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Chapter 7: Lung Microbiota and its Impact on the Mucosal Immune Phenotype

Benjamin G. Wu* and **Leopoldo N. Segal**

Department of Medicine, NYU Division of Pulmonary, Critical Care, & Sleep Medicine, New York City, NY, 10016

INTRODUCTION

In 2007, the Human Microbiome Project (HMP) was added to the National Institutes of Health (NIH) Roadmap for Medical Research and since then over \$200 million US dollars has been invested in the exploration of the human microbiome. Several sites on the human body, including the nares, oral cavity, skin, gastrointestinal tract, and urogenital tract have been studied by the HMP(1). The gastrointestinal tract remains the most thoroughly investigated organ-microbiome interaction studied thus far and its role in shaping the host immune response is rapidly becoming defined within the context of inflammatory response(2–4). Observations have noted associations of specific microbes and the gut microbiome in obesity(5–8), coronary artery disease(9–12), *Clostridium difficile* colitis(13, 14), type 2 diabetes(15, 16), and inflammatory bowel disease(4, 17, 18). While the nares and oral cavity were included as locations to be studied for the HMP, the lower airway respiratory system was not included as a location of interest. The microbial community of the oropharynx has been well described even before the advances of next-generation sequencing and multiplexed data(19, 20). Microbiome approaches to the upper and lower respiratory system created a deluge of associations between host and microbes in health and disease(21–23). Over the past few years, our understanding of the airway microbiome has shifted, upending the old adage of the lungs being a sterile field(24) to a new paradigm of a continuous organ system with a rich and vibrant mucosal surface that embodies complex interactions between the microbiome and its host that can propagate or resist disease(21, 24). Moreover, little is known regarding how the respiratory microbiome interacts with the host immune response. While multiple research projects have focused on the effects of gut microbiota on the immune response of the gastrointestinal mucosa(25–27), few papers have focused on the effects of the lower airway microbiota on the respiratory mucosa that it inhabits(23, 28–30). Some of the unique challenges studying the lower airway microbiome include the technical difficulty sampling the lower respiratory tract, the extremely low bacterial burden in healthy lung leading to low signal to high noise ratio, and the lack of animal models to study the lung microbiome(31, 32). Shifts in gastrointestinal microorganism composition in organ systems that contain high bacterial biomass, such as the gastrointestinal tract, can lead to broad observations resulting in disease states(33). However the lung microbiome is a low bacterial burden organ where transient perturbations

*Corresponding author: Benjamin G. Wu, wub02@nyumc.org.

of its microbiome can have dramatic effects on the diversity of its composition and the host inflammatory response to dysbiosis(23). Thus, the upper and lower respiratory tract microbiome is unique as it represents an organ with a significant biomass gradient, moving from a high bacterial burden reservoir in the upper respiratory tract to a very low bacterial burden area in the lung microbiome(32, 34). We will first discuss the microbiome of the upper and lower respiratory tract, then the possible mechanisms for microbial migration and colonization, followed by the recent finding of the host-immune response to the microbiome, the inflammatory pathways that the microbiome may impact, and finally address possible microbial targets or markers for specific diseases.

The airway microbiome can only be understood as the sum of its individual parts. In the upper airways, the nasal and oral cavities contain a very distinct microbiome. In the nasal cavity, the microbiota is characterized by enrichment with *Streptococcus*, *Acinetobacter*, *Lactococcus*, *Staphylococcus*, and *Corynebacterium*. In the oral cavity, the microbiota is characterized by enrichment with *Prevotella*, *Streptococcus*, *Fusobacterium*, *Neisseria*, *Leptotrichia*, and *Veillonella*(35). Further defining the qualities of each location, the upper airways are characterized by a constant exposure to airborne microbes and microbes ingested as part of each individual's diet. Although the respiratory system and its mucosa is a continuum, the lower airway microbiota has very distinctive features that set it apart from the upper respiratory tract. The boundary between what and where we define as upper versus lower airways is classically defined by anatomical structures with the vocal cords being analogous to a natural 'dam' against aspiration(36, 37). Similar to the water held by a dam, the upper airways hold a high bacterial burden while lower airway microbiota has about 100 to 10,000 times less bacterial burden based on 16S rRNA copies(32, 38). Microaspiration occurs in normal individuals(39) and its prevalence is higher in several lung diseases including Chronic Obstructive Pulmonary Disease (COPD), asthma, obstructive sleep apnea, cystic fibrosis, and lung infections due to atypical (such as non-tuberculous mycobacteria) and typical microorganisms(40–45).

Charlson and colleagues, who in initial studies, examined bronchoscopy results from six healthy patients and concluded that the lower respiratory tract resembled the microbiome of the upper respiratory tract (32). Further microbiome work from cell-free bronchoalveolar lavage (BAL) have noted that bacteria characteristic of the oral cavity is found in about 45% of relatively healthy subjects, the remaining subjects had a lung microbiome that resembled the background taxa(23, 30). Other studies, have found bacteria characteristic of the oral cavity at higher prevalence, although it is unclear if this is related to sample type utilized (acellular BAL vs. whole BAL vs. airway brush) or differences in cohorts(32, 46–48). Importantly, a dichotomized view of the lower airway microbiota (enriched with upper airway microbes vs. not) is likely an oversimplification of a more likely physiological continuum of similarity between the lower and upper airway microbiota. Of note, older studies using radiotracer have found that a similar proportion of healthy individuals microaspirate (39, 49). Using culture independent techniques, various studies have shown that bacteria commonly found in the upper airways are also frequently found in multiple other disease states such as: asthma(29, 50–58), cystic fibrosis(59–62), Human Immunodeficiency Virus (HIV) infection(47, 63, 64), and advanced COPD(21, 23, 28, 65, 66). Considering the continuity of the airway mucosa, it is no surprise that the upper airway

microbiota with its high bacterial burden is the major source of microbes to the lower airways. However, even when the lower airways are enriched with microbes found in the oral cavity, differences can still be noted between the upper and lower airway microbiota(30, 32). This suggests that there are other important factors that affect the dynamic homeostasis of the lower airway. Several questions remained unanswered: 1) what other microbiota from different mucosa contribute to the “cross pollination” of microbes to the lower airways, 2) what selection pressures do microbes suffer as they enter the lower airways, and 3) how does this selection pressure change in different compartments of the lower airways.

Unique Challenges with Evaluating the Lung Microbiome

One particular challenge that research in the lung microbiome has yet to resolve is the microbiome signal to background noise ratio during health and early disease (Figure 1) (31). While this might also be important in advanced disease states, the signal to noise ratio is highly unfavorable in early stages of disease where there is low microbial biomass in lower airway samples. Functionally, the lungs have 100 to 10,000 times lower bacterial burden than the upper airways(23, 38). Thus, the low-bacterial burden nature of the pulmonary system pose unique challenges: signal to noise ratios that are characterized by high background noise signals(31), lack of standardized methods for background removal, and limited ability to detect signal from low abundance taxa. These challenges confound and make the lung microbiome difficult to study. One consistent finding in multiple studies is that background environmental samples, such as sterile saline and pre-bronchoscopic wash through the bronchoscope (prior to the procedure), contain microbial DNA that is characterized as a diverse environmental background microbiota(30, 31). This is understandable and predictable, since instruments are “clean” but not necessarily DNA free. Furthermore, reagents utilized for DNA isolation and library preparation may also contribute to background DNA(67). A large percentage of healthy subjects have a lower airway microbiota enriched with microbial DNA that is characteristic of background samples(21, 23). Thus, background microbiome may confound a low biomass host microbiome in approximately 60% of healthy subjects that will have a microbiome enriched with background predominant taxa (also referred as pneumotype_{BPT})(23, 30). Even in these subjects, bioinformatic approaches that identify contribution of background taxa (e.g. SourceTracker) to a sample showed that close to half of the composition of a BAL sample was not present in background samples (e.g. bronchoscope)(30). This data suggests that there is still a significant amount of information to be gained from these low biomass samples. Consider for comparison the microbiome of the gastrointestinal tract. This high bacterial burden and diverse microbial community is subjected to multiple microbial challenges (e.g. food intake, medications, etc.). In this setting, the background microbiota is rarely seen as an important source of contamination. However, the background microbiota may influence the recovery of rare or less abundant microorganisms or in the setting of gross contamination, may play a role in obscuring the bacteria composition. Microbial changes due to different microbial challenges or active disease processes are more likely to be detected in the gastrointestinal tract (Figure 1A and 1C). Conversely, the low bacterial burden found in the respiratory tract makes the microbial assessment using culture independent techniques commonly influenced by the background microbiome. Thus, when microbial challenges that disturb the underlying lung microbiome occur, they may not be

apparent due to the low signal that needs to overcome a high background noise (Figure 1B and 1D). This model also suggests that the signal can be transient and subjected to the dynamics that dictate the microbial host interaction in the lower airways. The low microbial load of the respiratory system may lead to large shifts in microbiome when faced with upper airway tract (URT) microbial challenges. The microbial challenges caused by microaspiration may then be seen as the enrichment of the lower airway microbiome with upper airway microbes (also referred as pneumotype_{SPT} for supraglottic predominant taxa) (23, 30). The lower microbial biomass and low signal noise ratio represents a distinct challenge when studying the lung microbiome. It is therefore important to include the study of the background microbiome in microbiome research involving low biomass samples.

Questions remain regarding what to do with background taxa when studying the lower airway microbiome. Curation of the sequencing data commonly includes removal of technical contaminating taxa that can be done as part of the upstream processing of the data. Neutral modeling is a technique that can be utilized to identify taxa preferentially enriched in on sample type(68). Taxa found that deviate from this model are the most likely to be true inhabitants of the area that was sampled. For example, this technique can then be used to remove taxa found in the bronchoscope to identify microbes that are resident in the lung. SourceTracker is another technique utilized to estimate the degree of microbial contribution that sources of background microbiota (e.g. bronchoscope) may have on a biological sample(69). While these methods have been utilized in lung microbiome research, there is a lack of consensus on how to standardize the analysis. One concern is that in samples with a predominance of background taxa (e.g. pneumotype_{BPT}) removal of background taxa may yield low reads and given the relative abundance nature of the microbiome data, will inflate the relative proportion of the taxa that was not removed. Also, taxa removal approaches have the potential of excluding taxa existing in both lung and possible contamination sources (e.g. bronchoscope) that might be meaningful to comprehend the lower airway microbiota. Finally, bacteria may have a significant pathogenic role in a disease state despite its low abundance in a microbial community and removal of background taxa may in fact change our understanding of the microbe's role in the lung(70). For example, sequence analysis of experimental diarrhea induced by enterotoxigenic *Escherichia coli* have shown that even low abundance of this organisms can cause significant disease.(71) Thus, in the lung, the high abundance of background noise represents a major challenge to detect a signal that occurs in microorganisms from low abundance taxa but may be biologically important to health and disease.

Sources of Microbial Challenge to the Lower Airways

Predictably, the most straightforward explanation for the source of microbes to the lower airways is the delivery of microbes from the upper respiratory tract through microaspiration. Although it is generally accepted that aspiration of oral cavity secretions likely affects the lung microbiota, other possible sources must be considered. The contribution of the airborne microbiome present in ambient air may be a significant contributor to the lower airways. The human body ventilates approximately 7,000 to 20,000 liters of ambient air per day(24, 72) potentially exposing the lower airways to microbes present in the air (airborne microbiota) in conjunction with other well-recognized pollutants, chemicals, dust, allergens, and other

particulate matter from the atmosphere. While there is significant knowledge on pathogens that are aerosolized and can transmit infections, such as mycobacteria(73, 74), much less is known about the presence and relevance of other microorganisms present in the air. It is possible that different airborne microbiota may also significantly contribute to the lower airway microbiome, a subject that needs further investigation.

There is an increasing understanding of the complex airborne microbiome and its contributions to the lower airway microbiome. Adams and colleagues found differences between indoor and outdoor environments that were dictated by human-associated microorganisms(75). In indoor environments they found an enrichment of *Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Propionibacterium*, *Lactococcus*, and *Enterobacteriaceae*. In outdoor environments there was enrichment with *Pseudomonas*, *Acinetobacter*, and *Sphingomonas*(75). Also, Meadow and colleagues identified a distinct “microbial cloud” that supports that in an enclosed space, ambient air can be impacted by its occupants(76). However, the effects of exposure to different airborne microbiota on the lung microbiome or the effects of the exhaled air microbiota on the airborne ambient microbiome have not been studied. The nasal microbiota is distinct than the oral cavity microbiota(35) and likely represents an important microbial reservoir to the lower airways that is enriched by inhalation of ambient air.

Culture independent methods in chronic rhinosinusitis have shown enrichment with *Haemophilus influenzae* (77, 78) and other pathogens that are frequently associated with development of pneumonia. Post-nasal drip is highly associated with cough and bronchial inflammatory diseases, but its effect on the lower airway microbiota has not been elucidated. The gastrointestinal tract represents another potential source of microbial challenge to the lung microbiome. Gastroesophageal reflux disease (GERD) is frequently present in many disease states. Presence of distinct microbiome in the gut dominated by *Helicobacter pylori* has been associated with decreased risk for asthma(79, 80). The use of proton pump inhibitors affects the composition of, not only the gastric microbiota, but also the lung microbiome(81). Further, the lower gastrointestinal tract should be considered as a potential source of microbial challenge to the lung. Animal models of sepsis have shown that germ free animals are protected from lung injury(82) while selective gastrointestinal tract decontamination reduces the development of multiple organ dysfunction syndrome in 50% of the subjects studied(83).

However, the most frequently observed microorganisms in the lower airways are those characteristically present in the oral cavity. Multiple culture independent investigations have shown that different subjects have varying degrees of enrichment of the lower airway microbiota with oral microbes such as *Prevotella*, *Veillonella*, *Rothia*, *Streptococcus*, and *Porphyromonas*(23, 30, 65, 84, 85). Thus, multiple potential sources may contribute to the lower airway microbiome through different routes of microbial challenge, e.g. aspiration, regurgitation, hematogenous, and aerosolized. These microbial challenges will then be subjects of unique selection pressures present in the lower airway environment. Although there is limited understanding of what these selection pressures are at individual levels, it is likely that they have a very significant effect on the structure of the microbial communities that may reside in the lower airways.

Unique Selection Pressures of the Lower Airway Microbiome

Several different theories have been adapted from biology, ecology, and environmental studies to explain the composition of the lung microbiome. One of the most purported has been the *Adapted Island Model*(86–88). Similar to islands, the lungs are influenced by a balance between immigration of microbes from the “main land” and their extinction(86, 87). Based on this theory, the lungs can be viewed as conceptual “islands” that receive immigration challenges, most commonly from the upper respiratory tract. Thus, the diversity in the lungs is mainly driven by the diversity from the sources of microbial challenges and the selection pressure on a microorganism’s viability.

The proximal airways, including the trachea and the main stem bronchi, may be viewed as large swaths of area that due to their location in relation to the upper airway more closely resemble that of the upper respiratory tract(32). As one progresses distally in the lower respiratory tract, the conducting airways may represent isolated islands(87, 89), potentially conforming a unique microbial niche. Thus, diversity is influenced by three main factors: the rate of immigration, the rate of elimination, and the rate of reproduction of the community members(86–88, 90). In health, diversity of these islands is mainly dictated by immigration from the upper respiratory tract and elimination by various mechanisms including the mucociliary ladder, bacteriophages, and alveolar macrophages. Frequent episodes of microaspiration observed in healthy individuals are likely to be the most dominant source of microbial airway challenge. In the event of disease, different factors may influence the lung microbiome. For example, in active pneumonia, chronic airway colonization in COPD, or cystic fibrosis the reproduction rate of the organism in the lower airway will offset mechanisms of immigration and elimination.

Furthermore, the airway mucosa has several unique features: it is a hollow system of mucosal lined tubes in constant exposure to ambient air with a role to perform gas exchange; it contains high levels of phospholipids in the form of surfactant(91); and as a result of its gas exchange properties, it contains gradients of oxygen and carbon dioxide tension(92). Thus, microbes, as they enter the lower airways, have to overcome or adapt to the changing environment of the lung. Recent investigations have demonstrated that by evaluating microbial genetic variations within a host, multiple lineages can coexist providing evidence of selective pressures in the lower airways(93). Several environmental factors present in the lower airways potentially shape the lower airway microbiota. The phospholipid-rich environment is not only important to maintain surface tension and aerated alveoli, but also has significant effects on microbial metabolism(94). Multiple antimicrobial peptides such as surfactant protein A, lactoferrin, and defensins are present in the airways(94). Experimental evidence shows that secretory immunoglobulin A (SIgA) has an important role on the airway microbiota and its deficiency leads to increased inflammation in response to resident lung microbiota and progressive airway remodeling and emphysema(95). Thus, the antimicrobial properties of the pulmonary mucosal represent a significant hurdle for microbial colonization. Microbes have developed and evolved specific mechanisms in order to evade or ameliorate the effects of the host-defense. As an example, biofilm constitutes its own microbial niche where anaerobes can find an optimal anaerobic niche(96). For example, *Pseudomonas aeruginosa* advantageously uses biofilm to create its own

microenvironment(97). Biofilms, close proximity to aerobes and anaerobes, and development of oxygen-depleted microenvironments may allow for microbial colonization within anaerobic conditions(98). Thus, although the lung is a highly aerated organ, anaerobes are frequently described in the lower airway microbiome, suggesting that biofilms may have an important role on the co-occurrence of aerobes and anaerobes in the lower airways(96, 98). Microbes with different adhesion properties are also affected differently by mechanical selection pressure such as mucociliary clearance(99, 100). Different bacteria express adhesins, pilin, and flagelin in order to overcome mucociliary removal from the lower airways(101, 102). These are important virulence factors for bacterial colonization and pathogenesis and not all bacteria express these advantages. Thus, the microorganisms found in the respiratory system likely reflect a highly specialized strain or species that are in constant balance with a highly immune active mucosa(103, 104). Experimental approaches are needed to study how these factors affect the selection pressures that shape the lower airway microbiome.

The lung environment ecosystem can also be thought of as a series of forward and feedback loops that suppresses or allows the underlying lung bacteria to grow(88). A major risk factor for the development of pneumonia in the hospital setting is the use of antibiotics(105). Evidence suggests that it is likely that the pathogen responsible for the development of pneumonia exists in the lung prior to the onset of disease. In a murine model of this theory, Poroyko and colleagues conducted a study of lipopolysaccharide (LPS)-induced acute lung injury and studied the changes in the murine lung microbiome(106). These mice notably had an increase in bacterial burden without an increase in the number of species. There was also an increase in abundance of the family of Proteobacteria and decrease in Firmicutes. These findings suggest that challenge with LPS may overwhelm the feedback mechanisms responsible for equilibrium in the lungs allowing the resident microorganisms in the lung to proliferate unchecked during times of stress and inflammation. Nutrition availability may also affect the alterations in the lung microbiome(107). Issues such as iron availability and nutritional immunity may select for microorganisms that are able to adapt to the lack of or abundance of certain nutrients. Moreover, co-infection with viruses may affect the equilibrium in the lung as well. Molyneaux and colleagues demonstrated that there are intrinsic changes in the lower respiratory tract microbiome when it is challenged with rhinovirus infection. In this study, healthy subjects and those with a history of COPD were challenged with rhinovirus and followed longitudinally over a period of forty-two days(108). Using induced sputum the authors showed that rhinovirus challenge led to an increase in Proteobacteria, mainly driven by an increase in *Haemophilus influenzae*(108). This may explain why lower airway pneumonia occurs post-viral infection. Changes in host immune system will affect the selection pressure on the lower airway microbiota. An example of this selection is the enrichment with *Tropheryma whipplei*, the etiological agent of Whipple's disease, in the lungs of treatment naïve HIV subjects and subsequent decline of the relative abundance of *T. whipplei* post-antiretroviral therapy(64). Importantly, since this microbe was not found in the upper airway samples or background controls, it seems to reflect a true inhabitant of the lower airways where growth is favored or promoted by the host's immunodeficient state. Recently, more broad microbial differences were noted in advanced HIV and partially corrected with immune reconstitution(109). Twigg and colleagues found

that *Streptococcus*, *Prevotella*, and *Veillonella* taxa were found in higher relative abundance in the lungs of the HIV infected patients after 1 year of being treated with Antiretroviral Therapy (ART) (63). Moreover, there is increasing evidence of the effects of immune deficiency on non-bacterial microbes in the lung such as in the mycobiome, the collection of fungi found in a community(110). The interaction of the host's respiratory system to this environmental and microorganism challenge is an integral facet to understanding the role of the upper and respiratory microbiome plays in health and disease(21, 23, 30).

Specific deficiencies in the host immune response play a significant role on the microbiome. Richmond and colleagues explored changes in mucosal immunity by studying the impact of specific secretory IgA (SIgA) immunodeficiency(95). The researchers developed mouse models that were deficient in the polymeric immunoglobulin receptor (pIgR) and were not able to produce SIgA on mucosal surfaces. The pIgR deficiency results in persistent activation of inflammation signaling due to lung microbiota invasion(95). Mice with a pIgR deficiency contained an increase in taxa (400 versus 194) in the lung. Discriminating taxa found in higher relative abundance in pIgR deficient mice compared to wild-type mice included *Veillonella*, *Prevotella*, *Neisseriaceae*, *Bacillus*, *Actinomycetaceae*, *Tissierellaceae*, and *Ruminococcus*(95). Furthermore, germ-free pIgR deficient mice did not develop the COPD-like phenotype compared to pIgR deficient mice raised in a conventional environment. Once the germ-free pIgR mice were removed from their germ-free environment and their microbiota conventionalized, similar levels of airway wall remodeling, emphysema, and inflammation were measured and found to be comparable to pIgR mice raised in conventional (non-germ free) conditions for the same duration of exposure(95). Upregulation of NF- κ B was found in the lungs of pIgR deficient mice when compared to wild-type age matched controls(95). This is important since structural abnormalities in COPD correlate with decreased expression of pIgR and a disruption of the protective mucosal barrier(111, 112).

The use of medications, especially corticosteroids, may also affect the host response to microorganisms. Host immune response determines host susceptibility to microbes thereby affecting the distinction between pathogen and commensals. In cases where immune response is inadequate or altered, microorganisms that may regularly colonize or be cleared by the host may have the potential to cause disease. Mucosal associated invariant T (MAIT) cells are a prevalent and unique T-cell population in humans with the capacity to detect intracellular infections with bacteria including *Mycobacterium tuberculosis*(113). MAIT cells recognize early intermediates in bacterial riboflavin synthesis that are able to be potent antigens for these cells(113). Although they are able to recognize a wide variety of bacteria microorganisms, bacteria that do not express riboflavin synthesis pathways do not stimulate MAIT cells(114). Hinks and colleagues demonstrated that in 11 patients who were exposed to inhaled corticosteroids, peripheral serum and bronchial biopsies contained less MAIT cells compared to healthy controls(115). In *in vitro* studies, non-typeable *Haemophilus influenzae* (NTHi) infection of macrophages obtained from healthy subjects were exposed to fluticasone or budesonide. In the presence of these corticosteroids significantly impaired the upregulation of MR1, a stimulatory receptor on macrophages for MAIT cells, as well as a decrease in NTHi-induced IFN- γ expression from MAIT cells was found in these

experiments(115). Therefore, steroid-induced suppression of MAIT cells may be responsible for the increased risk of pneumonia in subjects with airway diseases(115).

Unique selection pressures of the lung may have profound impact on the lung microbiome. There is increasing evidence that the *Adapted Island Model* may help model the microbial challenges to the lower airways from microaspiration. Once in the lower airways, the oral microorganisms are likely exposed to multiple selection pressures that dictate their persistence or transient nature in the lungs. Microbes that evolutionarily develop advantages to mitigate and avoid the host immune response are more likely to persist in the lower airway microbiome. Their ability to overcome the innate and adaptive immune system may serve to guide future studies to address new pathways that are regulated by microbes and particular molecular patterns that may dictate these interactions. Most importantly, host responses and medications deserve particular attention, as these mechanisms remain largely unexplored in current literature in relation to their effects on the lung microbiome.

The Lung Microbiome in Disease States

Morris and colleagues studied the microbiome of healthy subjects and healthy smokers in a study of the lung microbiome (85). They found that the most common genera in both oral wash and bronchoalveolar lavage (BAL) were microorganisms commonly found in the oral cavity (*Prevotella*, *Streptococcus*, and *Veillonella*). Although the study did detect organisms found more abundant in the lung than in the oral cavity, including *Haemophilus*, *Enterobacteriaceae*, and *Tropheryma whippelii*, differences in the lung microbiota between smokers and non-smokers were not found(85). However, despite no differences found in the lung microbiome, microbiota differences were found in the oropharynx of smokers as compared with non-smokers. *Neisseria*, *Porphyromonas*, and *Gemella* were depleted in the oral washes of their subjects(85). Several other studies have shown that smoking alone is sufficient to change the upper airway microbiota(20, 35) but not the lower airway microbiota(23, 85).

The exposure to tobacco smoke may also greatly increase your risk of developing Chronic Obstructive Pulmonary Disease (COPD). In contrast to exposure to tobacco smoke, subjects with advanced stages of COPD have significant alterations within the underlying lung microbiome. Erb-Downward and colleagues described these differences in lung microbiome in the lungs of end-stage COPD patients(84). Importantly, different areas of the lung have different microbiota. Presumably, changes in the underlying lung architecture and decreased mucociliary clearance lead to enrichment with *Pseudomonas* and *Streptococcus*(84). Other studies have also described differences in the lung microbiome of COPD(28, 65, 116). However, most COPD microbiome studies have focused on GOLD Stage IV COPD and samples collected from explanted lungs, where other confounders such as use of inhaled steroids and frequent courses of antibiotics, are commonly present and may confound the lung microbiome. Hilty and colleagues also described a disordered lung microbiome in patients with asthma and moderate COPD(58). Notably 60% of these patients were on inhaled corticosteroid therapy.

Dysbiosis may also play a role in COPD exacerbations. Huang and colleagues studied a group of patients (n = 8) who were admitted to their hospital for COPD exacerbations. All of

the patients studied were mechanically ventilated, intubated, and received antibiotics(117). The subjects studied had variation in their interpersonal bacterial richness, with subjects with decreased richness having more *Pseudomonaceae* and *Enterobacteriaceae*, and subjects with increased richness possessing higher relative abundance of *Clostridiaceae*, *Lachnospiraceae*, *Bacillaceae*, and *Peptostreptococcaceae*(117). One challenging issue with interpreting some of these results may be that the subjects in the study received differing lengths and types of antibiotics at the time of the study. Thus, changes in the lung microbiome that may be related to a subject having a COPD exacerbation may be lost early on in the disease and the changes seen may be due to an antibiotic effect on the lung microbiome(117).

Pragman and colleagues addressed the lung microbiome of moderate and severe COPD subjects with relatively stable disease and compared them to healthy subjects. They found that while the moderate and severe COPD groups shared more OTUs in common, very few OTUS (approximately 13%) were shared among all three groups(116). Notably, the study found several anaerobes within the lung consisting of Bacteroidetes, *Fusobacteria*, and the genus *Clostridium* extending observations that anaerobic bacteria are found in an aerobic environment, however it is not known if these anaerobic bacteria are metabolically active(116). In sub-analysis of the study, the authors found that the most discriminant factor for a distinct lung microbiome was the use of inhaled steroids. Emphasizing the effects of medications on the microbiome, a recent investigation showed that in subjects with early emphysema, the use of azithromycin affects the diversity of the lower airway microbiome and increased stress related microbial metabolites that might have anti-inflammatory properties(118). This data further supports the role of the lower airway microbiome on the immune phenotype of the lower airway mucosa and illustrates the selective pressures of medications.

Using core biopsies from explanted lungs, Sze and colleagues also found differences in advanced COPD with an increase in relative abundance of Firmicutes including *Lactobacillus* and Proteobacteria(65). However, in a follow up study, the same researchers found that there was a relative expansion of the phylum Proteobacteria and a reduction of the phylum Firmicutes when they compared the lung specimens from COPD subjects to lungs from transplant donors(28) reinforcing that it remains difficult to define a consistent “core” COPD microbiota. One important finding of this last study was that there were several taxa that were positively and negatively associated with the host immune response including neutrophil infiltration, eosinophil infiltration, and B-cell infiltration(28).

Despite studies that have examined the lung microbiome in GOLD Stage IV COPD, early COPD, moderate COPD, and active smokers remain difficult to study and represent a knowledge deficit in lung microbiome research. Attention should be given to these populations due to several challenging issues including: a need to study early events in the disease process, a way to control and limit possible confounders such as inhaled corticosteroids, and a focus on longitudinal changes in the lung microbiome.

In asthma, there is evidence of enrichment with Proteobacteria, including the *Haemophilus* genera(29, 119). Importantly, distinct immunological phenotypes have been associated with

changes in lower airway microbiome. The respiratory tract of asthmatic patients tends to have higher bacterial burden compared to subjects without asthma(119). Exposure to diverse allergens and microbes (from animal or environmental exposure) in youth proves to be protective for developing asthma thus supporting the “Hygiene Hypothesis”(120, 121). In contrast, several taxa in the lung microbiome including *Streptococcus pneumoniae* and *Haemophilus influenzae* have been identified as increasing the susceptibility of developing asthma once colonization is established in a neonatal host(122). Huang and colleagues studied asthmatic patients with a disordered lung microbiome. They identified that greater airway diversity was strongly associated with increased bronchial hyper-responsiveness(119). Moreover, they found that the taxa that were strongly associated with bronchial hyper-responsiveness primarily belonged to the Proteobacteria phylum. The treatment of these patients with clarithromycin showed that those with improvement with their bronchial reactivity following clarithromycin treatment had an increase in their Shannon Diversity index (α diversity)(119).

In severe asthmatics, Huang and colleagues also sought to characterize the microbiome associated with in subjects with different phenotypes of asthma. They identified that enrichment with *Bacteroidetes* and the Firmicutes phylum (families including *Prevotellaceae*, *Mycoplasmataceae*, *Lachnospiraceae*, and *Spirochaetaceae*) was associated with obese subjects who were also asthmatics. They also identified certain taxa associated with worsening asthma symptoms such as the Proteobacteria phylum (families including *Pasteurellaceae*, *Enterobacteriaceae*, *Neisseriaceae*, *Burkholderiaceae*, and *Pseudomonadaceae*)(123). The researchers demonstrated that different asthma phenotypes might harbor distinct microbiomes supporting an important aspect of the lung microbiome on the host immune phenotype (29). In cystic fibrosis, disordered airway clearance is a result of dysfunction of the sodium chloride channel, leading to thickened secretions and progressive microbial airway colonization and infection. Culture-based data has identified clinically significant bacteria including: *Pseudomonas aeruginosa*(124), *Burkholderia cepacia* complex(124), methicillin-resistant *Staphylococcus aureus*(125), and *Mycobacterium avium* complex(126) in the airways of cystic fibrosis patients. Early microbiome studies in the cystic fibrosis identified a possible core microbiome represented by 15 taxa from 7 genera including *Catonella*, *Neisseria*, *Porphyromonas*, *Prevotella*, *Pseudomonas*, *Streptococcus*, and *Veillonella*(127). The lungs of cystic fibrosis subjects may contain high spatial heterogeneity(128). In a study consisting of 269 cystic fibrosis patients conducted by Coburn and colleagues, the researchers studied a broad range of ages and disease stages(128, 129). The study reaffirmed particular species as belonging to a possible core microbiome including *Streptococcus*, *Prevotella*, *Rothia*, *Veillonella*, and *Actinomyces*(129). More typical cystic fibrosis-related microorganisms (e.g. *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, and *Achromobacter*) were less prevalent, but when present in sputum specimens, tend to dominate the relative abundance within the samples(129).

Furthermore, Coburn and colleagues showed that community diversity and lung function were greatest in patients less than ten years of age, reaching a plateau at age 25, subsequent decreasing community diversity in following years correlated with worsening lung function(129). Bacteria that once thought to be oral contaminants may be important oral

microorganisms in lung ecology(90, 130) and may also represent a potential source of inflammatory response by the host in cystic fibrosis(131).

Lung-Gut Axis and its Role in Disease

The gut is a major reservoir for the microbiome within the human body. Evidence suggests that the gastrointestinal tract plays an important role for the immunological priming of the host. This immunological function is not limited to the gut mucosa or systemic circulation but there is growing evidence of its relevance to the lung through immunological cross talk between the gut and lung. Thus, the gastrointestinal tract not only may have direct interaction (e.g. aspiration) with the lower airways, but also may serve as an immune modulating organ with ability to crosstalk with other organs contributing cells related to the innate and adaptive immune system (dendritic cells and macrophages) and possibly inflammatory cytokines and chemokines in times of disease to the respiratory tract(60, 132, 133).

Microbiome studies of asthma have also identified possible lung and gut interactions. The exposure to livestock or canines in the youth significantly decreases the risk of developing asthma(134, 135). Until recently, the mechanism that protects the subject from the development of asthma remained elusive. Fujimura and colleagues explored this association with experiments using dust collected from households with canines(25). Mice exposed to canine-inhabited house dust exhibited protective responses to allergens. In particular, the mice that received canine household dust had elevated relative abundance of *Lactobacillus johnsonii* in their gastrointestinal tract(25). Oral supplementation of this single species of *Lactobacillus* decreased broncho-responsiveness to allergens and respiratory syncytial virus challenge. Furthermore, authors purport that while they were able to detect *L. johnsonii* in cecal samples, they did not detect any in the lung samples providing evidence that the gastrointestinal microbiome affects the local and systemic inflammatory response(25).

Cystic fibrosis murine models show that there is an appreciable modulation of the lung microbiome due to changes from the intestinal microbiome in response to antibiotic treatment(136, 137). Bazett and colleagues identified that with streptomycin treatment, an antibiotic that is not systemically absorbed from the gut in a murine cystic fibrosis model, that the treatment with the antibiotic was associated with a decrease in pulmonary IL-17 and $\gamma\delta$ cells T cells compared to an increase seen when wild-type mice were treated with streptomycin(136). Thus, gut microbiome also plays an important immune-modulating role in cystic fibrosis (138, 139).

Changes in the gut microbiome have also been associated with pulmonary complications in the setting of allogeneic hematopoietic cell transplantation in humans(140). Gammaproteobacteria-domination of the fecal microbiota, which *Klebsiella pneumoniae* and *Klebsiella oxytoca* are members, was associated with pulmonary complications and doubling the risk for mortality(140) suggesting that the gut contributes to the translocation of gut bacteria early in transplantation or is able to contribute to indirect lung injury from microbiota-induced local or systemic inflammatory pathways. The gut microbiome may also attenuate and protect against sepsis(141–143). Eradication of gastrointestinal microbiota with antibiotics is associated with a dramatic reduction in survival in mice intranasally

challenged with pathogenic train of *Streptococcus pneumoniae*, where cytokine production of alveolar macrophages such as IL-1 β , IL-6, and CXCL1 were up-regulated while IL-10 and TNF- α were down-regulated(144).

The full extent of gut-lung interactions still requires further clarification. There is clear cross talk between the two organs and both organs represent large areas of mucosal surfaces that likely share similar immune pathways. Thus, the gut and lung interactions expose shortcomings when describing the immunological effects of the lung or gut microbiome in isolation. New evidence is beginning to suggest that the gut-lung axis represents an area of communication and sharing of information between these two organs. The future direction of research is to define and refine these interactions and to take advantage of them to benefit the host.

Microbiota Effects on the Activation of Inflammatory Pathways of the Lower Airway Environment

The interaction of the host's respiratory system to the environmental and microbiome challenges is an integral component of understanding the role of the upper and lower respiratory microbiome plays in health and disease(21, 23, 30). Although examples are currently scant in pulmonary microbiome literature, several manuscripts have demonstrated the role of the gastrointestinal microbiome in the maturation of the Th17 response in the mucosal immune system(2, 145–147). Within the gastrointestinal literature, there is evidence that the gastrointestinal microbiome plays a significant immune-modulating role in health and disease(2, 146, 147). In particular, the Th17 response is of interest on mucosal surfaces, Ivanov and colleagues demonstrated that segmented filamentous bacilli (SFB) appear to play a role in the differentiation of Th17 cells in the gastrointestinal tract(2, 147). Furthermore, Suzuki and colleagues demonstrated that in the IgA-deficient mouse model, aberrant and uncontrolled immune responses were associated with proliferation of SFB in these mice(148). In all, gut microbiome was found to play a necessary role in the maturation of the immune response. However, the lung microbiome may also play a significant role in a mucosal-based immune response.

In the lung mucosa, changes in microbiome in healthy patients are associated with sub-clinical inflammation(23). In a dichotomized grouping of the lung microbiome, two distinct lung microbiomes (pneumotypes) could be identified: one characterized by enrichment with supraglottic predominant taxa (pneumotype_{SPT} – Figure 2); and another pneumotype characterized by enrichment with background predominant taxa (pneumotype_{BPT} – Figure 2) (23). The degree of similarity with upper airway can also be used to characterize the lung microbiome, where lower UniFrac distance (a measurement of dissimilarity based on relative abundance and a phylogenetic tree) occurs in BAL samples enrichment with supraglottic predominant taxa (pneumotype_{SPT}), however the metabolic activity of these taxa is currently unknown(93, 149). Moreover, the organization of the cohorts into these two pneumotypes (Figure 2) was supported by contributed phage data(30). The fact that phage data was recovered implies that different phages selected for different taxa in the lungs of our subjects suggesting that the ecological gradients of the lung may affect the virome as well as the microbiome. The phage data from the cohort also supports the existence of an

actively replicating phage population, therefore in order to have an active phage population implies that there also is an actively replicating microbial community(30). Importantly, the lower airway microbiome enriched with oral taxa is associated with a distinct genomic potential and associated with several metabolites of microbial origin. This provides evidence of active microbial metabolism in the lower airways. The host is not only a bystander in its interaction to the lower respiratory tract microbiome; it can also be affected or benefited from the byproducts of bacterial metabolism. A recent investigation has shown that the use of azithromycin in early emphysema promotes the bacterial production of anti-inflammatory metabolites(118) suggesting that some of the anti-inflammatory effects of macrolides might be mediated by its effect on the microbial metabolism in the lower airways.

Further, differences in lung microbiome are associated with a distinct immunological phenotype, demonstrating that the lung microbiome is not a bystander commensal but rather interacts with the host immune system. Specifically, enrichment with supraglottic predominant taxa (pneumotype_{SPT}) is associated with Th17 inflammatory phenotype (Figure 2). Lower airway microbiome is most likely an active participant in pneumotype_{SPT} and samples that resembled the URT were also associated with Th17-chemoattractant cytokines: IL-1 α , IL-1 β , Fractalkine, and IL-7(30). Furthermore, transcriptome analysis of airway brushes showed that factors important for a Th17 differentiation, such as STAT3 and TSLP, were upregulated in association with pneumotype_{SPT}. Given that these microbiome signals were found in both healthy volunteers and those who were exposed to cigarette smoke suggests that enrichment of the lower airways must occur with and without the context of disease (Figure 2).

Other investigations have also has found microbial signatures associated with a Th17 phenotype in severe asthma(29). The mechanism by which this phenotype occurs is still not clear in the lung. Huang and colleagues' study of severe asthmatic patients found that Proteobacteria correlates with Th-17 associated gene expression. Members of the Proteobacteria taxa include *Pasteurellaceae*, *Enterobacteriaceae*, and *Bacillaceae*. Although FKBP5, a gene associated with steroid responsiveness in asthma, and genes-associated with Th17 inflammation both correlated with taxa from the phylum Proteobacteria, particular families did not significantly overlap suggesting different microorganisms, despite sharing a common phylum, are responsible for different effects on the lower airway(29). Thus, distinct airway microbiota may promote Th17 associated inflammation, but may not be associated with FKBP5 related steroid-induced effects(29). The identification of microorganisms that are associated with Th17 inflammation may represent another pathway of inflammation that is independent of a Th2 and eosinophilic response. Th17 inflammatory phenotype may represent an important immunomodulatory target in some asthmatics, especially those with a neutrophilic phenotype(29). Th2 phenotype, however, was not significantly correlated with the microbiome data(29).

Moreover, in research by Yadava and colleagues with an experimental mouse model found that exposure to LPS and elastase leads to a dysbiotic lung microbiome with an increase of IL-17A expression due to an increase in $\gamma\delta$ + T cell phenotype(150). This murine inflammatory phenotype is associated with airway abnormalities and emphysematous changes that resembled human COPD. BAL obtained from mice challenged with LPS and

elastase demonstrated decreased α diversity and an increase of relative abundances of *Pseudomonas*, *Lactobacillus*, and *Chryseobacterium*(150). The researchers found that microbiota enhanced the production of IL-17A by $\gamma\delta$ + T-cells by using microbiota-depleted mice. Importantly, researchers recapitulated the upregulated IL-17A immunological phenotype by transfer of enriched microbiota from LPS and elastase treated mice and concurrent challenge with LPS and elastase into antibiotic-treated mice(150). This study demonstrated experimentally that the lung microbiome has a functional role on an immunological phenotype however the individual components of this role still need to be clearly elucidated. The use of LPS and elastase in this study raises questions, similar to Poroyko and colleagues' study, of whether it is living complex microbial communities or pathogen-associated molecular patterns (PAMPS) that is required to induce a Th17 phenotype. Furthermore, LPS may have variable effects depending on the source organism, commonly being *E. coli*, and thus may not be derived from microbes frequently found in the lower airways(106, 150). Given the complexity of the lower airway microbiome it is likely that other microbial products also play an important role on the host immune phenotype in the lower airways(106, 150).

The presence of the Th17 phenotype and upregulation of IL-17 has important implications for microbial communities in the lower airway. The lower respiratory tract microbiome, instead of being a non-responsive passenger, stimulates and attenuates the development of the innate and adaptive immune response. Dysbiosis, here defined by a microbiome that resembles the upper respiratory tract microbiome, is associated with a sub-clinical pro-inflammatory phenotype(30). Complex metabolic interactions are present in relationship with this dysbiotic microbiota. Further, there is also evidence of activation of counterregulatory mechanisms are triggered by a distinct lung microbiome. Pulmonary alveolar macrophages collected from subjects who had a lower airway microbiome characterized as pneumotype_{SPT} were associated with a blunted TLR4 response with decreased production of TNF- α , MDC, IL-6, and GM-CSF in response to LPS stimulation(30). This attenuated innate immune response in this distinct lung microbiome may therefore be an important counterregulatory mechanism present in alveolar macrophages needed to attenuate the inflammatory immune response in the lung mucosa(3, 30, 145, 147). This is in line with multiple other counterregulatory mechanisms found in other organ systems including the gastrointestinal tract(3, 145–147).

Th17 inflammatory pathway and IL-17 upregulation are not the only pathways that may be upregulated in a host with lung dysbiosis. Richmond and colleagues' study also demonstrated in pIgR deficient mice with the loss of mucosal immunity resulted in microorganism invasion into the epithelium(95). In contrast, the percentage of bacteria present in the lumen of the airways did not differ between the wild-type mice and pIgR deficient mice, suggesting that the effects were due to invasion of microorganisms alone. Mice with pIgR deficiency also demonstrated activation of the NF- κ B pathway with associated NF- κ B-dependent chemokine keratinocyte chemoattractant (KC) in BAL fluid(95). Taken all together, this mouse model of mucosal immunodeficiency is an example of another pathway that is activated by the interaction between innate microorganisms in the lower airway and represents a target for study.

Metabolites and products of bacterial metabolism will also have immunomodulating properties on the lung microbiome. Chronic inflammation typifies the effects of cystic fibrosis on the pulmonary system. Mechanisms of inflammation revolve upon colonization of typical cystic fibrosis microorganism, but also anaerobic metabolism from the facultative anaerobes that make up a smaller portion of the microbiome composition(90). The microorganisms capable for facultative anaerobic metabolism are frequently found in the mouth and have been identified in the lower airway of subjects with cystic fibrosis patients. Short chain fatty acids (SCFAs), a byproduct of anaerobic metabolism, have been shown to alter and influence the inflammation in cystic fibrosis(131).

The lung mucosal surface is an important interface with the exterior world with its role in the innate and adaptive immune responses that scavenge and respond to microbes, providing alveolar macrophages that maintain an important role for presenting antigens from opsonized microbes, and its ability to regulate signaling cytokines and chemokines(151). The data described above show that there are multiple lines of evidence that support that the lower airway microbiome has an important role in maintaining the immunological tone of the lower airway mucosa. This response may be part of a “healthy” immunological tone required to prime the immune system or may play an important role on the inflammatory process of several inflammatory diseases of the airways.

Microbiome studies have uncovered a complex and diverse microbial community that inhabit within us. With the use of multiomics, we can evaluate the dynamics between the microbes and the host. This new vision of a complex microbial community interacting with the host immune system forces us to reconsider classical Koch’s Postulates for our understanding of disease. The great challenge is how to recognize that complex communities of microbes may have a mutualistic and active role in influencing their microenvironment, including the host immune response(152). The effects of communities of microorganisms causing disease represent a challenge to Koch’s Postulates as they are commonly used to understand how a single microbe is the cause of a disease. These postulates have been used to categorize microbes as pathogens (153). In the context of microbial communities interacting with the host and affecting its environment, it is likely that the “pathogenic” role of a microbe and its community will have to be re-defined (154, 155). It is then possible that pathogenicity should be viewed in the context of the interaction between the microorganisms and the host, an interface affected by the microorganism’s own virulence factors, the associated microbiota, and the host immune response.

Our understanding of many lung diseases is currently limited by association studies and functional studies have yet to be done in human and mouse models. These different interactions and mechanisms can be further elucidated with the study of the metagenome, metabolomics, metatranscriptomic, metaproteomic, viromics, and other ‘-omic’ studies(22, 23, 30, 155). The use of these approaches will be important to understand how the microbiome communities affect health and disease and to challenge the classical approach to Koch’s Postulates by addressing the community’s role in health and disease as a whole(155).

CONCLUSION

The respiratory mucosa represents a broad and encompassing border between the human host and the environment. The differences between the gastrointestinal tract and the respiratory tract such as the concentration of microbes, length of organ, ease of sampling, and differences in physiological parameters represent challenging nuances in the study of the lung microbiome. Attention has shifted from understanding the ecological composition of the respiratory tract microbiome to several key issues including: The colonization and persistence of particular communities of microbes, inflammatory pathways that are upregulated and downregulated in the presence of particular communities, and the functional and mechanistic causes of these inflammatory pathways(23, 30).

The lungs see multiple microbial challenges through different mechanisms such as aerodigestive reflux and microaspiration during the course of the day and night(38). These repetitive microbial challenges may play an important role in the host immune response(23, 30, 156). The effects on the host immunological tone may represent beneficial immunological priming or contribute to a pathogenic immunological process relevant for disease development.

Future directions of the lower respiratory lung microbiome will require focus on dissecting the microbial community function, understanding microbe-microbe interactions, and developing experimental models to uncover causality. Ultimately, the goal is to find potential microbial targets for a more precise manipulation of the lower airway microbiota, such as refined use of antibiotics, phage therapy, or molecules that target specific microbial metabolic pathways, in order to alter the natural course of pulmonary disease processes.

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Figure 1. Conceptual Model: Signal to Noise Ratio

Figure 1 Panel A, represents the healthy gastrointestinal microbiome where there is an organ of healthy biomass and background “noise” or signal amplified by background (e.g. background microbiota present in the colonoscopy) that does not represent the gut microbiome. This background microbiome overwhelmed by the large biomass present the sample. In Figure 1 Panel B, represents the healthy lung microbiome where there is relatively low biomass and background signals tend to overwhelm the lung microbiome signal. Figure 1 Panel C, represents a diseased gastrointestinal microbiome where the pathogenic signal (dysbiosis) will eventually overcome the high underlying biomass. The pathogenic signal will overpower the background microbiome and be apparent given the high amount of biomass present in the gut. Figure 1 Panel D, represents a diseased lung microbiome. Unlike the diseased gut microbiome, the pathogenic signal may be confounded by the background noise and may not be apparent until sufficient progression of disease supports the altered dysbiosis.

Figure 2. The Host-Microbiota Interaction in the Lung

This schema represents the normal lung microbiome and its dysbiosis. In this model, enrichment with background taxa (represented as blue bacteria) in pneumotype_{BPT} occurs in a lung with preserved mucociliary clearance of microorganisms and minimal inflammatory signals within the lung. In the presence of enrichment of the lower airway microbiome with oral taxa (represented as red bacteria) in pneumotype_{SPT}, there will be upregulation of the Th17 inflammatory phenotype and recruitment of neutrophils and lymphocytes.