A Brave New World: The Lung Microbiota in an Era of Change

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Abstract

The development of culture-independent techniques has revolutionized our understanding of how our human cells interact with the even greater number of microbial inhabitants of our bodies. As part of this revolution, data are increasingly challenging the old dogma that in health, the lung mucosa is sterile. To understand how the lung microbiome may play a role in human health, we identified five major questions for lung microbiome research: (1) Is the lung sterile? (2) Is there a unique core microbiome in the lung? (3) How dynamic are the microbial populations? (4) How do pulmonary immune responses affect microbiome composition? and (5) Are the lungs influenced by the intestinal

immune responses to the gut microbiome? From birth, we are exposed to continuous microbial challenges that shape our microbiome. In our changing environment, perturbation of the gut microbiome affects both human health and disease. With widespread antibiotic use, the ancient microbes that formerly resided within us are being lost, for example, *Helicobacter pylori* in the stomach. Animal models show that antibiotic exposure in early life has developmental consequences. Considering the potential effects of this altered microbiome on pulmonary responses will be critical for future investigations.

Keywords: lung; microbiome; antibiotics; immune responses; inflammation

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With the advances in culture-independent techniques that have occurred, there has been renewed interest in searching for microbes in the lung. New observations are challenging the old dogma that in health, the lung mucosa is sterile (1, 2). With the logarithmic improvements in sequencing technology and bioinformatics analysis, the study of the lung microbiome has received increasing interest. The changing research environment represents a new frontier to address important long-standing problems in human health.

Key Questions about the Lung Microbiome

1. Is the Lung Sterile?

For many years, this question did not seem relevant, given the failure of culture-dependent

techniques to describe residential bacteria in the healthy lung. Among various factors, the low culturable bacterial burden in the lung, contamination with upper airway microbiota during sampling, and the difficulties in growing fastidious bacteria have contributed to the presumption that the lung is sterile (3, 4). With the use of culture-independent techniques, there is now substantial literature identifying the presence of bacteria products in the lower airways of normal individuals (5–8). Data from quantitative PCR for 16S ribosomal RNA (rRNA) genes confirm that the bacterial burden in the lower airways is less than in the upper airways. However, a diverse microbiome has been described in the lung of healthy subjects. A common finding in the lung is the presence of taxa often represented in the oral cavity (5, 8–11). Because sampling of

the lower airways is usually performed by bronchoscopy, there is controversy as to whether these microbes represent bronchoscopic carryover of upper airway microbiota and/or microaspiration (Figure 1). The decrease in bacterial loads observed in subsequent samples has been suggested as supporting a significant carryover effect (5). However, this has not been a consistent finding in all studies, which suggests substantial technical heterogeneity (8). In contrast, it is well known that microaspiration is a common phenomenon among healthy persons, and that it is exacerbated in several respiratory disease states (12–14). The finding of low bacterial burden in the normal lung supports the view that although the healthy lung has the ability to clear microaspirated microbes, a residual microbiota coexists in the lung

Figure 1. Key questions involving the lung microbiota.

mucosa. The ability to clear microorganisms also is commonly impaired in lung diseases (15, 16). Both carryover and microaspiration can variably contribute to the observed enrichment of the lower airway microbiome with upper airway taxa. Bronchoscopic techniques that are better able to minimize the contribution of carryover are needed. Further, considering the low bacterial burden in most lung samples, a high background microbiome signal commonly interferes with the interpretation of lung microbiome data (8). It is therefore expected that an unfavorable signal-to-noise ratio will commonly occur when trying to evaluate the lung microbiome of airways with low bacterial burden. New computational background subtraction techniques based on mathematical modeling, such as a neutral model, single-sided outlier test and SourceTracker, may account for carryover and have the potential to identify true lung taxa (7, 17, 18).

One final dilemma in addressing this question concerns determining the viability of the lung microbiome. Classically, culture techniques have been used to address this question. Using 16S rRNA PCR techniques, current lung microbiome data in healthy lung may reflect only bacterial DNA fragments (5–9, 17, 19, 20). With the current data available and given the high

sensitivity of powerful high-throughput sequencing technology, we may not be ready to answer this question. However, this limitation does not invalidate the relevance of determining the characteristics of a microbiome composed mainly of dead bacteria: the taxonomic composition may reflect a specific conglomerate of various microbial-associated molecular patterns (MAMPs) from nonviable bacteria that can elicit significant immunomodulatory effects on the host.

2. Is There a Core Microbiome?

Are the organisms found in the lung unique? One challenging observation is that the lower airway microbiome is more similar to the upper airway microbiome of the same individual than it is to the lung microbiome of a different person (17). Although part of this might be explained if significant carryover had occurred, most data suggest substantial diversity in healthy individuals (7–9, 19). Therefore, it is important to establish the degree of overlap between different individuals. Conceptually, the search for a shared core is consistent with the presumption that the lung environment imposes selection pressure on the microbial inhabitants in the lung. As an example, data have shown the presence of *Tropheryma whipplei* in the lung of normal subjects (6).

Importantly, this microorganism was not found in upper airway samples when 500 sequences per sample were evaluated. This observation suggests that *T. whipplei* reaches the lung by either hematogenous spread or by microaspiration. In either circumstance, the data suggest that the lung is a true niche for *T. whipplei*. This tropism for the lung might explain why it is easier to find *T. whipplei* in the lower airways than in upper airway samples. The evidence that colonization of the lower airways with this microbe was enhanced in immunodeficiency further suggests a dynamic state for the host–microbiota interaction. We therefore postulate that two components of the microbiome may elicit synergistic host responses: one in which unique taxa find a special niche in the lung environment, and a second in which MAMPs exert an immunomodulatory role. These two components of the lung microbiome deserve further exploration.

3. What Are the Dynamics of the Lung Microbiome?

With the common use of antibiotics and antiinflammatory drugs, a significant impact on the lung microbiome can be expected. Disturbances of the lung microbial community can lead to either no change (resistance), an altered microbiota that may

return to its original composition (resilience), a permanently altered microbiota that could be functionally similar (functional redundancy), or a microbiota permanently altered in both composition and function. These dynamic changes might be relevant for our understanding of pathogenesis in pulmonary health and disease. In the lung, microaspiration commonly is a repetitive phenomenon (12). As discussed previously, this might explain the frequent observation of oral taxa such as *Prevotella* and *Veillonella* in the lower airway microbiome. Such periodic exposure of the lower airways to upper airway microorganisms represents an important seeding mechanism that may influence microbial selection in the lower airways. As an example of this selection pressure, *S. pneumoniae*, a minor component of both the upper and lower airway microbiomes, causes more than half of all cases of community-acquired bacterial pneumonia. Further, dynamic changes in lower airway microbiota may reflect changes in bacterial tropism related to the development of lung injury, and/or immunosuppression states (such as in cystic fibrosis, bronchiectasis, alcoholism, or HIV infection). Several examples of dynamic changes in airway microbiota have been proposed to substantially affect disease progression. In chronic obstructive pulmonary disease, exacerbations are commonly caused by acquisition of new strains and associated with increased inflammation and decreased lung function (21). In cystic fibrosis, changes in bacterial diversity are associated with disease progression and consequent colonization with pathogens (22). Diversity of airway microbiota in cystic fibrosis has been found to decrease significantly with disease stage (22, 23). Also, the composition of the airway microbiota before an exacerbation event may be relevant to determine the microbial constituents involved in the exacerbations (24). Lower airway microbiota diversity (as measured by Shannon diversity indices) and a *Pseudomonas*-dominated microbiome preceding the exacerbation are predictive of larger changes in microbiome structure during exacerbations. These data indicate that a dynamic change from a “healthy” to an “unhealthy” airway microbiota leads to a susceptible microbial environment necessary for pathogen enrichment and host injury. Investigation of the dynamic

changes in the lung microbiome will require prospective and longitudinal studies.

4. What Are the Mucosal Inflammatory Implications of the Lung Microbiome?

The relationships between our human cells and our inhabitant microorganisms are not accidental, but likely have been programmed over long periods of time (25). The role of the gut microbiota in shaping the mucosal immune system is well understood (26, 27). The composition of the intestinal microbiota regulates the balance between helper T type 17 cells and T-regulatory cells (Th17:Treg balance) in the small intestinal lamina propria (26). Compared with conventionally colonized animals, germ-free mice have defective development of immunity, with fewer CD4⁺ and CD8⁺ T cells (28, 29). However, this symbiosis between the gut microbiome and the intestinal mucosa relies on barrier mechanisms that exclude most bacteria, preventing invasion and leading to tolerance to the continuous exposure by bacterial products. Loss of these important mechanisms may lead to bacterial invasion and chronic inflammatory processes, such as inflammatory bowel disease (30). In the lung, little is known about the effects of the airway microbiome on immune maturation and the types of tolerance that occur in the lower airway, which may in fact be relevant to the symbiotic interactions. Data from two large European studies showed that specified environmental exposures could be protective against development of asthma and atopy (31, 32). The use of molecular techniques to evaluate specific patterns of exposure to farm-related bacteria or fungi in two cohorts (PARSIFAL: Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle; and GABRIELA: Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community [GABRIEL] Advanced Study) has further characterized certain environmental microbiomes as protective against asthma (33). Increased environmental exposure to microbial products has been shown to be protective factor against allergy (34, 35). This microbial exposure occurs in early childhood and is determined by mode of birth delivery, farm habitation, or antibiotic use, all of which have been shown to be

factors conferring protection or conversely increasing risk of asthma later in life (33, 36–40). This has renewed interest in the “hygiene hypothesis,” which states that changing diets, improved sanitary conditions, and increased use of antibiotics prevent the exposure to microbes needed for adequate immune maturation in early life. This results in T-cell subset imbalances, such as Treg cell deficiencies, that may predispose to immune diseases.

In asymptomatic adults, enrichment of the lung microbiome with oral-characteristic taxa is associated with increased inflammatory cells and exhaled nitric oxide, suggesting that there are specific inflammatory responses to the airway microbiome (8). Although it might seem obvious that the microbiota plays a role in stimulating a host immune response, it remains unclear whether the observed associations are causal or not. Furthermore, it is likely that this is a bidirectional association and that the characteristics of the airway microbiota are determined by the host immune response as well. This is suggested by the observation that subjects with immunodeficiency have a lung microbiome enriched with *T. whipplei* and that there is subsequent reduction of the relative abundance of *T. whipplei* with antiretroviral therapy (6). Other potential roles of the airway microbiome may be related to metabolic processes (e.g., drug metabolism), control of surfactant production, or gas exchange, areas for which more investigation is needed.

5. Is There Cross-Talk between the Intestinal Microbiome–Mucosa Interaction and the Lung?

Analyses of the intestinal tract show that gut microbiota composition is relevant for the education of the immune system. Furthermore, there is increasing evidence that the gastrointestinal mucosa is the predominant site of microbiota–host interaction, and can play a role in the development of immune responses at distal mucosal sites. Several lines of investigation support the concept of cross-talk between the intestinal microbiome–mucosa interaction and the lung. In mice, disruption of the gastrointestinal microbiota leads to abnormal airway allergic responses (41, 42). In other mouse models, oral ingestion of various strains of *Lactobacillus* and ingestion of bacterial

products modulate allergic pulmonary inflammation (43–45). More importantly, there is evidence that children whose stomach is colonized with *Helicobacter pylori* are 40–60% less likely to have childhood-onset asthma than children who are not carriers (46). Similarly, reduced intestinal microbiome diversity in infants is associated with increased risk for allergic rhinitis and peripheral blood eosinophilia (47). Taken together, these data support the hypothesis that the gut microbiota shapes the systemic immune system, thereby affecting the lung mucosa. Alternatively, changes in gut microbiota might also reflect changes in the oropharyngeal microbiota, which may directly affect the lung microbiota and host immune response through microaspiration. Ultimately, both the gut and lung mucosa may function as a single system-wide organ and share the physiological function of immune surveillance and shaping of host responses. More investigation is needed to determine the immunological mechanisms that link these two mucosal sites.

Evidence That the Human Microbiome Is Changing, and Potential Consequences

Ancient, Vertical

Animals have had residential microbes colonizing them ever since animals first appeared on this planet, about 500 million years ago. These microbes live in specific niches in and on their hosts, and often are persistent for sizeable fractions of the host's

lifetime. Importantly, a large fraction of these residential microbes, which can be considered the normal microbiome of their host, can be vertically transmitted from mother to offspring (48). This has been true for countless millions of years, and includes all mammals, such as humans.

Onslaught

In the period since World War II, there have been sharp rises in the rates of many important diseases including asthma, type 1 diabetes mellitus, and obesity (49). We have focused our research efforts on obesity, because of the rising epidemic in the United States (Figure 2A) and other developed countries.

Birth

The transfer of microbiota from mothers to human babies is facing unprecedented constraints. At the outset, it is fair to say that we do not know the magnitude of this problem, whether the constraints are large or trivial, but they are extensive. About half of all pregnant women in the United States are receiving antibiotics while they are pregnant. These can lead to extinctions in their microbiota just before the transfer to their offspring. Especially if received just before birth, for example, to prevent group B *Streptococcus* infection, the antibiotic also may be present in the baby's blood and the mother's milk. These would create further selection for organisms resistant to the antibiotic and change the environment in the baby for the receipt of the hand-off of organisms from both mother and environment. In addition, about one-third

of all babies born in the United States are delivered via Caesarian section. In Brazil and urban China, the C-section rate approaches 50%, and even in developing countries, such as Ecuador and Iran, the rates exceed 40%. One consequence of C-section is that the baby no longer transits the vagina, and is not coated with the vaginal microbiota (50). In consequence, the initial microbiota in C-section and vaginally delivered babies at all sites—skin, mouth, and intestinal tract—differ immediately after birth (48). When and if the microbiota ever fully return to a normal developmental pathway is not yet known.

Data suggest that the ability to mount a balanced immune response, fundamental for managing healthy airways, is influenced by early exposure to pathogenic bacteria (51, 52). Upper airway colonization with *Moraxella catarrhalis* and *Haemophilus influenzae* in early life is associated with Th2 and Th17 inflammatory patterns that may recruit and activate eosinophils and neutrophils while counteracting Th1 responses (52). Further, respiratory syncytial virus infection early in life affects regulatory T-cell phenotypic plasticity, lowering their suppressive capacity and rendering the host more susceptible to asthma later in life (53). This airway exposure to pathogenic microbes in early life may contribute to the development of chronic inflammation and increased risk for asthma.

Failure to establish a healthy gut microbiota early in life also alters maturation of the immune system, promoting altered allergic responses in

Figure 2. (A) Obesity trends in U.S. adults: evidence of changing physiology. Source: CDC behavioral risk factor surveillance system. Available from: <http://www.cdc.gov/obesity/data/adult.html>. (B) Outpatient antibiotic usage rates by region of the United States in 2010. Reproduced by permission from Reference 62.

adulthood (54, 55). In a mouse model, oral vancomycin causes an exacerbated allergic response, whereas streptomycin does not, suggesting that microbial composition can influence allergic sensitization (56). In a murine model of allergic airway disease, germ-free mice exhibit more severe disease than conventionally housed controls, an effect that could be ameliorated by recolonization with conventional microbiota (57). Similar results were found when disturbance of the gut microbiome was induced by administration of antibiotics in drinking water or by an antibiotic–fungal microbiota combination (42, 58). Microbial colonization early in life regulates mucosal invariant natural killer T-cell tolerance and can impact immune responses at mucosal sites later in adulthood (59). Regulatory T-cell–mediated tolerance mechanisms may be disrupted when specific gut microbial populations, such as *Clostridium* species, have been lost or altered (27, 56). Therefore, gut colonization early in life plays an important role in guiding immune development and maintaining mucosal homeostasis throughout life (60, 61).

The onslaught on the inherited microbiota does not end at birth. Young children frequently receive antibiotics. It is clear that there is substantial overuse of antibiotics, especially for upper respiratory infections including otitis media. There have been some small improvements in reducing this problem; nevertheless, Centers for Disease Control and Prevention data (62) indicate that children are receiving more than one course of antibiotics on average in each of the first 2 years of life (Table 1). Extrapolating from the prevalence data in 2010 across the United States suggests that the average child may be receiving nearly 3 courses of antibiotics by the time they are

2, 11 courses by the time they are 10, and 17 courses by the time they are 20 years old. Such data are consistent with more limited studies in other developed countries (63–65).

In fact, antibiotic usage in the United States shows important regional differences (Figure 2B). In 2010, 258 million courses of antibiotics were prescribed to outpatients in the United States, representing 833 courses per 1,000 people. In the Northeast and Midwest, rates were similar to the national averages, but the rates in the West (638/1,000 people) were about 50% lower than in the South (936/1,000 people). Because the incidence and severity of bacterially induced infectious diseases of Southerners and Westerners probably do not differ by 50%, the differences in antibiotic use likely indicate differences in medical custom and practice. Interestingly, the 2010 maps on obesity prevalence and antibiotic use show striking parallels. Such observations raise the hypothesis that excess antibiotic usage is one of the drivers of the current obesity epidemic.

Consequences on the Farm

Support for this idea comes from observations on the farm, beginning in the 1940s. At that time, it was learned that feeding livestock with low doses of antibiotics (called subtherapeutic antibiotic treatment, which we abbreviate as STAT) promoted the growth of farm animals (66, 67). There were three important observations:

1. STAT was effective across a broad range of livestock, from chickens to cows, representing a broad swath of vertebrate evolution.
2. The effects were seen with a broad range of antibacterial agents, regardless of

chemical structure, drug classification, mechanism of action, or targeted organisms. Antiviral and antifungal agents were not effective.

3. The younger in life the treatment was begun, the greater the effect from STAT.

These last observations suggested the hypothesis that the STAT regimens were affecting the development of the animals. As such, we sought to address this question in a mouse model (68).

Mouse Models

Initial work focused on C57/BL6 mice. Providing a variety of antibiotics to mice in a STAT model showed that each regimen tested changed the development of adiposity in the mice, as well as early-life bone development (68). Studies of the microbiota showed a shift in composition as well as a change in gene abundances related to short-chain fatty acid synthesis, and there was evidence in the liver of widespread changes in expression of genes involved with intermediate carbohydrate metabolism and with lipid production and transport. On-going work has confirmed and extended the findings in terms of the windows of exposure, and the combined effects of dietary and antibiotic interventions. These studies provide a model system for exploring the role of early life antibiotic exposures on host development, and may provide clues about the etiology of the obesity epidemic.

Effects on the Lung

Is widespread antibiotic use having effects on the formation and dynamics of the lung microbiome? At this point, it is clear that we just do not know. Antibiotics could affect the lung microbiome either directly through their selective force, or indirectly through

Table 1. Cumulative outpatient antibiotic use, by age

Patient Age Group (yr)	Number of prescriptions (millions)	Prescriptions/1,000 People	Average Number of Courses	
			During Period	Cumulative
0–1	16.6	1,365	2.73	2.73
2–9	29	1,021	8.17	10.9
10–19	28.9	677	6.78	17.68
20–39	55.4	669	13.38	31.06
40–64	81.6	797	19.93	50.98
≥65	41.1	1,020	—	—
Total	258	833		

Adapted by permission from Reference 62.

effects on the gut microbiome and its downstream effects on immunity. Our “other” genome, as the human microbiome has been considered, is facing an era of major changes, induced by lifestyle, diet, and antibiotic use. The use of culture-independent 16S rRNA and metagenomic approaches provides a new vision to our

understanding of the lung as a unique mucosal niche exposed to a complex microbial environment. We are just learning about associations involving the microbiome of the gastrointestinal tract and the airways, as well as the immunological consequences of the human microbiome. The combination of modern changes in

microbial environment and technological developments that allow understanding the human microbiome provides a unique opportunity to explore mechanisms of disease in this brave new world. ■

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