

Microbiota in Allergy and Asthma and the Emerging Relationship with the Gut Microbiome

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Asthma and atopy, classically associated with hyper-activation of the T helper 2 (Th2) arm of adaptive immunity, are among the most common chronic illnesses worldwide. Emerging evidence relates atopy and asthma to the composition and function of the human microbiome, the collection of microbes that reside in and on and interact with the human body. The ability to interrogate microbial ecology of the human host is due in large part to recent technological developments that permit identification of microbes and their products using culture-independent molecular detection techniques. In this review we explore the roles of respiratory, gut, and environmental microbiomes in asthma and allergic disease development, manifestation, and attenuation. Though still a relatively nascent field of research, evidence to date suggests that the airway and/or gut microbiome may represent fertile targets for prevention or management of allergic asthma and other diseases in which adaptive immune dysfunction is a prominent feature.

The Human Microbiome

The field of microbiology owes its genesis to observations made by Antonie van Leeuwenhoek, a Dutch microscope lens maker who, in 1676, first described microscopic “animalcules” in dental plaque (Porter, 1976). For much of the ensuing history of the field, research has largely focused on culture-based studies of individual microbial species, with an emphasis on understanding the basis of microbial pathogenesis. However, over the past 30 years, the sub-specialty of microbial ecology has driven development of molecular methods for microbial detection that obviate the necessity for microbial culture. These efforts have revealed the existence of a vast diversity of microbes that inhabit natural systems and demonstrated that these organisms rarely exist in isolation, but instead occur in multi-species, multi-functional communities.

Pioneered by Dr. Carl Woese, the use of molecular techniques to classify microbes without recourse to laboratory culture-based approaches led to the subsequent development of the 16S rRNA gene as a widely used bacterial biomarker for profiling bacteria present in a mixed-species community. This gene, which is exclusive to and ubiquitous among bacteria (Fox et al., 1977; Winker and Woese, 1991; Woese, 1987), is a useful molecular target for bacterial identification due to the presence of regions of sequence that are highly conserved across the majority of known bacteria. These conserved sequences flank hyper-variable regions, reflective of varying evolutionary rates across bacterial species. The conserved 16S rRNA sequence regions permit design of PCR primers and amplification of the gene (or a portion thereof) from the majority of known bacterial species. The amplified hyper-variable region is typically sequenced to permit classification of amplicons into discrete taxonomic groups, generating a fingerprint of bacterial phylotypes within a given community. Application of ecological statistics and theory to these profiles allows for analyses and interpretation

of the composition of microbial communities. Distinct biomarkers, e.g., the internal transcribed spacer region 2 (ITS2) of the fungal rRNA gene, are used for mycological community profiling, which is performed in a similar manner. More recent advances and declining costs associated with high-throughput sequencing platforms have permitted more expansive microbial biomarker sequencing efforts, leading to identification of novel microbial species, expansion of the known microbial tree of life, and an enhanced appreciation of the diversity of microbes that inhabit natural systems. This increase in sequence capacity has also led to the development of complementary microbiome analyses tools, including shotgun sequencing approaches to determine the functional genes encoded or expressed by a microbial community (metagenomics or metatranscriptomics, respectively; Figure 1). Parallel advances in mass spectroscopy platforms have led to improvements in methods for detecting microbial products, including the dominant proteins (metaproteomics) or metabolites (metabolomics) collectively produced by microbial members within a community (Figure 1). As the field continues to rapidly evolve, efforts are becoming more focused on integration of these approaches to produce a more comprehensive view of microbial community composition and function.

Though microbial ecology research has traditionally focused on terrestrial and aquatic ecosystems, more recently, predominantly biomarker-based microbial profiling techniques have been applied to the study of the human microbial ecosystem. The results have led to a more comprehensive view of the healthy human body as a series of ecosystems, each with its own particular microbial composition, when considered at the broadest (phylum) level of classification. It has been estimated that microbial cells outnumber human cells by approximately 10-fold (Savage, 1977) and that healthy humans possess 500–1,000 distinct bacterial phylotypes (Claesson et al., 2009; Frank et al., 2007). Though much research has focused on bacterial communities

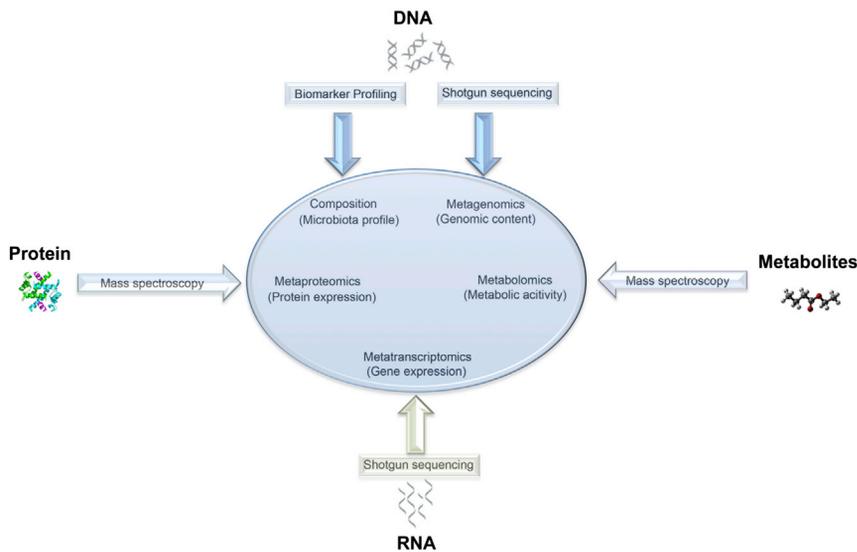


Figure 1. Molecular Tools for Interrogating Microbiome Composition and Function

DNA, RNA, protein, and metabolite fractions of the samples may be interrogated using next-generation sequencing and mass spectroscopy platforms to assess microbiome composition and function.

in the human host, fungi, viruses, and archaea have also been detected in these assemblages. The genetic capacity of such a diverse microbial ecosystem is immense; the pan-genome of the healthy human microbiome is estimated to encode approximately 100 times the number of genes found in the human genome. Though the genomic capacity of the healthy gut microbiota, where much research has focused to date, is predominantly encoded by bacteria, fungal and archaeal genes are also present in this consortium, though their functional contribution to host health status is as yet largely undefined (Qin et al., 2010).

Microbial members of the human super-organism contribute critical functions to their host, including enzymic digestion of complex carbohydrates; gastrointestinal microbes digest fermentable fiber to produce short-chain fatty acids (SCFAs; Roediger 1980), an essential energy source for the epithelial cell lining of the gut (Kaneko et al., 1994; Flint et al., 2008). Production of SCFAs also acidifies the local gastrointestinal microenvironment, making it less hospitable for colonization or overgrowth of pathogenic species such as *Escherichia coli* and *Salmonella* spp. (Cherrington et al., 1991). Commensal microbes also engage in competitive colonization, which provides further protection against pathogen overgrowth. The gut microbiome also biosynthesizes essential vitamins and hormones (Yatsunenko et al., 2012), as well as a range of anti-inflammatory compounds (Herbst et al., 2011; Mazmanian et al., 2008; Round and Mazmanian, 2010; Sokol et al., 2008). Our reliance on microbial function is evident from the critical role microbes play in mammalian development; germ-free mice are both immunologically and physiologically aberrant, a phenotype that can only be rescued through introduction of commensal bacteria (Atarashi et al., 2011; Smith et al., 2007). Beyond early-life development, evidence has emerged that the microbiome influences the tone of host immune response, particularly that of the adaptive arm. Specific *Clostridium* species belonging to clade IV or XIVA as well as members of the *Bacteroides* exhibit the capacity to induce T regulatory (Treg) cells, which are essential for immune tolerance and abrogation of chronic inflammatory or autoimmune disease (Atarashi et al., 2011; Ochoa-Repáraz et al.,

2010). In addition, murine studies have demonstrated the capacity of specific species in the gut microbiome to induce discrete arms of the adaptive immune response. Black 6 mice whose ileum is overtly colonized by segmented filamentous bacteria (SFB) exhibit robust proliferation of IL-17- and IL-22-expressing T helper 17 (Th17) cells (Ivanov et al., 2009). This phenotype could be conferred to Th17 deficient mice by co-housing with SFB colonized mice or by transfer of fecal material from SFB mono-colonized animals.

SFB, an unculturable member of the *Clostridiales*, was identified as the species responsible for induction of Th17 cell proliferation through culture-independent microbiome profiling (Ivanov et al., 2009). However, perhaps more importantly, these studies demonstrate a direct role for microbiome members in the promotion of distinct adaptive immune responses associated with protection against or development of a range of chronic inflammatory and autoimmune diseases. These pertinent observations in mice raise the possibility that diseases associated with overactive arms of adaptive immunity may owe their genesis to microbiome dysbiosis and related dysfunction. It also raises the possibility that diseases currently associated with “inappropriate immune activation” may in fact represent perfectly appropriate host immune responses to specific pathogenic microbiome compositions and their related activities that promote immune activation.

The Asthmatic Airway Microbiome

Asthma is estimated to affect approximately 300 million individuals worldwide, incurs significant health care expenditure (Accordini et al., 2013; Barnett and Nurmagambetov, 2011), and is one of the most common chronic diseases. Given increases in disease prevalence over the last several decades, it is predicted that the number of individuals affected worldwide will increase by 100 million people by 2025 (Masoli et al., 2004). In the United States, the risk of developing asthma is highest for children during the period between birth and four years of age (Jackson et al., 2014); disease prevalence is also higher among women, families below the poverty line, and people of multiple races compared to other groups (Akinbami et al., 2009). Though risk alleles have been associated with asthma development (reviewed in Ober and Yao, 2011), the rapid increase in prevalence over the recent decades, particularly among children, points to environmental factors playing a key role in disease development (Cookson and Moffatt, 1997). Atopic sensitization (allergy), characterized by elevated levels of total and allergen-specific IgE in the serum and typically by positive skin-prick tests to at least one of a panel of common allergens, is considered the strongest risk factor for

childhood asthma development in westernized nations (Simpson et al., 2010), and its rise is associated with a parallel increase in asthma prevalence (Masoli et al., 2004). More recently, an unsupervised statistical approach to pediatric subject clustering, based on the range and degree of their atopic sensitization, revealed that subjects that were predominantly multi-sensitized to common food and aero-allergens had the highest risk of subsequently developing asthma in childhood (Havstad et al., 2014; Simpson et al., 2010).

Allergic asthma is a heterogeneous and complex disease, clinically defined as reversible airflow obstruction and immunologically by hyper-activation of the T helper 2 (Th2) arm of the adaptive immune response and the overexpression of the pro-inflammatory cytokines IL-4, IL-5, and IL-13, as well as eosinophilia and mast cell infiltration of the airways (Adcock et al., 2008). Asthma is difficult to diagnose because of inherent heterogeneity across asthmatic patient populations and the multiple contributory factors such as risk alleles, environmental exposures, and lung function (reviewed in Chung et al., 2014). Patients are commonly stratified using a range clinical parameters including bronchial hyper-responsiveness to methacholine (a drug that promotes bronchoconstriction), as well as medication use and frequency of exacerbation. Recently, unsupervised statistics have been used to improve diagnosing asthma and its severity (Chung et al., 2014).

However, emerging evidence indicates that other distinct immune phenotypes exist among discrete asthmatic patient subpopulations; recent studies have demonstrated an association between T helper 1 (Th1) cells specifically in obesity-related (Rastogi et al., 2012) and smoking-induced asthma (Tsoumakidou et al., 2007). Additionally, among asthmatic populations, a recent study distinguished two patient subgroups, Th2-high and Th2-low, which evenly divided the cohort under study ($n = 22$ and $n = 20$, respectively; Woodruff et al., 2009). Th2-high patients exhibited significantly increased expression of three biomarker genes (POSTN, CLCA1, and SERPINB2) and of the Th2 cytokines IL-5 and IL-13, but not IL-4. They exhibited increased mucin stores (as indicated by increased MUC5AC and MUC2 expression) and more severe airway hyper-responsiveness, as measured by the provocative concentration of methacholine necessary to reduce the forced expiratory volume in one second by 20% from baseline, more commonly known as the PC₂₀ test. Th2-low individuals consist of both asthmatic and non-asthmatic subjects and characteristically exhibit low levels of both Th2 cytokines and the three-gene biomarker expression. While Th2-high and Th2-low asthmatics do not differ in disease severity, they react distinctly to inhaled steroids; only Th2-high asthmatics respond to corticosteroids, a common treatment for asthma. These findings clearly require validation in multiple large cohorts; however, they suggest that Th2 heterogeneity exists among asthmatics and may represent a critical approach to stratifying patients. Given the existing evidence for microbial activation of T cell subsets in the gastrointestinal tract, the current hypothesis is that the overall heterogeneity in asthma-associated immune dysfunction is related to the presence of distinct microbial populations in the airways. While several groups have long postulated that asthma is an infectious disease caused by bacterial pathogens (reviewed in Kraft, 2000), it has only been the relatively recent application of culture-inde-

pendent microbiome profiling approaches to asthmatic airway samples that has revealed the diversity of microbes that exist in the asthmatic airway and that relationships do exist between microbial members within these communities and features of the disease.

Multiple studies of asthmatic patient cohorts using distinct microbiome platforms (next-generation sequencing and phylogenetic microarrays) have reported the presence of a diverse microbial community in the airways of these patients. These microbiota are commonly enriched for members of the *Proteobacteria* (Goleva et al., 2013; Hilty et al., 2010; Huang et al., 2011). When compared to healthy subjects (few of which possessed sufficient bacterial material for 16S rRNA-based microbiome profiling), asthmatics possessed significantly higher bacterial burden and diversity in their lower airways (Huang et al., 2011). The finding of a characteristically low bacterial burden in the lower airways of healthy subjects was supported by a separate study of bronchoscopic lower airway samples, which found that the lower airway microbiome differed from the upper by having lower biomass but being compositionally similar, consisting of members of the *Staphylococcaceae*, *Propionobacteriaceae*, *Corynebacteriaceae*, *Streptococcaceae*, *Veillonellaceae*, and *Prevotellaceae* (Charlson et al., 2011). Marri et al. (2013) reported a slightly different community for non-asthmatics based on induced sputum samples that consisted of members of the *Lachnospiraceae*, *Staphylococcaceae*, *Streptococcaceae*, *Carnobacteriaceae*, *Fusobacteriaceae*, and *Peptostreptococcaceae*. This compositional differential between the two studies may be a function of sampling distinct compartments within the lower airways (i.e., induced sputum versus bronchoalveolar lavage [BAL]).

The airway microbiome composition of asthmatic patients is highly correlated with the degree of bronchial hyper-responsiveness (a characteristic feature of allergic airways), thus raising the possibility that specific bacterial members within the asthmatic airway microbiome may potentiate airway allergic responses. Additional evidence of a relationship between airway microbiome composition and disease characteristics comes from a study of corticosteroid-responsive (CS) or -resistant (CR) patients. Goleva and colleagues (2013) demonstrated the presence of distinct lung microbiomes in CS and CR patients. CR patients were enriched in *Proteobacteria*, including *Neisseria* and *Haemophilus*, which possess lipopolysaccharide (LPS) with short acyl chains known to have high endotoxic activity and an enhanced capacity to trigger Toll-like receptor 4, leading to pro-inflammatory IL-8 production. Conversely, CS patient microbiomes were predominantly populated with *Bradyrhizobium* and *Fusobacterium* members, possessing longer acyl chain LPS purported to confer relatively lower endotoxicity with reduced capacity to induce innate immune host responses. How these distinct microbiome features relate to steroid responsiveness remains unclear, though it is tempting to hypothesize that discrete functional features of these compositionally distinct airway communities, such as steroid degradation capacity, account for this dichotomy in steroid responsiveness.

Emerging evidence also suggests that the bacterial microbiota that colonizes the respiratory mucosa may also impact host response to viral infection. Severe respiratory viral infection with respiratory syncytial virus (RSV) and rhinovirus (RV) is

associated with increased risk of developing asthma, and as such, these viruses are considered asthmagenic. Mice nasally exposed to two distinct strains of the commensal bacterial species *Lactobacillus rhamnosus* (LR05 and LR06) were protected against subsequent RSV infection. Protection was associated with increased levels of IFN- β , IFN- γ , IL-6, and TNF- α in both BAL and serum samples, which contributed to viral clearance. In parallel, animals exhibited elevated levels of the anti-inflammatory cytokine IL-10, which reduced inflammation-associated lung tissue damage (Tomosada et al., 2013). In this study the authors demonstrated that the specific strain of *L. rhamnosus* mattered; strain Lr05 was found to be more effective in increasing IFN- γ and IL-10 compared with strain Lr06, but Lr06 was more efficient in increasing IFN- β , TNF- α , and IL-6, thus emphasizing the capacity of distinct strains of the same species to elicit discrete host responses.

Other studies have demonstrated that the presence of specific pathogenic species in the airway potentiates RV infection in children (Kloepfer et al., 2014). Risk of asthma exacerbation significantly increased when RV was found concurrently with airway *Moraxella catarrhalis*, *Streptococcus pneumoniae*, or *Haemophilus influenzae* compared with children with neither bacterial nor viral pathogens. In addition, the odds of experiencing illness symptoms for more than 2 days were significantly higher when *Moraxella catarrhalis* or *Streptococcus pneumoniae* was detected concurrently with RV when compared to children who did not possess these bacterial or viral pathogens. Detection of airway pathogens did not predict RV presence, but discovery of RV increased the chances of detecting these bacterial pathogens; RV infection was associated with increased burden of each of three pathogenic bacteria examined, suggesting RV may change the airway environment in a manner that permits outgrowth of these respiratory pathogens, which in turn potentiate the effect of this respiratory viral infection. A previous independent study demonstrated that the same three bacterial species and viruses were associated with wheezy episodes in children (Bisgaard et al., 2010). However, this study included eight additional viruses, which may have masked the interactive effect of RV and bacteria.

Development of the nasopharyngeal microbiome in the first year of life has been linked to the risk of asthma development (Teo et al., 2015). In a longitudinal study of postnasal aspirates of 234 children in Australia, six distinct bacterial microbiota types were identified. Each was characterized by the presence of a particular dominant genus: *Moraxella*, *Streptococcus*, *Corynebacterium*, *Staphylococcus*, *Haemophilus*, or *Alloicoccus*. Those genera that have previously been associated with airway disease, e.g., *Moraxella*, *Streptococcus*, and *Haemophilus*, were also linked in this study to increased risk of asthma development. Airway communities dominated by these three specific genera during an airway respiratory infection in infants who were later diagnosed with atopy (at age 2) was associated with increased risk of lower airway infection and febrile symptoms. Airway microbiota dominated by *Streptococcus* were significantly associated with both lower respiratory infection at a younger age and chronic wheeze. Also, the age of bacterial colonization appears important, since the risk of developing wheeze at age 5 was highest when a high abundance of *Streptococcus* was found at age 7 weeks compared to 8 or 9 weeks of age. Moreover, the study

demonstrated that the composition of the nasopharyngeal microbiome of these children is related to early-life exposures such as day care and antibiotic administration, indicating that airway mucosal colonization, like the gastrointestinal tract (see below), is influenced by local exposures during the critical period of microbiome development in neonatal life and early infancy. This large study of several hundred children demonstrates relationships between patterns of early-life bacterial colonization and respiratory infection, recurrent wheeze and asthma, strengthening the hypothesis that microbial mucosal colonization plays a critical role in airway disease susceptibility. A more comprehensive review of the microbiome and respiratory infection is covered by Vissers and colleagues (2014).

While the lower airways do not appear to be overtly colonized in healthy humans, the oropharynx supports a thriving and diverse microbial ecosystem (Huttenhower et al., 2012; Lemon et al., 2010). Early-life perturbations to this community have been linked to childhood asthma development. Neonates whose oropharynx were colonized by a high burden of *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Moraxella catarrhalis* within the first month of life had an increased risk for recurrent wheeze and asthma development, significantly higher counts of eosinophils, and elevated levels of total serum IgE (Bisgaard et al., 2007). However, infants colonized by these same species at 12 months of age did not incur the same risk for disease development, suggesting that very-early-life microbial colonization patterns may strongly influence immune development in a manner that is skewed toward allergic response, and that the activities of these species is contextual, and perhaps dependent on age-dependent changes in the local microbiome in this niche (Figure 2). Support for this hypothesis comes from early-life airway studies in mice. Recently, the lower respiratory microbiome was also shown in a murine model to play a role in the allergic immune response development against house dust mites (HDM; Gollwitzer et al., 2014). Programmed death ligand 1 (PD-L1) is necessary for the development of T regulatory cells, which lack expression of the Helio transcription factor (Helio⁻ Tregs). These cells, which reside in the lungs, are inversely related to aeroallergen response to HDM. Within the first 2 weeks of life, PD-L1 expression peaked in the murine lungs; this occurred in parallel with changes in bacterial burden and composition. However, this temporal gene expression pattern is absent in germ-free animals, suggesting that the presence of microbes is necessary for PD-L1 expression and expansion of Helio⁻ Tregs. Collectively, these data point to an immune maturation process in the respiratory mucosa that parallels that which occurs in the gut, both of which are intimately dependent on (appropriate) microbial colonization.

Early-Life Gut Microbiome and Childhood Allergic Asthma

While the airway microbiome has naturally formed the focus of asthma studies, a growing body of epidemiological and microbiological literature supports the hypothesis that the genesis of allergic disease, and by extension asthma development, may lie at least in part in the communities of microbes that exist in the gastrointestinal tract. Support for this phenomenon comes from factors that have been identified as related to allergic disease development in childhood. These include early-life

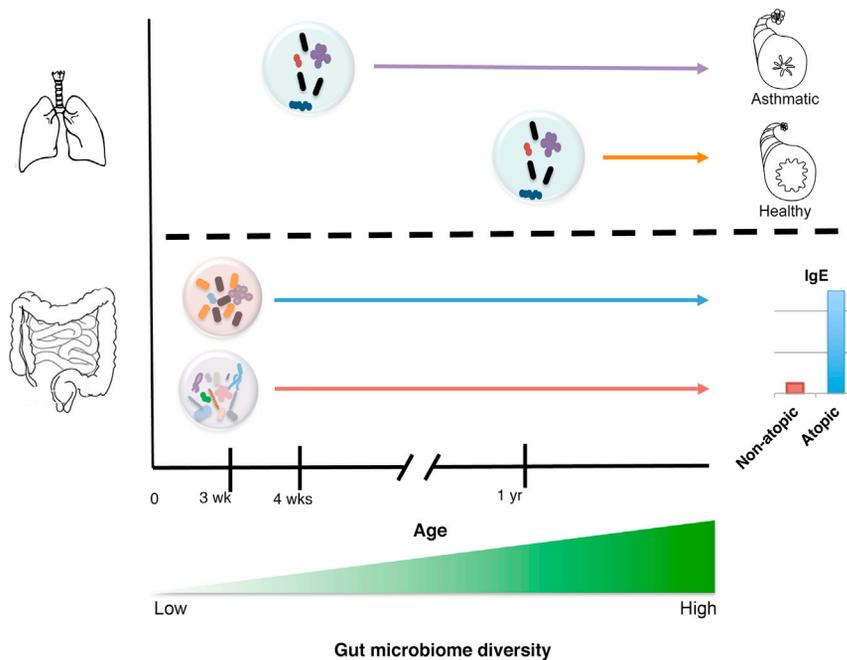


Figure 2. Very-Early-Life Exposures Influence Asthma and Allergy Development

Oropharyngeal colonization of neonates by *Streptococcus*, *Haemophilus*, or *Moraxella* species at 4 weeks of age is associated with development of recurrent wheeze. Infants colonized at 12 months with the same species were not at higher risk. Dysbiotic neonatal (3-week-old) gut microbiomes enriched with *Escherichia coli* or *Clostridium difficile* are at significantly higher risk for childhood atopy development, which is typically characterized by elevated serum IgE levels.

nguez-Bello et al., 2010). In comparison, Caesarian-born infants (who are at significantly higher risk of allergic disease development [Renz-Polster et al., 2005]) typically begin life with a microbiome enriched with *Staphylococcus* and *Streptococcus* spp. and a microbiome most similar to that of the maternal skin microbiome (Dominguez-Bello et al., 2010). Ecologically, development of the gut microbiome follows the central tenets of primary succession, a well-established

series of events that occur during the initial colonization of a previously pristine environment. Pioneer species, i.e., those that initially colonize and ecosystem, frequently determine ecosystem conditions and dictate the types of organisms that co-colonize the niche. This raises the possibility that early aberrant microbial colonization in the gut, as described by Penders et al. (2006) and Kalliomäki and colleagues (2001) (or indeed, the airway), may lead to dysbiotic microbiomes lacking commensal species necessary for appropriate physiological and immune development and subsequent maintenance of immune homeostasis. Hence, early-life microbiome development may prove a critical factor in dictating both immune maturation and childhood allergic disease development.

antimicrobial exposure (Johnson et al., 2005), Caesarian birth (Renz-Polster et al., 2005), formula feeding (Friedman and Zeiger, 2005; Harmsen et al., 2000), lack of maternal exposure to pets or livestock during pregnancy (Ownby et al., 2002), and maternal consumption of antimicrobials during pregnancy (Stensballe et al., 2013). From a microbiological standpoint, these risk factors have the capacity to influence microbiome composition; this has been clearly demonstrated for antimicrobial administration, which acutely depletes gut microbiome diversity (Dethlefsen and Relman, 2011), while formula feeding has been shown to deplete commensal *Bifidobacteria* populations in the infant gut (Balmer and Wharton, 1989; Bullen et al., 1976; Yoshioka et al., 1983). That the identified risk factors are focused around the pre- and early post-natal period suggests that development of childhood allergic asthma may owe its genesis, at least in part, to microbiome perturbations in early life during the critical period of microbiological and immunological development. In support of this hypothesis, independent studies have demonstrated that 3-week-old neonates who possessed significantly higher fecal burden of *Clostridium difficile* and a higher *C. difficile* to *Bifidobacteria* ratio had a significantly higher rate of atopy development (Kalliomäki et al., 2001). In a separate study, infants with a high abundance of fecal *Escherichia coli* developed IgE-associated eczema (Penders et al., 2006).

Support for this hypothesis comes from studies of mice colonized in early life by a cocktail of 46 spore-forming *Clostridium* clade IV and XIV species (Atarashi et al., 2011) or, in an independent study, by a diversity of bacteria (Cahenzli et al., 2013). In both studies, early-life bacterial colonization resulted in significantly decreased circulating IgE concentrations (elevated serum IgE is a hallmark of atopy) in adulthood. However, supplementation of adult animals with these same bacterial cocktails did not lead to protection. CD4⁺ Foxp3-expressing Treg cells represent an important subset of T helper cells with respect to allergic asthma, because of their capacity to mitigate pro-inflammatory adaptive immune response via the production of IL-10 (Barnes and Powrie, 2009; Rubtsov et al., 2008). In addition to demonstrating reduced IgE responses, Atarashi and colleagues (2011) demonstrated, using an IL-10 reporter mouse, that supplementation of mice with *Clostridia* IV and XIV members promoted Treg proliferation in the colon and decreased IL-4 concentrations in the airways in response to ovalbumin (OVA) challenge, indicating that appropriate gastrointestinal microbial colonization in early life leads to induction of immune tolerance (Figure 3).

To understand how these early-life risk factors may be related to disease development, it is first necessary to consider microbiome development in early infancy. Recent studies have described a placental (Aagaard et al., 2014) and cord blood (DiGiulio et al., 2008)-associated microbiome, raising the prospect of microbial exposure in utero. However, the birthing process appears to represent a strong microbiological influence on the neonate. At birth, vaginally delivered infants are colonized at various sites (skin, mouth, gut) by a microbiome enriched for *Snethia* and *Lactobacillus* spp., which is most similar in composition to that found in their mother's vaginal tract (Domi-

In an independent study, Geuking et al. (2011) also showed that colonization of germ-free mice with Altered Schaedler Flora (ASF),

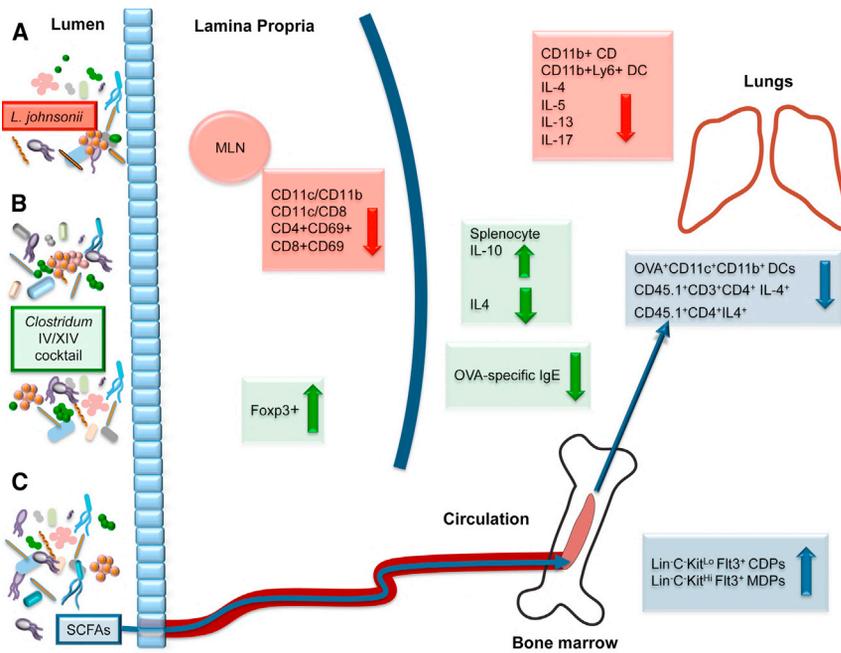


Figure 3. Adaptive Immune Modulation in the Gut and Airways through Gastrointestinal Microbiome Manipulation

(A) *Lactobacillus johnsonii* supplementation leads to decreased numbers of activated dendritic cells (DC) in the mesenteric lymph nodes (MLN) and a parallel decrease in Th2 cytokine expression (IL-4, IL-5, IL-13, IL-17) and DCs in the lungs.

(B) Mice supplemented by a cocktail of *Clostridium* IV and XIV species exhibit an increase in colonic T regulatory cells and the anti-inflammatory cytokine IL-10 and were protected against ovalbumin-induced allergic diarrhea in part due to decreased levels of serum OVA-specific IgE and IL-4.

(C) Feeding propionate to mice resulted in circulation of short-chain fatty acids (SCFAs), associated with increased common and macrophage DC precursor cells (Lin⁻ C Kit^{lo} FcγR1⁺ CDPs and Lin⁻ C Kit^{hi} FcγR1⁺ MDPs, respectively), which is associated with reduced DC proliferation and Th2 reactivation in the lungs.

a defined mixture of eight bacterial species (two *Clostridium* species, ASF356, ASF502; *Lactobacillus* sp., ASF360; *L. murinus*, ASF361; *Mucispirillum schaedleri*, ASF457; *Eubacterium plexicaudatum*, ASF492; *Firmicutes* sp., ASF500; and *Parabacteroides* sp., ASF519; Wannemuehler et al., 2014) induced de novo Treg proliferation in the colonic lamina propria (LP). Tregs produced in the periphery (not in the thymus) are known as induced Tregs (iTreg) and are principally stimulated in the mesenteric lymph nodes (MLN), Peyer's patches, and LP of the small and large intestines. Mice deficient in iTregs spontaneously develop Th2-type pathologies characterized by higher percentage of CD4⁺ T cells producing IL-4, IL-13, and IL-5 cytokines in the MLN, IL-4 in the LP of the large intestine, and IL-13 and IL-5 in the LP of the small intestine (Josefowicz et al., 2012). iTreg-deficient animals possess an altered gut microbiome, enriched with members of the TM7 phylum, a relatively recently described candidate division consisting of uncultivated bacteria, as well as *Alistipes* species, and also exhibit macrophage and neutrophil infiltration in the airways coupled with increased goblet cell numbers, mucin hyper-secretion, smooth muscle hyperplasia, fibrosis, and poorer lung function (Josefowicz et al., 2012). That the presence of specific bacterial stimuli have been shown to be critical to the proliferation of this T helper subsets in mice suggests that failure to populate the developing gastrointestinal microbiome with such species represents at least one pathway by which allergic disease may develop.

Invariant natural killer T (iNKT) cells represent another important subset of IL-4- and IL-13-producing (Akbari et al., 2003) lymphocytes that accumulate in the colonic LP and lungs of germ-free mice. iNKT cells accumulate soon after birth in germ-free animals, resulting in persistence of the associated allergic pathology into adulthood. Significantly increased allergic response in response to airway OVA challenge in germ-free mice is only abrogated through maternal colonization (and thus neonatal exposure) when animals are co-housed with specific

pathogen-free animals (Olszak et al., 2012). Neonatal protection was due, in part, to the capacity of commensal organisms to block methylation of chemokine CXCL16 in the colon and lungs, which is

crucial for iNKT recruitment (Olszak et al., 2012). Colonization of adult germ-free mice with commensal bacteria did not abrogate their disease phenotype, indicating that early-life microbial colonization influences epigenetic modification of host DNA in a manner that produces life-long effects on the host immune response. These studies highlight the importance of appropriate early-life microbiome development in Th2 and allergy-associated phenotypes and suggest that interventions aimed at correcting dysbiotic microbiomes or promoting appropriate colonization in the neonatal phase of life may prove highly effective in preventing childhood allergic disease.

Environmental Influences on the Developing Microbiome

Due to the rapid increase in prevalence over the past several decades, primarily in westernized nations, allergic asthma is considered a disease that is strongly related to environmental exposures. Emerging evidence suggests that environmental factors play an influential role in shaping human-associated microbial communities and immune responses. A recent study of immune cell population frequencies, cytokine responses, and serum proteins of 210 healthy twins indicated that 77% of these immunological parameters were dominated (> 50% of variance) and 58% almost completely determined (> 80% of variance) by non-heritable influences (Brodin et al., 2015). The study also demonstrated that immune responses diverged across twin pairs over time (Brodin et al., 2015), supporting the concept of a primarily reactive and adaptive immune response in healthy humans, presumably in response to local environmental exposures.

Although previous studies have reported that family members typically possess gut microbiomes that are more similar to each other than to others (Lee et al., 2011; Turnbaugh et al., 2009; Yatsunen et al., 2012), their similarity was attributed to environmental factors, since the bacterial compositions of monozygotic twins were no more alike than dizygotic twins (Turnbaugh et al.,

2009; Yatsunenکو et al., 2012). Diet has been shown to play a significant role in shaping gut microbiome composition. A recent murine crossover study highlighted the strong selective pressure diet exerts on the microbiome. Successively transferring genetically diverse adult mice from high-fat/low fiber to a low-fat/high fiber diet three times during the course of the study led to similar shifts in microbiome composition despite the genetic diversity of the animal population studied (Carmody et al., 2015). The strong influence of diet on the gut microbiome is also supported by a study of individuals from Malawi, Venezuela, and the United States, which demonstrated that the gut microbiome of adult human subjects in the developing nations was significantly distinct from that of residents in the United States. While geography and ethnicity represent potential contributory factors, diet was considered a primary driver of these compositional and functional differences; developing nation diets were primarily plant polysaccharide based, while those of U.S. participants was enriched in animal products. Functionally, Malawians and Amerindians were enriched for genes encoding glutamate synthase, associated with herbivores, while gut microbiomes of U.S. participants were enriched for the capacity to degrade glutamine, a function commonly associated with carnivores. Undoubtedly diet represents a strong selective pressure on the gut microbiome, a factor that likely explains the increased risk of allergy associated with formula feeding. Indeed, studies dating back to 1936 have reported lower incidences of eczema (Grulee and Sanford, 1936) and asthma (Friedman and Zeiger, 2005) in breastfed infants. The lasting effects of breastfeeding have been reported for children 8 years old, and in an independent study, infants breastfed for at least 4 months had reduced risk of developing asthma (Kull et al., 2010).

More recently, early-life microbial exposure in the built environment has been linked to allergic asthma development. In western nations where the burden of allergic asthma is greatest, exposure to microbes has decreased substantially, most strikingly in the post-industrial revolution era (Rook, 2010). Proposed in the late 1980s, the hygiene hypothesis encapsulates this altered human exposure to microbes and posits that a lack of exposure to commensal microbes represents a driving force in the increased prevalence of allergic asthma and other chronic inflammatory disease in westernized nations (Strachan, 1989). Inhabitants of westernized nations spend almost 90% of their time in the built environment, with approximately 70% of time spent in their personal residences (Klepeis et al., 2001). This represents a significant departure from the environment in which the human microbiome evolved and has led to the hypothesis that residential microbial exposures in early life influence microbiome development, immune maturation, and consequently the propensity to develop allergic asthma. In the United States, children raised in inner-city environments are more likely to develop asthma and atopy (Crain et al., 1994; Weiss et al., 1992). This observation was seen to contradict the hygiene hypothesis since the inner city had always been assumed to represent an environment of enhanced microbial exposure (Kay, 2001). However, until recently, no study had comprehensively examined the composition of bacterial communities in inner-city households and whether differences in early-life bacterial exposure related to allergic asthma development in childhood. Using culture-independent profiling, Lynch and colleagues (2014) examined the

bacterial composition of house dust samples collected during the first year of life from residences in a study of full-term infants born in low-income, inner-city urban neighborhoods in New York, Baltimore, St. Louis, and Boston. Children were followed prospectively, and clinical outcomes of atopy and recurrent wheeze were assessed at 3 years of age. The investigators demonstrated that healthy children in the study (who developed neither atopy nor recurrent wheeze by age 3) were exposed in the first year of life to richer (increased number of bacterial types) and more diverse bacterial communities that were of significantly distinct composition compared to those in households where the children developed either atopy or both recurrent wheeze and atopy. Consistent with the hygiene hypothesis, healthy inner-city children were exposed to a greater diversity of bacterial species in early life, characterized by significant enrichment of approximately 80 bacterial taxa belonging to 16 distinct families. Among those enriched were members of the *Bifidobacteriaceae*, which are critical commensal colonizers of the human gastrointestinal tract, whose loss in early life is associated with allergy development (Penders et al., 2007).

Further evidence for the role of local household microbial exposures in early life in protecting against allergic disease development comes from an independent study of microbial exposures associated with pet ownership. Exposure to dogs and, to a lesser extent, cats in infancy protects against allergic disease development in childhood (Ownby et al., 2002). To determine whether a microbial correlate of protection existed in these households, Fujimura et al. (2010) examined both bacterial and fungal communities in house dust collected during the course of a prospective birth cohort study centered around the Detroit metropolitan area. Bacterial communities in household dust from residences with dogs or cats present were significantly richer and more diverse compared to those with no pets present and, as in the inner-city studies, a large majority of bacterial taxa enriched in the protective environments have previously been detected in the human gut microbiome (Fujimura et al., 2010). These findings are consistent with previous lower-resolution studies in which environments with no pets present are depleted of bacteria (Gereda et al., 2000; Hesselmar et al., 1999; Ownby et al., 2002). Moreover, the investigators noted that bacteria-impooverished residences that conferred a higher risk of allergic disease development possessed a wider range of fungal species (Fujimura et al., 2010). Since fungal species have long been associated with allergic disease development (Bush and Portnoy, 2001; Gravesen, 1979; Kurup et al., 2000), this study suggests that increased local environmental exposure to fungal species in early life may play a role in shaping the neonatal microbiome and subsequent allergic disease development.

High-resolution microbiome profiling of the residential environment in early life has demonstrated a significant relationship between early-life bacterial and fungal exposure and allergic asthma development in childhood. It has been hypothesized that one mechanism by which these exposures protect against or contribute to disease development is by influencing the composition of the gut microbiome, which in turn influences allergic immune responses. In an attempt to address this hypothesis, Fujimura and colleagues (2014) used a murine model of airway allergen challenge. In the study, groups of mice were supplemented either with house dust from residences with two dogs

or no pets present, prior to and during airway challenge with cockroach allergen. Only animals exposed to dust from homes with dogs present exhibited significantly reduced airway expression of Th2 cytokines, IL-4 and IL-13, and the mucin-associated gob-5 gene. Lung histology supported these findings; protected animals exhibited pristine airways with no evidence of inflammatory infiltrate or mucin hyper-secretion. Comparative analysis of the gut microbiome of protected and unprotected animals revealed significant differences in their taxonomic content. Protected animals possessed a gut microbiome enriched in members of the *Firmicutes*, including taxa belonging to the *Lachnospiraceae*, *Bacillaceae*, *Peptococcaceae*, and *Lactobacillaceae*. Using selective media, the authors isolated and confirmed that the dominant *Lactobacillus* in protected animals was *Lactobacillus johnsonii*, a species that coincidentally becomes highly enriched in the human vaginal tract immediately prior to birth (Aagaard et al., 2012) and is thus transferred to the vaginally born neonate. This suggested that this species may play a crucial role in shaping gut microbiome composition. Using this single species, the authors performed subsequent supplementation experiments using distinct airway allergen challenges (cockroach and OVA) and demonstrated that animals who received *L. johnsonii* were protected against allergen challenge. Again protection was associated with significant downregulation of airway Th2 responses (IL-4, IL-5, and IL-13), both at the mRNA and protein level. The study also demonstrated that the protective effect of *L. johnsonii* supplementation extended to viral infectious agents, a critical finding since children hospitalized due to respiratory infection with respiratory syncytial virus (RSV) have a significantly increased risk of developing allergic asthma (Darville and Yamauchi, 1998; Stein et al., 1999). Using this viral pathogen, the investigators demonstrated that oral supplementation with viable *L. johnsonii* protected against RSV infection (heat-killed organisms did not confer protection; Fujimura et al., 2014). Protection was again associated with a significant reduction in both bronchial hyper-activity and the expression of Th2 and Th17 cytokines (IL-4, IL-5, IL-13, and IL-17) in the airways. However, these animals did not demonstrate significant differences in airway expression of IFN- γ compared to control animals, suggesting that they maintain the capacity to effectively clear the virus, but significantly downregulated the adaptive immune responses that are largely responsible for RSV-associated pathology. Protected animals exhibited a significantly distinct gut microbial community (from that of susceptible animals) and significantly reduced activation of dendritic cells (DCs) in the mesenteric lymph nodes, indicating that compositional (and presumably parallel functional) changes in the gut microbiome influence the capacity of antigen-presenting cells to activate local T cell populations, a mechanism by which allergic responses may be inhibited (Figure 3).

More mechanistic evidence for how the gut microbiome may prevent allergic response comes from studies demonstrating the ability of the murine gut microbiota to regulate colonic T cell populations via production of short-chain fatty acids (SCFAs) such as butyrate (Furusawa et al., 2013), acetate, and propionate (Smith et al., 2013). SCFAs are anti-inflammatory and have been shown to increase significantly with induction of colonic CD103⁺ Foxp3⁺ cells and IL-10 production (Furusawa et al., 2013; Smith et al., 2013). SCFAs, which are also an impor-

tant energy source of gastrointestinal colonocytes, specifically bind the G protein-coupled receptor 43 (GPR43; also known as free fatty acid receptor 2 [FFAR2]), which also mediates their effect on colonic Tregs (Smith et al., 2013). Indeed, *Gpr*^{-/-} mice exhibit lower IL-10 expression compared to wild-type controls (Smith et al., 2013) and develop more severe inflammation when their airways are challenged with OVA (Maslowski et al., 2009). Microbial-derived SCFAs are produced through the fermentation of complex carbohydrates. The importance of these microbial fermentation products in allergic disease was recently illustrated by Trompette et al. (2014). Mice fed a low-fiber diet followed by nasal exposure to house dust mite (HDM) extract exhibited significantly increased IL-4, IL-5, IL-13, and IL-17A in lung tissue as well as increased airway mucus production and goblet cell hyperplasia. In parallel, these animals possessed higher levels of circulating IgE and significantly higher amounts of total HDM-specific IgG1. In contrast, mice fed a high-fiber diet prior to HDM exposure were protected, exhibiting significantly lower cytokine concentrations and a normal mucin phenotype. Fiber intake impacted the gut microbiome of animals; a low-fiber diet promoted the abundance of specific members of *Firmicutes*, especially those belonging to the *Erysipelotrichaceae* family, while animals on a high-fiber diet were enriched for *Bacteroidaceae* and *Bifidobacteriaceae*. Supplementation of animals with propionate increased Foxp3⁺ CD25⁺ CD4⁺ Treg cell numbers and enhanced hematopoiesis of DC precursors. This suggests that SCFAs not only induce iTreg populations, but also modulate bone marrow-derived antigen-presenting cell precursors, indicating a mechanism by which gut microbial-derived metabolites may reprogram the immunological tone of the human ecosystem. Collectively, these studies provide evidence that gut microbiome composition and activities influence not only local immune activity, but also that at remote mucosal sites (Figure 3), suggesting that the microbial communities resident in the gut may represent a key target for prevention or management of allergic asthma.

While gastrointestinal microbiome manipulation of mice clearly influences airway immune responses, direct microbial stimulation of the respiratory mucosa has also been shown to be protective against Th2-associated allergic responses. Nembrini and colleagues (2011) demonstrated that inhalation of non-pathogenic *Escherichia coli* was protective against allergic inflammation (Nembrini et al., 2011). Compared to untreated animals, mice treated nasally with *E. coli* prior to OVA allergen challenge exhibited fewer eosinophils in their lower airways, a parallel abrogation of IL-4 and IL-5 production, fewer goblet cells, and pristine lungs as evidenced by histology. These animals also exhibited airway induction of $\gamma\delta$ T cells, which protected mice from airway hyper-responsiveness, but did not appear to impact cell recruitment. In parallel, innate immune responses were also impacted. Protected animals exhibited induction of Toll-like receptor 4 (TLR4), which resulted in a suppressive lung environment, altered DC function, and reduced Th2 effector function in the airways. Other intranasal instillation studies using a bacterium isolated from cowsheds, *Acinetobacter lwoffii*, also conferred protection against Th2 allergic airway responses. In this case, protection was associated induction of the Th1 cytokine IL-12 and a concomitant attenuation of Th2 responses following allergen challenge (Debarry et al.,

2007). In a separate study, Conrad and colleagues (2009) demonstrated that the capacity of *A. lwoffii* to protect against allergic response could be passed from mother to offspring. Pregnant mice who received *A. lwoffii* intranasally during gestation exhibited significantly increased circulating IL-6 in response to this microbial exposure (Conrad et al., 2009). Placental concentrations of this cytokine were reciprocally related to the concentrations found in the maternal circulation and offspring were less susceptible to airway allergen challenge compared to those whose mother was not supplemented with *A. lwoffii*. In this case, protection appeared to be mediated via innate immune responses, since the offspring of *TLR2/3/4/7/9*^{-/-} mice supplemented with this species were susceptible to airway OVA challenge. This latter study opens up the possibility that maternal microbial exposures during pregnancy may influence the susceptibility to allergic disease development, an area that clearly merits further intensive study.

In summary, asthma and atopy are complex diseases, which affect and are affected by the communities of microbes that colonize the gut and respiratory tracts; these communities in turn are influenced by an array of environmental influences from diet and antimicrobial administration to early-life local microbial exposures. Though many of the studies to date have been performed in mice, they serve as proof of the existence of a gut-airway axis. This opens up the possibility that strategies aimed at manipulating the airway and/or gut microbiome, particularly in the earliest stages of life when the immune response is maturing and the microbiome appears most plastic and receptive to exogenous microbes, may serve as a novel strategy for prevention of allergic asthma, as well as a host of other diseases in which adaptive immune dysfunction is a prominent feature.

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