 HDM; n=2 HDM + Amoxicillin; n=3 HDM + Antibiotic Mix.
Figure 24: Continuous Antibiotic Exposure Prevents Resolution of Eosinophilia and Leukocytosis
Figure 24. Continuous Antibiotic Exposure Prevents Resolution of Eosinophilia and Leukocytosis.

BAL fluid was assessed for cellular populations using May-Grunwald stain (A) and counted. From that, the proportion of eosinophils (B) was determined. The total number of eosinophils (C), number of leukocytes/white blood cells (WBCs) in the BAL (D), number of leukocytes/WBCs in the lung (E), and number of leukocytes/WBCs in the HLN (F) were also determined. Data in B-F represent the mean ± the SEM. n=5 per group except for HDM + Antibiotic Mix HLN, where n=4. a:p < 0.01, HDM + Antibiotic Mix vs HDM and HDM + Amoxicillin; * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 25: Continuous Antibiotic Exposure Reduced Populations of Regulatory T and B Cells

Figure 25. Continuous Antibiotic Exposure Reduced Populations of Regulatory T and B Cells. Single-cell suspensions of the HLN and Lung were stained with antibodies targeting CD3, CD4, CD5, CD19, B220, and Foxp3 and assessed using flow cytometry. Regulatory T Cells (CD3+CD4+Foxp3+) were identified in the HLN (A) and lung (C). Regulatory B Cells (CD3-CD19+B220+CD5+) were identified in the HLN (B) and lung (D). Data represent the mean ± the SEM. n=5 per group except for HDM + Antibiotic Mix HLN, where n=4. * p < 0.05; NS, not significant.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Innovation and Significance

The present body of work includes three important advances in our understanding of asthma and its relationship to the microbiome. First, we show that the development of inhalational tolerance to HDM is not a consequence of concurrent oral tolerance. Indeed, oral exposure to HDM not only failed to induce tolerance but failed to produce any changes in the gut microbiome. Second, we report that short, repeated ABX exposures early in life are sufficient to produce increased severity of experimental asthma later in life. This finding is in contrast to other reports in the literature which suggested that ABX exposure must either occur immediately following birth or be accompanied by an additional insult (such as fungal inoculation) to produce increased severity of disease. Third, we show that it is possible to disrupt the development of inhalational tolerance through disruption of the microbiome using ABX. This is consistent with a large body of literature concerning oral tolerance, where lack of microorganisms or ABX treatment can impair tolerance development. Finally, for the latter two points we have also shown that increased severity of experimental asthma and disruption of tolerance are associated with a loss of regulatory T cells in the lung or lung-associated lymphatic tissue. This links the present work to the extensive investigation of the role of Tregs in asthma undertaken by the Thrall laboratory since its first report on inhalational tolerance in the late 1990s.

These findings, particularly the second, add much to our understanding of how the microbiome influences asthma pathogenesis and provide tools for future studies of the same. Abundant clinical evidence has linked early-life ABX exposure to increased risk of developing asthma, but hitherto models of ABX exposure and asthma required exposures that had little relevance to those seen in clinical medicine, such as many weeks of ABX exposure or exposure beginning prior to
birth and continuing through early life. The model described in the present work, while by no means perfect,

**Future Directions and Concluding Discussion**

The lab has long studied the mechanisms underlying asthma pathophysiology and those which contribute to disease resolution and tolerance. However, the present work marks the first foray of the lab into the contribution of the microbiome to both elements. Accordingly, and unsurprisingly for a PhD dissertation, these studies have seen far more stories begin than end. In prior chapters, we have discussed the next steps for a number of the findings presented. Here, we highlight a select few of the broader concepts that the present work has begun to uncover but which await future efforts to fully elucidate.

**How does the microbiome contribute to inhalational tolerance to HDM?**

In the recent study of endotoxin in farm dust[180], the ability of dust to induce tolerance was directly linked to endotoxin levels within the dust. Our data may suggest that, at the least, simple endotoxin encounter is not the entire story. HDM itself contains endotoxin[32], yet when administered in the context of lifelong ABX it fails to produce complete tolerance and resolution of disease. The presence of LPS can restore oral tolerance to OVA in GF mice[306], yet it is unable to do so for inhalational tolerance in the face of ABX. This suggests that there is something about a healthy microbial community early in life that is critical for the development of proper immune responses in the lungs, as suggested by recent work examining the lung microbiome of mice early in development[179].

Dissecting this relationship could be attempted using GF mice. By inoculating GF mice with microbiome of varying composition, we could explore whether certain organisms are critical (as suggested by recent clinical evidence[97]) or whether overall diversity of the microbiome is what matters, as our data for ABX and AAD would indicate. It may be that endotoxin (perhaps through
the A20 mechanisms indicated by Shuijis) is absolutely required for inhalational tolerance, but it may simultaneously be true that the immune system is incapable of properly responding to endotoxin in the absence of the microbiome at a critical time of immune maturation.

**Can we undo the damage wrought by ABX?**

We have shown in the present work that asthma can be made more severe through perturbation of the microbiome. A natural question to follow these studies is if the reverse is possible. As previously discussed, probiotics are not likely to prove effective in reversing the effects of ABX. While they have shown great effect in mice, evidence remains decidedly mixed that they have any beneficial effect in on humans asthma[189]. A better option is fecal microbiome transplant (FMT), the only microbiome-based therapy with demonstrated clinical efficacy[190–192]. Using the model described in Figure 6 of the Materials and Methods, we attempted to restore the microbiome of ABX-exposed mice using donor feces from animals never exposed to ABX.

Foremost amongst the questions remaining is if the transplant we attempted was successful. 16S analysis of the community following transplant will provide that answer. If the transplant was not successful, that could explain why no effect was seen. However, if the transplant was successful, that raises a second question: why was no protection seen? Here, the answer may lie in timing. As discussed in the prior section of this chapter, the key concept of microbiome-immune interactions is timing. Microorganisms must be present at precisely the right moment in development to provide the proper signals for health immune function[325]. It is possible that the transplant strategy selected simply acts too late to have any benefit. A more appropriate strategy may be to attempt to restore the microbiome using FMT immediately following each ABX exposure (Figures 26 and 27). If we can prevent the ABX-induced loss of diversity that we have linked to a loss of immune regulators, The childhood antibiotic course of the future may feature an FMT pill as the final dose.
**How can we move beyond 16S?**

16S rRNA sequencing is a useful tool to obtain a snapshot of the composition of the microbial community, but it is limited in what it can teach us. If an organism is present, 16S sequencing tells us nothing about what it is doing. Answering this question requires one or a combination of three tools: metagenomics, metatranscriptomics, and metabolomics.

Metagenomics, or the study of all of the genes of all of the microorganisms within the microbiome, is a snapshot of what could be expressed. Are genes associated with virulence present? Does ABX exposure lead to a decrease in the relative proportion of genes related to fermentation of dietary fiber and SCFA production? Such a change could suggest less SCFA production and less microbiome-driven induction and maintenance of Tregs.

However, presence of genes does not guarantee expression. One need only consider a genetically female mammalian cell to realize this: two X chromosomes and their respective genes are present, but only one is active in any given cell. Determination of active gene expression requires assessment of RNA, and metatranscriptomics is the tool to do so for the microbiome. Perhaps ABX treatment does not eliminate all organisms capable of inducing Tregs, but these organisms may be expressing few to no genes associated with the metabolic products that promote Treg formation. Perhaps a small population of organisms, and a correspondingly small proportion of the total metagenome, are highly transcriptionally active. Metagenomics will not tell you this, and 16S sequencing certainly will not.

The shortcoming of both metagenomics and metatranscriptomics is that they require some knowledge of what you are looking for. One may discover that a certain bacterial gene is abundant, or abundantly expressed, but if nothing is known of what that gene does (not an uncommon situation when so many of the microorganisms we study have yet to even be cultured), this tells us little of mechanism. Metabolomics is the next step, looking at metabolic products of the microbial community. Are certain classes of lipids more or less abundant as tolerance
develops? Are byproducts of uric acid metabolism (potentially important for asthma, as discussed in Chapter 1) accumulating following ABX therapy? Metabolomics can begin to answer these and many similar questions.

Such efforts studies are critical to determine how the microbiome is affecting immunity in the context of AAD and tolerance, particularly to identify pathways that we can target to promote tolerance, erase the harmful effects of ABX, and end the asthma epidemic. To future students will be the task of applying these new and rapidly-advancing analytical tools to the microbiome-asthma relationship.

**Concluding Remarks**

One of the most important lessons of this project was not scientific or mechanistic. That lesson was that even a hypothesis which subsequently fails can inspire great insight if the studies to test it are designed appropriately and if one always remembers to follow the data. The original hypothesis guiding this work was that HDM exposure leads to the formation of a regulatory microbiome and that this regulatory microbiome promotes the induction of Tregs and tolerance. This hypothesis was clearly not sustained by the evidence gathered in the present work, but the investigations to validate it were directly responsible for new insights into oral tolerance, the effects of early-life ABX exposure, and the importance of the microbiome to inhalational tolerance generation.

Despite recognition of the innumerable small actors inside of us 130 years ago[61], we still understand so little of their importance to our health. Every new relationship we uncover between the microbiome and the host immune system answers another vexing question (e.g. why did the rise in allergic diseases seem to correlate with elimination of peptic ulcer disease?), but creates many more. Such fields in science are the most exciting and the most promising, but also the most challenging for even the accomplished investigator. What we have reported herein is only the beginning of the story, and perhaps in another 130 years we will see ourselves near its end.
Figures

Figure 26: Fecal Microbiome Transplant Following ABX Treatment Does Not Protect Against Eosinophilia

Figure 26. Fecal Microbiome Transplant Following ABX Treatment Does Not Protect Against Eosinophilia. BAL fluid was assessed for cellular populations using May-Grunwald stain (A) and counted. From that, the proportion of eosinophils (B) was determined. n=4 per group.
Figure 27: Alternative FMT Model Featuring Post-ABX Corrective Dosing

Figure 27. Alternative FMT Model Featuring Post-ABX Corrective Dosing. Magenta arrows indicate fecal gavage dates, occurring one day following the cessation of ABX therapy.
APPENDIX

Appendix A: QIIME Analysis Script

#!/bin/bash

#PBS -l nodes=1:ppn=25
#PBS -N hpcl_27_mar_2016_hdm_pbs_2dplots
#PBS -V

# Number of processors to request. On HPC1, do not request more than 40 or
# you may kill a node
#torque_procs=25

# Flag for processing multiplexed, single-index data
# Demultiplexed, dual-index data is assumed if the orig_files directory is
# present
# Accepts 1 for yes and 0 for no
#multiplexed=1

# References
reference_seqs=/home/CAM/aadami/references/gg_13_8_otus/rep_set/97_otus.fasta

# Script variables
data_source=/home/CAM/aadami/datasets
preprocess_directory=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/preprocess
work_directory=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/split_libs_out
chimeric_seqs_out=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/split_libs_out,chimeras_out
otus_out=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/otus_out
map_file=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/merged_map.txt
map_file_2dclean=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/merged_map_2dclean.txt

START=`date +%s`

# Modified from script provided by Kendra Maas of the University of
Connecticut Microbial Analysis, Resources, and Services (MARS) Facility and
from scripts referenced in Nelson et al 2015 PLoS ONE DOI:
10.1371/journal.pone.0094249

# Move into main analysis directory
cd $work_directory

cd $work_directory

# Clear the preprocessing directory of any content
# Remove any intermediate directories created by future steps
# This is useful only if you had a previous failed run and wish to retry it
# It will clear all existing work from previous runs in the working
directory!
xm -rf *.fna
rm -rf usearch_*
rm -rf $preprocess_directory
rm -rf $otus_out
rm -rf $split_libs_out

# Actions for multiplexed data
if test $multiplexed -eq 1; then
    # Combine samples from multiple runs (pre-MARS dual indexing) using only the forward read
    $work_directory/R1_map.txt,$work_directory/R2_map.txt,$work_directory/R4_map.txt -q 19
fi

# Actions for demultiplexed data
if test -d orig_files; then
    # Make and move into the preprocessing directory, copying all original files to it
    mkdir $preprocess_directory
    cp -rf orig_files/* $preprocess_directory/
cd $preprocess_directory

    # Loop through data folders and move files, renaming them by sample
    mkdir $preprocess_directory/fastq
    for i in */Data/Intensities/BaseCalls/*.*; do mv $i "fastq""/"${i%%-*.fastq}" "fastq""/"$i""/basename $i"; done

    # Merge all paired ends by sample
    mkdir qiime
    multiple_join_paired_ends.py -i . -o $preprocess_directory/qiime/

    # Remove any fastq files that failed to join
    mkdir nonjoin
    find qiime/ -name "fastqjoin.un*" -print -exec mv {} nonjoin/ \;
    find qiime/ -size "0" -print -exec mv {} nonjoin/ \;

    # Merge all demultiplexed libraries by sampleID using filenames, retaining only QScores above 19
    multiple_split_libraries_fastq.py -i $preprocess_directory/qiime/ -o $preprocess_directory/qiime/ --demultiplexing_method_sampleid_by_file --include_input_dir_path -p $work_directory/qiime_parameters.txt

    # Remove extra BaseSpace info from sequence names in seqs.fna
    cp $preprocess_directory/qiime/seqs.fna
    $preprocess_directory/qiime/demultiplexed_with_basespace_info_seqs.fna
    sed 's/_S.*_L001.*join.fastq//g'
    $preprocess_directory/qiime/demultiplexed_with_basespace_info_seqs.fna > $preprocess_directory/qiime/basespace_cleaned_seqs.fna

    # Count sequences: what did we get?
    echo "Sequences after demultiplexing\n"
    count_seqs.py -i $preprocess_directory/qiime/basespace_cleaned_seqs.fna
# Move into main analysis directory

cd $work_directory

# Concatenate the two fasta files

cp $preprocess_directory/qiime/basespace_cleaned_seqs.fna .
cat $split_libs_out/seqs.fna >> basespace_cleaned_seqs.fna

# Count sequences: what did we get?

echo "Sequences after concatenation"
count_seqs.py -i basespace_cleaned_seqs.fna

# Length filter sequences to between 245 and 260 bp
# This command
# 1) checks to see if the current line begins with a fasta sequence
# identifier, which always starts with >. In other words, says if the
# beginning of the line (^) is NOT (!) followed by >
# 2) stops all activity and moves to the next line
# 3) if it passes 1 and 2, grabs the current line and copies it into the
# variable seq
# 4) asks if the length of seq is between 245 and 260 bp inclusive, and
# finally 5) prints a newline and then the contents of seq if the line met the
# length conditions.
# Note that this will fail to generate the expected output if your input
# FASTA file has sequences that are not represented on a single line!
awk '!/^>/ { next } { getline seq } (length(seq) >= 245 && length(seq) <= 260) { print $0 "\n" seq }' basespace_cleaned_seqs.fna > length_filtered_seqs.fna

# Count sequences: what did we get?

echo "Sequences after length filtering"
count_seqs.py -i length_filtered_seqs.fna

# Check for chimeric sequences using usearch61
identify_chimeric_seqs.py -i length_filtered_seqs.fna -m usearch61 -o $chimeric_seqs_out/ -r $reference_seqs
filter_fasta.py -f length_filtered_seqs.fna -o seqs_chimeras_filtered.fna -s $chimeric_seqs_out/chimeras.txt -n

# Count sequences: what did we get?

echo "Sequences after chimera checking"
count_seqs.py -i seqs_chimeras_filtered.fna

# Pick OTUs using usearch61 in an open reference manner (i.e. reference based
# and then de novo on unmatched sequences)
pick_open_reference_otus.py -o $otus_out/ -i seqs_chimeras_filtered.fna -r $reference_segs -m usearch61 -a -0 $torque_procs

# Summarize OTU table information (i.e. how many sequences do we have for
# each sample)
biom summarize-table -i $otus_out/otu_table_mc2_w_tax_no_pynast_failures.biom -o $otus_out/otu_table_mc2_w_tax_no_pynast_failures_summary.txt

# Calculate alpha and beta diversity metrics (metrics specified in parameters
# file), rarefying to 10000 reads
core_diversity_analyses.py -o $otus_out/diversity/ -i $otus_out/otu_table_mc2_w_tax_no_pynast_failures.biom -m $map_file -t $otus_out/rep_set.tre -p $work_directory/qiime_parameters.txt -e 10000 -c "Timepoint,TimepointExtra,GroupTimepoint" -a -O $torque_procs

# Calculate alpha and beta diversity metrics (metrics specified in parameters file), rarefying to 5000 reads
core_diversity_analyses.py -o $otus_out/diversity5k/ -i $otus_out/otu_table_mc2_w_tax_no_pynast_failures.biom -m $map_file -t $otus_out/rep_set.tre -p $work_directory/qiime_parameters.txt -e 5000 -c "Timepoint,TimepointExtra,GroupTimepoint" -a -O $torque_procs

# Set mapfile and preference variables
map_file_2dclean=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/merged_map_2dclean.txt
map_file_gp35=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/merged_map_gp35.txt
map_file_gp27=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/merged_map_gp27.txt
map_file_gp06=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/merged_map_gp06.txt
plot_prefs=/home/CAM/aadami/analysis/plot_prefs.txt
twodplots_out=/home/CAM/aadami/analysis/2016-03-24_hdm_pbs_analysis/otus_out/diversity/2dplots

# Make plots
make_2d_plots.py -i $otus_out/diversity/bdiv_even10000/unweighted_unifrac_pc.txt -m $map_file -b 'Timepoint' -o $twodplots_out/TE/ -p $plot_prefs
make_2d_plots.py -i $otus_out/diversity/bdiv_even10000/unweighted_unifrac_pc.txt -m $map_file_2dclean -b 'Timepoint' -o $twodplots_out/TE_clean/ -p $plot_prefs
make_2d_plots.py -i $otus_out/diversity/bdiv_even10000/unweighted_unifrac_pc.txt -m $map_file_gp35 -b 'Timepoint' -o $twodplots_out/TE_gp35/ -p $plot_prefs
make_2d_plots.py -i $otus_out/diversity/bdiv_even10000/unweighted_unifrac_pc.txt -m $map_file_gp27 -b 'Timepoint' -o $twodplots_out/TE_gp27/ -p $plot_prefs
make_2d_plots.py -i $otus_out/diversity/bdiv_even10000/unweighted_unifrac_pc.txt -m $map_file_gp06 -b 'Timepoint' -o $twodplots_out/TE_gp06/ -p $plot_prefs

# End script
echo ''
END="[date +%s"
RUNTIME=$(($END - $START ))
echo "This job took $RUNTIME seconds to complete"
echo "Began"
date -d @$START
echo "\n"
echo "Ended"
date -d @$END
exit
Appendix B: QIIME Settings

split_libraries_fastq:phred_quality_threshold 19
alpha_diversity:metrics PD_whole_tree, chao1, observed_otus, shannon, simpson
beta_diversity:metrics
  bray_curtis, binary_ochiai, euclidean, unweighted_unifrac, weighted_unifrac

Appendix C: Oral Exposure to HDM Does Not Produce Inhalational Tolerance

We have discussed the impact of inhaled HDM exposure on the gut microbiome and whether any changes resulting therefrom may correlate with the development of tolerance (refer to Chapter 3). It is well recognized that inhaled substances can be swallowed, and multiple groups have shown that intranasal instillation of substances ranging from viruses to microspheres sees a proportion, although not a majority, subsequently make its way to the gut[326–328]. To explore the possibility that the generation of inhalational tolerance to HDM could be a product of oral tolerance due to swallowed HDM, we gavaged mice in a manner identical to intranasal HDM exposure in terms of timing and allergen delivery. We further sampled and assessed the gut microbiome during the course of gavage to assess for HDM-specific changes in the composition of the gut microbiome. Refer to the Materials and Methods for a detailed description of the gavage procedure and timing.

Results

Eosinophilia but No Change in Leukocyte Numbers Accompany HDM Gavage

HDM gavage led to increased relative eosinophilia in the BAL as compared to PBS gavage (Figure 28A-B). This eosinophilia was accompanied by a relative decrease in the proportion of macrophages in HDM-gavaged animals as compared to PBS-gavaged animals (Figure 28A). Absolute numbers of eosinophils did not differ between the groups (Figure 28C). BAL leukocytosis was significantly elevated in HDM-gavaged animals as compared to PBS-gavaged
animals, although this difference was primarily driven by a single sample (Figure 28D). Cell counts in the HLN (Figure 28E) and the lung (Figure 28F) did not differ significantly between treatment groups.

**Eleven Weeks of HDM Gavage Does Not Induce Oral Tolerance**

Following eleven weeks of HDM or PBS gavage, both groups of animals were subjected to two weeks of intranasal HDM exposure to induce AAD. Robust AAD was induced in both treatment groups, with over 50% of airway leukocytes being eosinophils (Figure 29A-B). These proportions are similar to expected values we have previously published in the model[32]. Similarly, the absolute number of eosinophils did not differ between the animals which had received HDM gavage and those which had received PBS gavage (Figure 29C). Furthermore, neither BAL (Figure 29D), lung (Figure 29E), nor HLN (Figure 29F) leukocyte counts differed significantly between the two treatment groups. Overall, the type of gavage received did not appreciably change AAD cellular profiles.

**The Fecal Microbiome of HDM-Gavaged Animals Does Not Differ from that of PBS-Gavaged Animals**

Fecal samples obtained from animals over the course of gavage and subsequent intranasal HDM were evaluated using QIIME. During the course of gavage, the community composition shifted in a similar manner between PBS- and HDM-gavaged animals (Figure 30A). The relative abundance of a group of unclassified organisms of family Rikenellaceae increased, as did the proportion of organisms of the genus Turicibacter. The relative abundance of genus Akkermansia decreased from nearly a quarter of all naïve fecal organisms to less than 10% of all organisms. Organisms of the genus Allobaculum first increased from less than 10% to nearly 20% of all organisms before decreasing to 10% or less by eleven weeks of gavage.
However, while much of the community changes were mirrored between HDM- and PBS-gavaged animals, one notable distinction did appear following two weeks of intranasal HDM. PBS gavage and intranasal HDM saw a large increase in organisms of genus Prevotella. These organisms were not absent from HDM gavaged and intranasal HDM animals, but the abundance in the latter group was less than 10% as compared to over 15% for PBS gavage and HDM intranasal animals.

PCoA of the unweighted UniFrac distances reinforced the similarity between the two groups (Figure 30B). Naïve samples began in the middle right of the principle coordinate plot, initially moving to the lower left after five weeks of both HDM and PBS gavage but subsequently moving to the upper left of the plot after both eleven weeks of HDM or PBS gavage and further intranasal HDM exposure. As a result, the primary shift by the end of the experiment was along principle coordinate 1. No distinct clustering was noted at any of the timepoints. This was reflected in the proportion of the variation explained by principle coordinates 1 and 2, as neither one exceeded 15% of the total variation amongst the samples.

Finally, the similarity of the effects of the two treatments on the fecal microbiome was evident by comparing the diversity of the fecal microbiome as gavage and intranasal HDM progressed (Figure 30C). Neither sample significantly differed from the other at any timepoint, with both tending to increase in diversity (as measured by Simpson’s diversity index) as time went on, although comparison at most time points was hampered by a lack of samples (e.g. 2 and 5 week gavage, 11 week gavage and HDM).

**Discussion**

The present study has two key results. First, these data demonstrate that HDM is distinct from OVA and many other antigens in that oral tolerance does not develop in response to exposure to HDM, at least not at the same timescale as it does when inhaled. Second, these results suggest that the inhalational tolerance which develops in response to sustained, inhalational
administration of HDM is not an unintentional byproduct of oral tolerance, as oral tolerance was unable to be induced following eleven weeks of oral exposure.

While we initially suspected that changes we saw in some groups exposed to inhaled house dust mite were due to the interaction of the gut microbiome with swallowed HDM, the present study tends to suggest this is not the case. Indeed, the similarity of animals gavaged or exposed intranasally to HDM or PBS was striking, with the same dominating taxa (unidentified order Clostridiales, Bacteroides family S24-7, and genus Akkermansia) present in all groups. At the least, this suggests that any differential changes in the gut microbiome due to HDM exposure are due less to swallowing of inhaled allergen and perhaps more to the interaction of the diseased lung with the gut (a relationship which has been defined by others as the gut-lung axis[329; 330]) and vice-versa.

A surprising finding from the gavage experiments was the complete failure of oral tolerance development. Both HDM- and PBS-gavaged animals had eosinophilia of expected severity following two weeks of HDM intranasal exposure, appearing as if the eleven weeks of gavage had no effect at all on the response to HDM. This is in sharp contrast to OVA, where oral tolerance is robust and consistently-inducible. Indeed, oral tolerance to OVA completely abrogates or highly attenuates disease on subsequent inhalational exposure[331; 332]. This difference is in spite of both antigens being capable of producing inhalational tolerance[32; 257], suggesting that the mechanisms mediating tolerance may differ between the two. This idea may not be entirely surprising. The two model allergens differ greatly in both composition and mode of disease induction. OVA is a peptide, while HDM is an assortment of peptides, lipids, carbohydrates, and other compounds derived from the dust mite and its associated microbial community. Inducing AAD with OVA requires sensitization with OVA and an adjuvant (such as alum), while HDM can induce AAD with no prior sensitization period.
This and other differences could explain why so upwards of 80% of human asthmatics have sensitivity to HDM[333; 334] and perhaps why oral tolerance fails for HDM. It also adds urgency to the question of precisely how inhalational tolerance to HDM develops, as mechanisms therein may provide a way to cure otherwise-intractable HDM-induced asthma. One recent mechanistic study of a different kind of dust could provide clues, and brings the story back to the microbiome. Schuijs and colleagues evaluated the means by which the farm environment can provide protection against HDM-induced AAD[180]. Exposing mice to farm dust, the group found that endotoxin in the dust promoted tolerance and non-reactivity of the pulmonary mucosal immune system, acting through suppression of NF-κB signaling by the protein A20. The idea that LPS and other bacterial products within dust could provide protection has much clinical evidence to support it. Multiple investigations of house dust have shown that higher levels of endotoxin, diversity of microorganisms, or some combination thereof within environmental dust (such as in mattresses) correlates with protection from asthma[86; 88; 89; 92; 94; 96]. While the level of LPS in the HDM extract utilized in the present study is low[32], it may be sufficient to induce a response similar to that produced by the endotoxin in farm dust.

One interesting finding from the gavage experiments was the presence of eosinophilia in some, but not all, HDM-gavaged animals following eleven weeks of gavage (unsurprisingly, no PBS-gavaged animals displayed eosinophilia). Perhaps the most likely explanation for this is simple aspiration of gavage materials by mice, inducing AAD in a similar manner to if the HDM had been instilled intranasally. It is likely that such aspiration did not occur with each gavage exposure, or it would conceivable have induced inhalational tolerance as if the HDM had been administered directly to the lung compartment.

One of the few appreciable differences between the gavaged groups following intranasal HDM was the presence of a sizable proportion of Prevotella in the PBS gavaged animals (over 15% of all organisms in PBS Gavage + HDM as compared to 5% or less in HDM Gavage + HDM, Figure
Interestingly, among the few studies of the lung microbiome in humans, Prevotella has been reported to be more abundant in control subjects than in asthmatics[173]. Furthermore, at least one investigation has suggested that some Prevotella species may have anti-inflammatory properties specifically in the context of lung inflammation[335]. It remains to be seen whether this difference is a pure chance effect (such as one driven by cage differences, although with multiple cages this is less likely) or a truly definitive characteristic of PBS gavage, but it clearly has no effect on the early induction of AAD, as these animals have disease at least as severe as HDM-gavaged animals.

To conclude, oral exposure to HDM in the same temporal manner as intranasal exposure does not induce oral tolerance to HDM and subsequent protection against AAD, in contrast to the robust oral tolerance seen with OVA. With respect to the gut microbiome, few changes are seen between HDM and PBS gavage save for an increase in the proportion of Prevotella in HDM-gavaged mice, the significance of which (if any) remains unclear. Much remains to be explored with respect to HDM gavage. Perhaps the amount of HDM gavaged is too high, and a smaller amount would have led to tolerance (e.g. equivalent to how much may be swallowed when it is instilled intranasally). Furthermore, we do not know if the lack of tolerance is simply in the lung or systemic, as HDM gavage could conceivably be protective against exposure to HDM elsewhere (e.g. footpad). These questions await future investigation and investigators to answer.
Figure 28: 11 Weeks of HDM Gavage Produces Slight Eosinophilia

BAL fluid was assessed for cellular populations using May-Grunwald stain (A) and counted. From that, the proportion of eosinophils (B) was determined. The total number of eosinophils (C), number of leukocytes in the BAL (D), number of leukocytes in the HLN (E), and number of leukocytes in the lung were
also determined. Data represent two independent experiments, n=10 per group except for PBS Gavage HLN cell counts, where n=9. a: p < 0.05, Eosinophil %, HDM Gavage vs PBS Gavage; b: p < 0.05, Macrophage %, HDM Gavage vs PBS Gavage; * p < 0.05.

**Figure 29: 11 Weeks of HDM Gavage Is Not Protective Against Subsequent Intranasal HDM Exposure**
Figure 29. 11 Weeks of HDM Gavage Is Not Protective Against Subsequent Intranasal HDM Exposure. BAL fluid was assessed for cellular populations using May-Grunwald stain (A) and counted. From that, the proportion of eosinophils (B) was determined. The total number of eosinophils (C), number of leukocytes in the BAL (D), number of leukocytes in the HLN (E), and number of leukocytes in the lung were also determined. Data in B-F represent the mean ± the SEM. Data represent two independent experiments, n=10 per group except for HDM Gavage + HDM Differentials, Eosinophil %, Eosinophil Counts, and HLN cell counts, where n=9.
Figure 30: HDM Gavage Produces No Change in the Composition of the Fecal Microbiome

QIIME was used to pick OTUs and analyze samples from mice that had received PBS or HDM gavage. OTUs picked at the 97% similarity level are represented as bars in (A). Bars represent the average abundance of each OTU. In (A), o__ refers to Order, f__ refers to Family, and g__
refers to Genus. OTUs lacking a lower classification (e.g. an organism identified at the Order level but not the Family or Genus level) refers to an unidentified OTU within that level, not all organisms at that level. For example, the OTU identified as Order Clostridales does not represent all Clostridales, merely one Clostridales at the 97% similarity level that is not classified in GreenGenes below the Order level. PCoA was performed on the distance matrix of the unweighted UniFrac distances and the two largest principal coordinates represented (B). Simpson’s diversity index was calculated for each sample and plotted (C). Data in (C) represent the mean ± the SEM except where no error bars are present. If error bars are not present, that point represents a single sample. The addition of “+ HDM” indicates two weeks of intranasal HDM exposure following the specified gavage (PBS or HDM). n=9, Naïve; n=5, 2 Week HDM Gavage; n=6, 2 Week PBS Gavage; n=1, 5 Week HDM Gavage; n=6, 5 Week PBS Gavage; n=3, 11 Week HDM Gavage; n=3, 11 Week PBS Gavage; n=1, 11 Week HDM Gavage + HDM; n=1, 11 Week PBS Gavage + HDM.
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