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The Microbiome and the Respiratory Tract

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Abstract

Although the notion that "the normal lung is free from bacteria" remains common in textbooks, it is virtually always stated without citation or argument. The lungs are constantly exposed to diverse communities of microbes from the oropharynx and other sources, and over the past decade, novel culture-independent techniques of microbial identification have revealed that the lungs, previously considered sterile in health, harbor diverse communities of microbes. In this review, we describe the topography and population dynamics of the respiratory tract, both in health and as altered by acute and chronic lung disease. We provide a survey of current techniques of sampling, sequencing, and analysis of respiratory microbiota and review technical challenges and controversies in the field. We review and synthesize what is known about lung microbiota in various diseases and identify key lessons learned across disease states.

INTRODUCTION

Since the dawn of germ theory, we have known that the lungs are constantly exposed to microbiota from inhaled air and from the upper respiratory tract. Recently, use of novel culture-independent techniques of microbial identification has confirmed that the lungs, in both health and disease, harbor diverse communities of microbiota. This revelation has challenged numerous long-held assumptions regarding respiratory health and the pathogenesis of infectious and noninfectious lung disease. Recognition that the respiratory tract is a dynamic ecosystem has infused the field of lung microbiology with fresh hypotheses and new conceptual models of disease pathogenesis.

This revolution in lung microbiology has prompted increased need for clarity and rigor in definitions. The term "microbiome" was first proposed in 2001 by Joshua Lederberg, who used it to refer to "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (1). The microbiome, as one of a series of -omes (e.g., "metagenome," "proteome," "metabolome"), initially referred only to the collective microbiota on and in a host. Unfortunately, the word was spelled identically to the ecological term used to describe "the microscopic biome" of a site: the micro-biome versus Lederberg's microbi-ome. For this article, we use the term "microbiota" to mean the collection of microbes from a site. By microbiome, we mean the community of microscopic cells, viruses, and phages that occupy a site and are under environmental pressures from that site. The microbiome includes microbial and host cells, as well as the biotic and abiotic factors that modulate the interactions of these cells. Relatively little is known about the contribution of viruses, phages, and fungi to the lung microbiome; we thus restrict our discussion largely to bacteria.

HISTORY AND EVOLUTION OF THE FIELD

Although the notion that "the normal lung is free from bacteria" (2) remains common in textbooks, it is virtually always stated without citation or argument. Although one is tempted to invoke Hitchens's razor ("What can be asserted without evidence can be dismissed without evidence"), the dogma of lung sterility is so entrenched that a brief review of its origin is warranted.

In retrospect, the claim that the lungs represent a bacteria-free compartment is a remarkable one: There is virtually no environment on earth so extreme in temperature, pH, salinity, or nutrient scarcity that microbial communities cannot be detected (3). The dogma of lung sterility posits that an exception to this ubiquity is the warm, moist mucosa mere inches below the human pharynx, topologically outside of the body. As Hilty et al. (4) stated in the seminal study in the field: "[I]t would be extraordinary if the lower airway were able to maintain sterility in the presence of high volume airflow through a damp open communication with the oropharynx." And indeed, of the more than 30 published studies that have tested the lower respiratory tracts of healthy subjects using modern molecular techniques of bacterial identification, none has demonstrated the absence of bacterial signal.

Origins of the Notion of Lung Sterility

Before the supposed sterility of the lungs was considered self-evident, it was a topic of vigorous scientific investigation. Louis Pasteur's refutation of spontaneous generation relied on the presence of viable microbes in air (5), of which healthy adults inhale more than 7,000 liters each day. The first published claim of lung sterility was in 1888 by Hildebrandt (6), who, using the available cultivation techniques of the time, identified minimal bacterial growth from the excised nasal and tracheal mucosa of rabbits. From this observation, the author inferred the sterility of the

lungs—and nasal passages!—of healthy humans. This inference was actively contested by a variety of arguments and approaches: the culture of viable bacteria from human lung specimens (7), cultivation of bacteria from the lungs of large animals (8) that resembled the microbial content of nearby bedding and hay (9), the calculation that between 1,500 and 14,000 organisms are inhaled each hour (10), and the observation that subclinical microaspiration of upper airway secretions is common among healthy adults (11). A century ago, conventional wisdom was not that "the normal lung is free from bacteria" but rather that it is under constant exposure to the microorganisms of inhaled air and the upper respiratory tract (9).

In subsequent decades, several conceptual errors gave rise to the ubiquitous and unsupported claim that healthy lungs are free of bacteria. These errors are considered in turn here.

The first conceptual error was the misapplication and misinterpretation of clinical microbiology testing. The most commonly employed tests of bacterial identification applied to respiratory specimens are the culture-based protocols of clinical microbiology laboratories. These protocols were developed and optimized for the specific purposes of identifying respiratory pathogens and discriminating acute infections from their absence (12); they were not developed to exhaustively identify the microbiota present in noninfected airways. These protocols selectively disadvantage the growth of anaerobes and bacteria with optimal growth temperatures of less than 37°C, which encompasses much of what is now described as healthy lung microbiota (13). Yet the absence of growth via these protocols has been widely misinterpreted as meaning the absence of bacteria rather than the absence of infection. In fact, when various culture conditions are applied to lung specimens from healthy subjects, more than 60% of taxonomic groups identified by sequencing can be identified by culture (14).

The second key conceptual error was the confusion of ecological contiguity with contamination. The lungs are in direct mucosal communication with the upper respiratory tract, their primary source of microbial immigration; thus, it is unsurprising that considerable overlap exists in their microbial communities (14, 15). Yet it is commonly and spuriously assumed that identification of bacteria in lung specimens must reflect contamination from the upper respiratory tract. The microbial communities of the stomach more closely resemble those of the upper respiratory tract than do those of the lung (15), yet microbiota of the upper gastrointestinal tract are never dismissed as reflecting contamination. The lungs are no more contaminated by the upper respiratory tract than is a tide pool by the ocean.

The final conceptual error that underlied the dogma of lung sterility was the conflation of two distinct concepts: sterility and the absence of resident microbes. Two observations, established since the early 1900s, demonstrated that the lungs are host to a constant, low-level immigration of microbes. Specifically, these observations were that (*a*) inhaled air contains diverse bacteria (16) and (*b*) subclinical microaspiration of oropharyngeal contents is ubiquitous among even healthy subjects (11, 17–19). The notion of lung sterility arose from the presumed absence of resident, reproducing microbes in the lung environment. Yet a microbial (or nonmicrobial) community can be exclusively defined by the balance of immigration and elimination, even in the absence of local reproduction of its members (20). By analogy, the human population of Antarctica is exclusively determined by immigration and elimination; no human reproduction occurs (21). Yet deeming the continent free of humans would be a fallacy.

The Culture-Independent Revolution

In the first decade of the twenty-first century, the advent of culture-independent techniques of microbial identification provoked a surge in interest in the human microbiome. When the National Institutes of Health launched the Human Microbiome Project in 2008, the lungs were

not included among the 18 body sites sampled. The first applications of these techniques to respiratory specimens were to patients with cystic fibrosis (CF) (22, 23), in whom the sterility of the lung was never entertained. These early studies demonstrated a wide diversity of bacteria in sputum specimens, undetected by conventional culture-based techniques.

In 2010, Hilty et al. (4) published the first application of these culture-independent techniques to the airways of healthy subjects. Using both bronchoalveolar lavage (BAL) and protected specimen brushings in healthy volunteers and patients with asthma, they made two key, subsequently validated, observations: (*a*) Healthy airways contain bacteria similar to but distinct from those of the upper respiratory tract, and (*b*) the airways of patients with respiratory disease contain distinct communities that are relatively enriched with the Proteobacteria phylum. Within several years, reports of altered lung microbiota in a variety of lung diseases were published.

The rate of publications in the field has grown exponentially and without plateau. Dozens of research groups from around the world have contributed to the field, with important contributions made from researchers with backgrounds in microbiology, microbial ecology, pulmonary pathophysiology, infectious disease, and bioinformatics. Whereas most early studies in the field were small, retrospective, and exploratory surveys of convenience samples, more recent studies are increasingly prospectively designed, appropriately controlled, and hypothesis driven. With increasing frequency, the lung microbiome is being studied as a secondary outcome and a potential mediator of benefit in prospective, interventional trials.

TECHNIQUES USED IN THE STUDY OF THE LUNG MICROBIOME

A comprehensive review of molecular techniques of microbial identification would exceed the scope of this manuscript; we provide, in the Literature Cited, sources for further reading (13, 24). We here review the basic principles of culture-independent analysis and discuss challenges specific to the study of respiratory specimens.

Culture-Independent Methods of Microbial Identification

The most commonly used modern approach to the study of bacterial communities employs high-throughput sequencing of amplicons of the 16S rRNA gene, a small and highly conserved locus of the bacterial genome. When sequenced using platforms such as 454 pyrosequencing and Illumina MiSeq, DNA isolated from a single specimen yields thousands of short genome sequences. These sequences are aligned, sorted, and classified according to publicly available taxonomic databases. Sequences are clustered by similarity; by convention, sequences sharing 97% homology are grouped as operational taxonomic units (OTUs), which are roughly comparable to genus- or species-level phylogeny. The result of this bioinformatic analysis is a population census for each specimen: a tally of how many times each taxonomic group was counted. Comparisons in total bacterial abundance (using quantitative PCR), relative abundance (how much of a specimen's community is composed of a single taxonomic group), and community features (such as diversity) can be made across specimens. These sequencing-based techniques do not rely upon the reproduction of microbes in the narrow growth conditions of conventional culture techniques, which fail to identify most human-associated microbes.

Role of Potential Sources of Contamination in Studies of the Lung Microbiome

Although the sequencing technology and bioinformatic analyses summarized above are common across microbiome studies regardless of body site, the study of respiratory specimens presents

unique technical challenges. Whereas the high bacterial burden of specimens such as stool overwhelms any potential contamination introduced by procedural sampling or by laboratory reagents, additional thought must be given to the design and interpretation of experiments when one is studying the relatively low biomass specimens of the respiratory tract.

Reagents. Due to the low levels of bacteria in the healthy lung, a key concern in the field is the potential contaminating influence of bacterial DNA present in laboratory reagents. Sterile laboratory reagents—including those found in DNA isolation kits—contain small amounts of bacterial DNA. Salter et al. (25) demonstrated a clear dose-response relationship between progressive dilution of bacterial DNA and contamination by sequences derived from laboratory reagents. With each serial dilution, an increasingly large fraction of the sequenced "community" was composed of contamination from laboratory reagents. This contamination was not random but rather was specific to the sequencing laboratory and to the extraction kit used. Removal of eukaryotic cells from BAL fluid further reduces the bacterial content of BAL fluid and alters community composition (26); thus, concurrent sequencing of laboratory reagents is of special importance if acellular BAL specimens are used. These findings underscore the need for thoughtful use of procedural control specimens in lung microbiome analysis. We recommend that multiple specimens of prominent potential sources of contamination (e.g., bronchoscope rinse, buffer from DNA isolation) be sequenced concurrently with respiratory specimens.

Upper respiratory tract. Most studies in the field to date have used BAL fluid or protected specimen brushings to access the microbiota of the lower respiratory tract. The passage of a bronchoscope through the upper respiratory tract introduces a theoretical risk of contamination via carryover of pharyngeal microbiota, but multiple lines of evidence suggest that this effect is minimal. Despite the markedly divergent microbiota of the mouth and nose, the route of bronchoscope insertion (oral versus nasal) has no detectable influence on BAL microbiota (**Figure 1**) (15, 27, 28). Several investigators have performed serial sampling experiments on healthy subjects to determine whether bacterial communities are diluted with serial lavages or brushings in the lower respiratory tract (15, 29, 30), as is expected if most bacterial signal is attributable to bronchoscopic carryover. None has found evidence that bacterial communities are diluted with serial sampling. These observations suggest a minimal influence of pharyngeal contamination on the microbiota detected via bronchoscopic specimens.

Sputum specimens have been extensively used in the study of some diseases [CF, bronchiectasis, and chronic obstructive pulmonary disease (COPD)]. Although use of sputum introduces an additional risk of upper airway contamination, features of microbiota identified in sputum have been significantly and consistently associated with numerous biologically and clinically meaningful indices: severity of illness (31), airway inflammation (32, 33), antibiotic exposure (34, 35), response to controlled viral exposure (32), and frequency of subsequent exacerbation frequency (36). The presence of oropharyngeal microbiota thus does not obscure the meaningful microbial signal in sputum that is correlated with established indices of lung health.

ECOLOGY AND TOPOLOGY OF THE HUMAN RESPIRATORY TRACT

To understand an ecosystem—and the biome of the respiratory tract is an ecosystem—one must consider both the conditions of its terrain and its population dynamics. In the study of the lung microbiome, our understanding of these factors benefits from a century of study of the anatomy, physiology, and pathophysiology of the human respiratory tract.

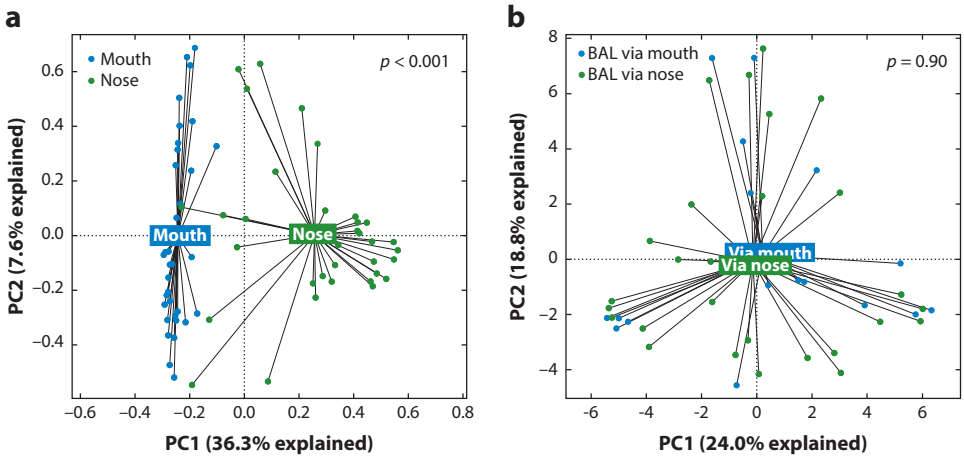

Figure 1

Lack of influence of upper respiratory tract microbiota on bronchoalveolar lavage (BAL) microbiota. Although the microbial communities of the mouth and nose differ significantly (*a*), the route of bronchoscope insertion (via mouth or via nose) has no appreciable effect on BAL microbiota (*b*). Panel *a* adapted with permission from data published in Reference 15. Panel *b* adapted with permission from Reference 27.

The Terrain of the Respiratory Ecosystem

The airways and alveoli of the human respiratory tract are topologically outside of the body; when the larynx is open, no additional physical barriers separate the most distal alveolus from the dense microbial ecosystem of the pharynx. Whereas the linear distance from nares to alveolus is only half a meter, the internal surface area of the lungs is 30 times that of the skin (37) and is larger than current estimates of the surface area of the gastrointestinal tract (38). Unlike the intestines, which are lined by a thick, protective mucus layer, the airways produce relatively little mucus in health (~100 mL per day) (39). A thin and selectively bacteriostatic (40) layer of lipid-rich surfactant lines the alveoli, preventing local collapse. Thus, the respiratory tract is in general a low-nutrient environment for microbes compared with the nutrient-rich environment of the gastrointestinal tract.

Population Dynamics in the Respiratory Tract

The total microbial burden and relative community membership of the lung bacterial microbiome are determined by the balance of three factors: microbial immigration, microbial elimination, and the relative reproduction rates of the members (**Figure 2*a***) (20, 28). Any alteration of lung microbiota must be attributable to some combination of these three factors. In health, the lung microbiome is determined largely by the balance of immigration and elimination, with relatively little contribution from the differential reproduction rates of its members (14, 29).

The primary routes of microbial immigration to the lungs are microaspiration, inhalation of air, and direct dispersion along mucosal surfaces. Of these, microaspiration is likely the dominant route of immigration on the basis of the overlap in community composition between oral and lung microbiota (14). Multiple studies since the 1920s have demonstrated that subclinical microaspiration is ubiquitous among healthy subjects (11, 17–19). Decades before the microbiome revolution, investigators who studied microaspiration wrote with confidence that aspiration "occurs commonly

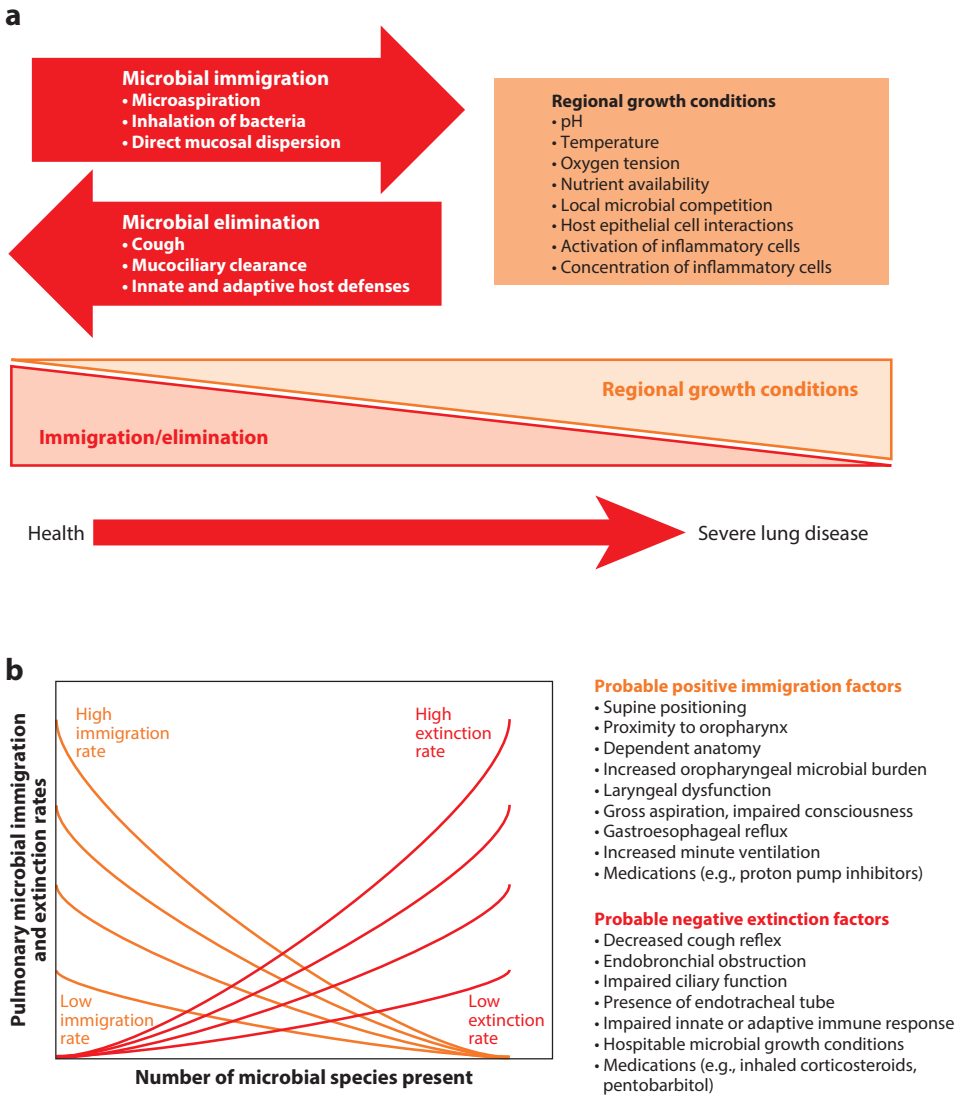

Figure 2

Ecological modeling of the respiratory microbiome. (*a*) The constitution of the respiratory microbiome is determined by three factors: microbial immigration, microbial elimination, and the relative reproduction rates of its members. In health, community membership is determined primarily by immigration and elimination; in advanced lung disease, membership is determined primarily by regional growth conditions. Adapted with permission from Reference 28. (*b*) The adapted island model of lung biogeography. Community richness in health for a given site in the respiratory tract is a function of immigration and elimination factors. Adapted with permission from Reference 20.

among healthy young men during sleep . . . the quantity aspirated is of an order of magnitude likely to contain bacterial organisms in physiologically significant quantities" (17). Thus, "normal adults are constantly contaminating their lower respiratory tract with bacteria: however, infection only develops when normal pulmonary defense mechanisms are either impaired or overwhelmed and the aspirated bacteria can rapidly multiply" (18). The primacy of subclinical microaspiration

among routes of microbial immigration to the lungs is thus thoroughly established and is entirely consistent with more recent microbial ecology validations (14, 29). Additionally, atmospheric air contains 10^4–10^6 bacterial cells per cubic meter, even before traversing the microbe-dense upper airways (41). Finally, direct contiguous spread of reproducing microbial colonies is a theoretical route of immigration, although to date no study has compared the composition of contiguous airway communities via serial brushings.

Elimination of microbes from the lungs is an active and continuous process. Healthy airways employ a mucociliary escalator that uses ciliated epithelial cells to constantly and steadily propel microbes cranially along the thin, basally secreted mucus layer (39). Even in the absence of infection, healthy subjects cough roughly once every other hour (42), propelling microbes into the laryngeal pharynx, where they are either swallowed or expectorated. The turbulent act of coughing likely homogenizes the luminal contents of the airways, which may partially explain the relatively low spatial variation observed in the microbiota of healthy human lungs (29). Finally, the lungs and airways possess a diverse arsenal of innate and adaptive immune defenses that selectively recognize, kill, and clear microbiota. The abundance and identity of host inflammatory cells are associated with features of the lung microbiome in health (30).

The local conditions relevant to microbial growth within the respiratory tract are heterogeneous. Considerable regional variation can be found in a single healthy lung in numerous environmental factors that impact bacterial growth: oxygen tension, pH, relative blood perfusion, relative alveolar ventilation, temperature, epithelial cell structure, deposition of inhaled particles, and concentration and behavior of inflammatory cells (20, 43–45). Despite considerable spatial variation in these environmental factors within healthy lungs, spatial variation within the lungs of healthy subjects is modest (29), confirming that in health, local selective pressure on bacterial growth in the lungs is less important than the balance of immigration and elimination in determining microbial community composition.

Borrowing a seminal conceptual model from the field of ecology (46), we have proposed the adapted island model of lung biogeography (**Figure 2*b***) (20). This model acknowledges that in health, the respiratory tract is a single ecosystem extending from the nares to the alveoli, comprising a continuous, and continuously varying, microbial topography that is subject to the constant immigration of microbes from a single source community (the upper respiratory tract). The number of microbial species at a given site in the respiratory tree is an integrated function of a number of immigration and extinction factors. In a published validation study (29), we demonstrated that, as predicted by the adapted island model, community richness decreases with increasing distance from the source community of the upper respiratory tract. The predicted effects of various anatomical, physiological, and clinical factors on the island model are listed in **Figure 2*b***.

Effects of Acute and Chronic Lung Disease on the Respiratory Ecosystem

The presence of lung disease alters both the population dynamics of microbial immigration and elimination and the terrain of the respiratory ecosystem and local growth conditions. The unique constellation of anatomical and physiological changes that define each lung disease translates into a unique constellation of environmental conditions and altered microbial communities (**Figure 3**). To borrow from Tolstoy: All healthy lungs are alike; every unhealthy lung is unhealthy in its own way.

Immigration of microbiota is accelerated by the presence of gastroesophageal dysfunction and reflux, which are extremely common (with >70% prevalence) among patients with severe lung disease (47, 48). Numerous diseases (e.g., CF, bronchiectasis, and chronic bronchitis) feature

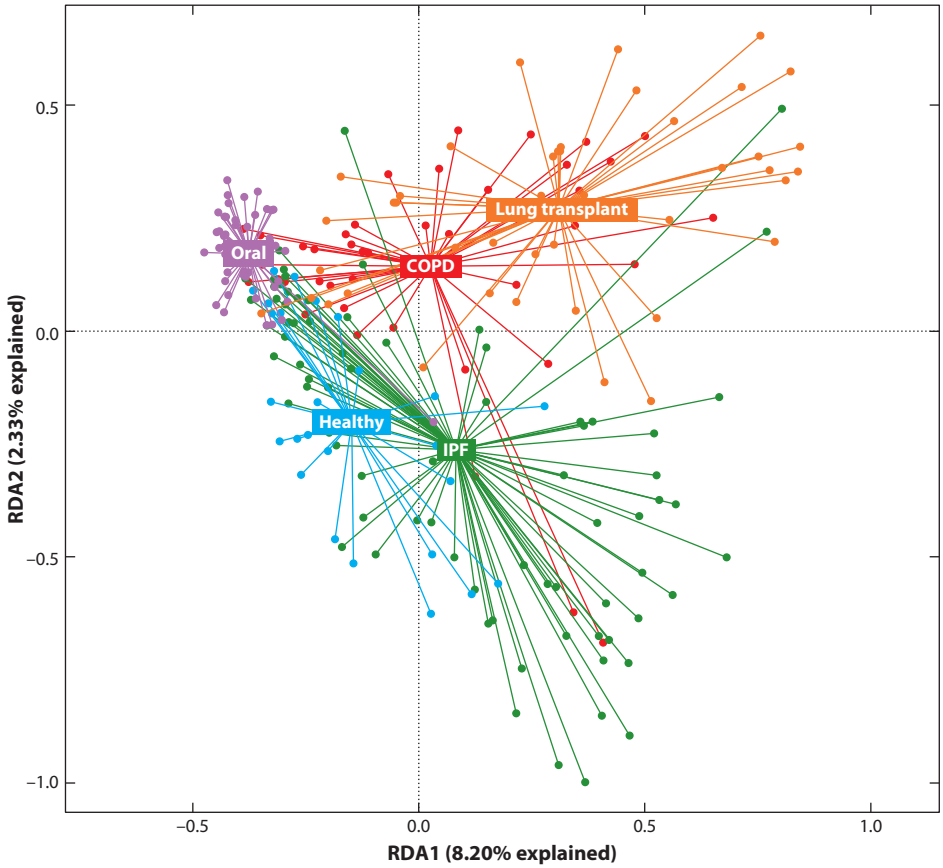

Figure 3

Lung microbiome communities vary by disease state. Each data point represents the bacterial community detected in a specimen acquired from the population labeled. The unique constellation of anatomical and physiological changes that define each lung disease translates into a unique constellation of environmental conditions and altered microbial communities. "All healthy lungs are alike; every unhealthy lung is unhealthy in its own way."

mucociliary clearance impairment, which inhibits the rate of microbial elimination. Microbial elimination is accelerated by the presence of acute or chronic cough and by the influx and activation of host inflammatory cells. The surface area of the lungs is decreased by as much as 90% in advanced destructive diseases such as emphysema and pulmonary fibrosis (49, 50). Thus, even if the rate of bacterial immigration is unchanged, the same absolute burden of microbes is distributed to a far smaller terrain; this may in part explain the increased bacterial burden observed in patients with severe destructive lung disease (51).

The presence of acute and chronic lung disease dramatically alters the local conditions of microbial growth in the respiratory tract. As the severity of lung disease worsens, the composition of the lung microbiome is determined more by regional growth conditions and the differential reproduction rates of its members than by the balance of immigration and elimination (**Figure 2*a***). This shift is evidenced by the long-acknowledged association between severity of lung disease

and the identification of persistent bacterial species (colonizers) via culture-based testing: These species represent community members well adapted to the specific environmental conditions of the injured respiratory tract. Injury and inflammation of the respiratory tract are associated with increased mucus production. Mucus production results in local pockets of anoxia (52) and increased temperature (53), both of which selectively favor the growth of certain community members. The inflammatory cells of the airways and alveoli increase in number and activation (54–56), killing and clearing bacteria with highly variable effectiveness (57) and creating selection pressure across the bacterial community. Numerous by-products of the host inflammatory response, including catecholamines (58), inflammatory cytokines (59–61), increased temperature (53, 62), and free ATP (53, 62), are known growth factors for select bacterial species. The nutrient supply of the airways, scarce in health, is abruptly increased by the presence of mucus and vascular permeability. In addition to the intrinsic changes to the respiratory ecosystem in disease, many therapies for lung disease have clear effects on microbial growth conditions. Supplemental oxygen, systemic and inhaled corticosteroids, and systemic and inhaled antibiotics likely have pleiotropic effects on the influx, efflux, and relative reproduction rates of lung microbiota.

THE MICROBIOME OF THE HEALTHY RESPIRATORY TRACT

The Origins and Development of the Lung Microbiome

In utero, the lungs are filled with amniotic fluid, which has historically been presumed to be free of bacteria. Recent use of culture-independent techniques has revealed that bacterial DNA is present in amniotic fluid and placental specimens (63, 64), raising the possibility that prenatal lung development occurs in the presence of microbial communities. Provocatively, the taxonomic distribution of these detected bacteria more closely resembles that of the oral cavity than it does other body sites (63). To date, no study has confirmed the viability of the placental microbiome via culture or transcriptomic approaches, and the clinical and developmental significance of this finding is undetermined.

Immediately following delivery, a newborn's microbiota resemble those of his/her mother and are specific to the route of delivery (65). The microbiota of newborns are relatively homogeneous across organ sites but very quickly (over days and weeks) differentiate into organ-specific communities (66). The intestinal microbiota of young children are highly dynamic before stabilizing at approximately 3 years of age to resemble those of adults (67), a pattern of community maturation that is recapitulated in the microbiota of the upper respiratory tract (68, 69). No analogous study of lower respiratory tract microbiota has been performed. The microbiota of the upper respiratory tract are influenced by exposures such as breast-feeding (68), day care attendance, and treatment with antibiotics (69).

Composition of the Healthy Respiratory Microbiome

Culture-independent studies of the healthy lung microbiome have been consistent in their account of the dominant taxonomic groups. The most abundant phyla are Bacteroidetes and Firmicutes; prominent genera uniformly include *Prevotella*, *Veillonella*, and *Streptococcus* (27, 30, 70). Microbiota of the lung more closely resemble those of the mouth (its primary source community) than those of other body sites (14, 15). Several reports have identified a relative enrichment of *Tropheryma whipplei* in healthy lungs relative to the mouth (29, 70, 71), implying site-specific reproduction and selective growth, but this organism is at most a small minority [~1% (71)] of the healthy lung bacterial community. Healthy subjects vary considerably in the similarity of the lung and

mouth microbiota (15, 30); it is undetermined whether this variation is persistent over time within subjects or is correlated with clinical phenomena such as esophageal reflux, laryngeal dysfunction, and oral hygiene. It is plausible but unproven that the bulk of physiological microaspiration occurs during sleep, when subjects are supine and protective laryngeal and cough reflexes are depressed (72). Mouth-lung microbial similarity may be circadian, peaking overnight and waning during the day, when immigration is slowed and elimination is enhanced.

Spatial Variation in the Human Respiratory Tract

Although the lung microbiota of subjects with advanced lung disease vary considerably within the lungs (and lobes) of a single subject (73–75), lung microbiota of healthy subjects are relatively uniform within a single respiratory tract (29). Considering that the healthy lung environment contains considerable spatial variation in bacterial growth conditions, the lack of spatially consistent spatial variation (e.g., a left-upper-lobe microbiome) implies that in health, lung microbiota are determined more by the balance of microbial immigration and elimination than by local growth conditions (**Figure 2*a***). As predicted by the adapted island model of lung biogeography (**Figure 2*b***), community richness decreases with increasing distance from the primary source community of the oropharynx (29).

Whether the lung microbiome of healthy subjects varies geographically has not been determined. A study analyzing lung microbiota from healthy subjects from eight US cities found no evidence of geographic clustering (70), and the most abundant community members detected in British volunteers resemble those of healthy American subjects (4, 51). The microbiota of the human gastrointestinal tract exhibit considerable geographic variation when studied internationally (67), but to date studies of the lower respiratory tracts of healthy volunteers have been restricted to Western Europe and North America.

THE MICROBIOME OF THE DISEASED LUNG

Asthma

From the numerous studies that have examined the lung microbiota of patients with asthma (76–80), two provocative observations have emerged. First, the development of allergic asthma is associated with altered microbiota in childhood. Second, differences in lung microbiota are associated with patients' clinical course and responsiveness to therapy and may define an understudied disease variant.

A clear and consistent association has been observed between childhood exposure to microbes, alterations in gastrointestinal and pharyngeal microbiota, and subsequent development of asthma (69, 81). In general, greater burden of bacterial exposure and increased bacterial diversity are protective against asthma development (81), although the presence of specific potentially pathogenic bacterial species in the respiratory microbiota of children has been positively associated with asthma (69). In animal models, manipulation of the gastrointestinal microbiome—by antibiotics or changes in diet—can profoundly alter the host's CD4 T cell–mediated allergic airway response (82, 83). The lines of causality in this web of pathogenesis—involving the microbiota of the gut and lung as well as the local and systemic immune response—are likely complex and bidirectional. But once asthma is established, its relationship with altered airway microbiota is inarguable. Adult asthmatics, even at clinical baseline (4, 76) and without treatment (78), exhibit altered lower respiratory tract microbiota, typically with increased community diversity and enrichment with members of the Proteobacteria phylum.

Among asthmatics, features of the respiratory microbiome correlate both with airway reactivity (76) and with physiological and clinical response to corticosteroids (77) and macrolide antibiotics (76). Recently, interest has grown in noneosinophilic asthma, a reactive airway disease that lacks the T_H2-associated airway inflammation found in most asthmatics. Compared with patients with eosinophilic asthma, patients with noneosinophilic asthma have more frequent severe exacerbations and respond more poorly to corticosteroids (84). Researchers have recently posited that this phenotype of asthma may be driven by a disordered microbiome (80), and animal models and human studies suggest that this phenotype may be especially responsive to macrolides (85, 86). Unsurprisingly, macrolide therapy alters the lung microbiota of asthmatics (79), although it is undetermined whether the conjectured benefit of macrolides in noneosinophilic asthma is attributable to a direct antimicrobial effect, host immunomodulation (85), or both.

COPD

The relationship between disordered airway microbiota and COPD differs from that of disordered airway microbiota and asthma. Whereas in asthma even patients with mild disease have detectably altered airway microbiota (4, 78), the airway microbiota of patients with mild and moderate COPD is indistinguishable from those of healthy volunteers (32, 51, 75, 87, 88). Yet once COPD has advanced in severity (generally with an FEV_1 of less than 40–50% of the predicted value), airway microbiota are consistently altered. Most studies of patients with advanced COPD have observed a shift in community membership away from the Bacteroidetes phylum (75, 89), often to Proteobacteria and its familiar potentially pathogenic members (e.g., *Pseudomonas* spp., *Haemophilus* spp.) (75, 89, 90) and sometimes to Firmicutes (90, 91). Garcia-Nuñez et al. (89) demonstrated an unambiguous relationship between severity of airway obstruction and decreased community diversity; the least diverse airway community in patients with FEV_1 greater than 50% predicted was more diverse than the most diverse airway community in patients with FEV_1 less than 50% predicted. Thus, unlike for asthma, for which airway inflammation and respiratory dysbiosis appear to be linked at every stage of severity, the respiratory dysbiosis of COPD may emerge only once a critical threshold in severity has been crossed.

Despite this difference, two intriguing observations have provided evidence that respiratory microbiota may be relevant even in early COPD. In a study using a human model of virus-induced COPD exacerbation, Molyneaux et al. (32) analyzed the sputum of subjects with mild COPD and healthy control subjects. The sputum microbiota of COPD subjects was similar to those of healthy subjects at baseline, but after viral exposure the COPD sputum had a significant shift toward the Proteobacteria phylum that was absent in the sputum of healthy subjects. Key bacteria that spiked in abundance during exacerbation exposure were detected in the same subjects at baseline before viral exposure as well as after clinical resolution of the exacerbation. Thus, although at baseline the lung microbiota of early COPD may be indistinguishable from those of healthy airways, the community's dynamic response to inflammatory insult may be disordered and may contribute to the pathophysiology of exacerbations. Separately, Sze et al. (88) studied the microbiota, histology, and inflammation of surgically excised lung tissue from patients with mild and moderate COPD as well as from patients with normal airways. Although the bacterial burden and community composition of the lung tissue were indistinguishable across patient groups, the presence of the glycerol dehydratase gene (found in certain *Lactobacillus* spp. and speculated to have a protective anti-inflammatory effect) was negatively associated with the presence of COPD and was inversely correlated with the local abundance of macrophages and neutrophils. This gene was absent in patients with more severe disease. These two studies illustrate how in early COPD, a grossly

normal microbiome may be functionally disordered via the presence—and absence—of important minor community members that respond to and mediate changes in host inflammation.

Several recent observations have raised the possibility that the presence of fungi in the respiratory tract—the so-called "mycobiome"—may be in part a cause or consequence of COPD. Chronic airway colonization with *Pneumocystis jiroveci* has been correlated with severity of obstruction in COPD (92), and a recent sequencing-based survey suggested that other fungal species are enriched in COPD airways (93). *Aspergillus fumigatus*, another familiar fungal pathogen, is commonly detected in the sputum of COPD patients; sensitization to *Aspergillus* is significantly associated with worse lung function (94).

Cystic Fibrosis

A relationship between respiratory microbiota and disease progression in CF has been uncontroversial since the early descriptions of the disease. By adolescence, nearly all CF patients grow select respiratory pathogens from sputum (95, p. 33), both during clinical stability and during the exacerbations that punctuate the disease's natural history. Although exacerbations have long been considered infectious events, two large trials have found no detectable association between patients' clinical response to antibiotic therapy during exacerbations and the in vitro susceptibility of their cultured organism to their administered antibiotic (96, 97). The clinical response of patients receiving antibiotics during exacerbations is unrelated to a concurrent change in culture-determined sputum bacterial burden (98). Thus, even prior to the advent of culture-independent techniques, evidence suggested that the relationship between airway microbiota and CF pathogenesis was more complex than that of simple monomicrobial airway infections. Early culture-independent studies revealed a far greater microbial diversity in sputum than had been appreciated via culture (23). The viability of many microbes was confirmed via both amplification of transcribed RNA (99) and advanced culture techniques (100). Community diversity of sputum microbiota decreases with age and disease severity (13, 31, 35), although this loss of diversity is most tightly correlated with cumulative antibiotic exposure (34, 35).

Several observations demonstrate the potential ways in which an understanding of the lung microbiome could alter our therapeutic and diagnostic approach to CF. In two separate clinical trials, administration of enteric probiotics to patients with CF significantly decreased the frequency of pulmonary exacerbations (101, 102). Although probiotics certainly influence the community composition of intestinal microbiota, what effect they have on respiratory microbiota is unknown. A longitudinal study of stool and pharyngeal specimens from children with CF found overlap and concordant shifts in community composition across the gut and respiratory tract (103); thus, both the enteric and respiratory microbiome may be viable therapeutic targets in this population. Studying daily sputum specimens in patients with CF has revealed suggested possible avenues for the enhancement of our diagnostic tools. In one large analysis of daily sputum specimens from multiple CF patients, sputum communities were temporally stable at clinical baseline; before or at the time of clinical exacerbation, a decrease in the relative abundance of dominant taxonomic groups was observed concurrent with a varied drop in community stability (104). The same research group, using liquid chromatography mass spectrometry, demonstrated the feasibility of characterizing the metabolomic activity of bacterial communities in sputum, including the detection of molecules that mediate quorum sensing in *Pseudomonas aeruginosa* (105). These complementary techniques may soon be leveraged for clinical purposes to rapidly determine the identity and metabolic activity of respiratory microbiota in CF via noninvasive and culture-independent means.

Bronchiectasis

The microbiome of the airways of non-CF bronchiectasis has not been studied as extensively as that of CF. A study comparing explanted lung tissue found comparable microbiota in CF and non-CF bronchiectasis lungs (106), but it is unknown how generalizable findings of CF studies are to this population. Similar to the microbiota of other chronic inflammatory diseases of the airways, the microbiota of bronchiectasis are relatively enriched with Proteobacteria (107), and the relative abundance of prominent community members (*Haemophilus* spp. and *Pseudomonas* spp.) is significantly associated with concentrations of matrix metalloproteinases implicated in the progression of irreversible airway disease (108). Importantly, the community composition of the microbiota of bronchiectasis patients at their clinical baseline is predictive of subsequent exacerbation frequency (36), an association that is undetectable when restricted to culture-based results.

Lung Transplantation

Lung transplantation dramatically alters the terrain and population dynamics of the respiratory ecosystem. Transplantation impacts immigration [via increased reflux and aspiration (109)], elimination (via an impaired/absent cough reflex and aggressive systemic immunosuppression), and regional growth conditions (via the selective pressure of *Pneumocystis* prophylaxis). The leading cause of death in the first year following transplant is infection, overtaken in subsequent years by chronic rejection in the form of bronchiolitis obliterans syndrome (BOS) (110). Multiple culture-based studies have consistently shown that airway colonization with *P. aeruginosa* is predictive of subsequent development of chronic rejection (111–113).

Four studies to date have examined the lung microbiome of transplant recipients using culture-independent techniques (27, 114–116). All have shown that the bacterial burden in transplant BAL is elevated compared with that of healthy control subjects. All have found community enrichment with Proteobacteria, often members of the *Pseudomonas* genus and other familiar lung-associated taxa. Although most of these studies have reported decreased community diversity in transplant BAL microbiota, we have observed that the decrease in community diversity is confined to patients with active respiratory symptoms, is not present when analysis is restricted to asymptomatic subjects, and is strongly associated with measures of acute infection (increased bacterial burden, BAL neutrophilia, and positive culture growth) (27). These associations illustrate a key lesson applicable to all lung microbiome studies: Subjects' clinical status—specifically the presence or absence of acute infection—must be considered a key potential confounder in microbiome analysis. Patients with respiratory infections should be either systematically excluded from study of chronic lung disease or analyzed separately from clinically stable patients.

Studies of the posttransplant lung microbiome have demonstrated that transplanted lungs are susceptible to the growth of multiple *Pseudomonas* species. One study found a surprising negative association between the presence of *Pseudomonas* spp. in lung microbiota and the development of BOS (116). In our own analysis of posttransplant BAL communities, we identified at least two distinct *Pseudomonas* species with profoundly divergent clinical associations (27). *P. aeruginosa*, when present, dominates respiratory communities and is strongly associated with evidence of acute infection (patient symptoms, high bacterial burden, BAL neutrophilia, and BAL culture growth). By contrast, a distinct *Pseudomonas* species, *Pseudomonas fluorescens*, was common among asymptomatic subjects, was not associated with acute infection, and was undetected by culture. The identities of the two pseudomonads were indistinguishable by initial 16S rRNA classification but were readily determined using complementary techniques of microbial identification (e.g., NCBI

BLAST, species-specific PCR, and phylogenetic tree generation). We subsequently identified *P. fluorescens* as an underappreciated colonizer of diseased airways in numerous disease states (117); its clinical significance in lung transplantation or other lung diseases is unknown.

Idiopathic Pulmonary Fibrosis

Although bacteria have not traditionally been implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF), recent clinical observations and culture-independent studies have sparked widespread interest in the hypothesis that the microbiome contributes to disease progression. A randomized controlled trial of cotrimoxazole found reduced mortality among IPF patients receiving antibiotic therapy (118), and the PANTHER trial demonstrated increased mortality in IPF patients receiving immunosuppression (119).

Two recent studies have demonstrated associations between the lung microbiome and prognosis in IPF. Han et al. (120) observed a positive association between the relative abundance of specific microbial community members (*Staphylococcus* spp. and *Streptococcus* spp.) and disease progression. Separately, Molyneaux et al. (121) found that total bacterial burden in BAL fluid was positively associated with mortality independently of all measures of disease severity. The increased risk associated with a high bacterial burden (hazard ratio 4.6) is comparable to the increased risk associated with 6-month decline in lung function, the current prognostic standard in IPF (122). BAL bacterial burden was decreased among patients with the rs35705950 polymorphism of the *MUC5B* mucin gene; this polymorphism has been associated with an increased risk of developing IPF and with improved survival among patients with established disease (123). These observations have sparked excitement in what may be an important and unstudied component of pathogenesis in this relentlessly progressive, incurable disease.

Pneumonia

The culture-based protocols of clinical microbiology laboratories were derived not to identify bacteria in healthy lungs, but rather to identify the presence of acute infection. Culture-independent comparisons have demonstrated that, with occasional exception, such protocols do this job well. We previously reported that the results of quantitative BAL culture (the closest approximation of a clinical gold standard in pneumonia) correlate well with culture-independent indices of infection: total bacterial burden, low community diversity, and community domination by a single pathogen (12). Culture-dependent and culture-independent analyses of BAL from pneumonia patients are usually well aligned, although we have identified rare instances of culture-negative pneumonia (12). Although an adequate depth of sequencing can identify numerous additional bacterial species in the BAL of pneumonia patients (124, 125), the dominant pathogenic species generally composes the vast majority (75% or more) of detected sequences. Some long-assumed beliefs about the microbiology of pneumonia are still true: Most pneumonia is caused by a single pathogen, and most (but not all) pathogens are effectively identified by bacterial culture.

Discovery of the lung microbiome has, however, upended our understanding of pneumonia pathogenesis (20). The conventional model of pneumonia pathogenesis reflects the assumption that lungs are sterile in health: An inoculum of a pathogenic species gains entry to the lower respiratory tract, overwhelms host defenses, and results in the rapid and unrestrained growth of a single bacterial species. The recognition that the lungs are a dynamic ecosystem has challenged this model. The identified pathogenic species in a given pneumonia is merely one of many with access to the lower respiratory tract, each with its own positive and negative growth factors. In numerous studies of healthy volunteers, potential pathogens have been identified in the lower respiratory

tract of asymptomatic, uninfected subjects (4, 32, 75). Rather than reflecting the invasion of a sterile space by a pathogen, pneumonia is instead an emergent phenomenon of low microbial diversity, high microbial biomass, and host inflammation that arises from a preexisting homeostasis of biodiversity (20).

In this ecological model of pneumonia pathogenesis, a key question is how the reproduction of a single community member is selectively favored, despite competition from dozens or hundreds of other bacterial species. A promising hypothesis, supported by a wealth of in vitro evidence, is that the growth of select potential pathogens in the lung environment is paradoxically promoted by features of host inflammatory defenses, establishing a positive feedback loop that propels the reproduction of these pathogens and the domination of lung microbiota. As an example, numerous in vitro studies have shown that catecholamines enhance the growth, biofilm production, and virulence of *P. aeruginosa* (58, 126) and *Streptococcus pneumoniae* (62). We previously observed that increased alveolar catecholamine concentrations are tightly correlated with indices of acute infection, as well as with the emergence of specific microbiome members as dominant species (127), making plausible the posited positive feedback loop of emergent pneumonia: Select bacterial growth provokes alveolar inflammation, resulting in increased intraalveolar catecholamines, which in turn further provoke selective bacterial growth among catecholamine-responsive species. Similar in vitro growth-promoting effects have been observed with TNF-α, IL-1, IL-6, and IL-8 (59–61), all of which are part of the acute host response to infection. With culture-independent methodology, we have the means of studying the emergence of pneumonia within an ecosystem and of unfolding how it is both blunted and accelerated by features of the host response.

KEY CONCEPTS AND MODELS

The Dysbiosis-Inflammation Cycle of Lung Disease

In every lung disease studied to date, the lung microbiome is altered compared with that of healthy subjects. A natural question is the directionality of causation behind these associations: Does an altered lung microbiome drive the progression of lung disease, or is it merely a secondary consequence of the altered growth environment of the lungs? We have proposed a model of the host-microbiome interface—the dysbiosis-inflammation cycle—that acknowledges the bidirectionality of this relationship between an altered lung microbiome and the host response (**Figure 4**) (28). Any source of inflammation in the respiratory tract provokes a cascade of host responses that alter the microbial growth conditions of the airways. Increased airway wall permeability and mucus production introduce a nutrient supply to the normally sparse lung environment. The generation of intraalveolar catecholamines and inflammatory cytokines promotes the growth of select bacterial species (e.g., *P. aeruginosa*, *S. pneumoniae*, *Staphylococcus aureus*, *Burkholderia cepacia* complex) (58–61, 126), whereas the recruitment and activation of inflammatory cells kill and clear bacteria with variable, species-specific effectiveness. Mucus introduces pockets of increased temperature and decreased oxygen tension, selectively favoring the growth of prominent disease-associated microbes (52, 53). These multifold effects on microbial growth conditions result in a disordered and dysregulated community of respiratory microbiota. The shift in community membership to species with enhanced immunogenicity exposes the airways and alveoli to pathogen-associated molecular patterns and microbial metabolites that provoke further inflammation, which in turn further alters airway growth conditions. Thus, a self-amplifying feedback loop perpetuates respiratory inflammation as well as the disordered microbiota that propel it. Tissue injury to the airways and parenchyma can occur via a combination of direct injury from prominent community members,

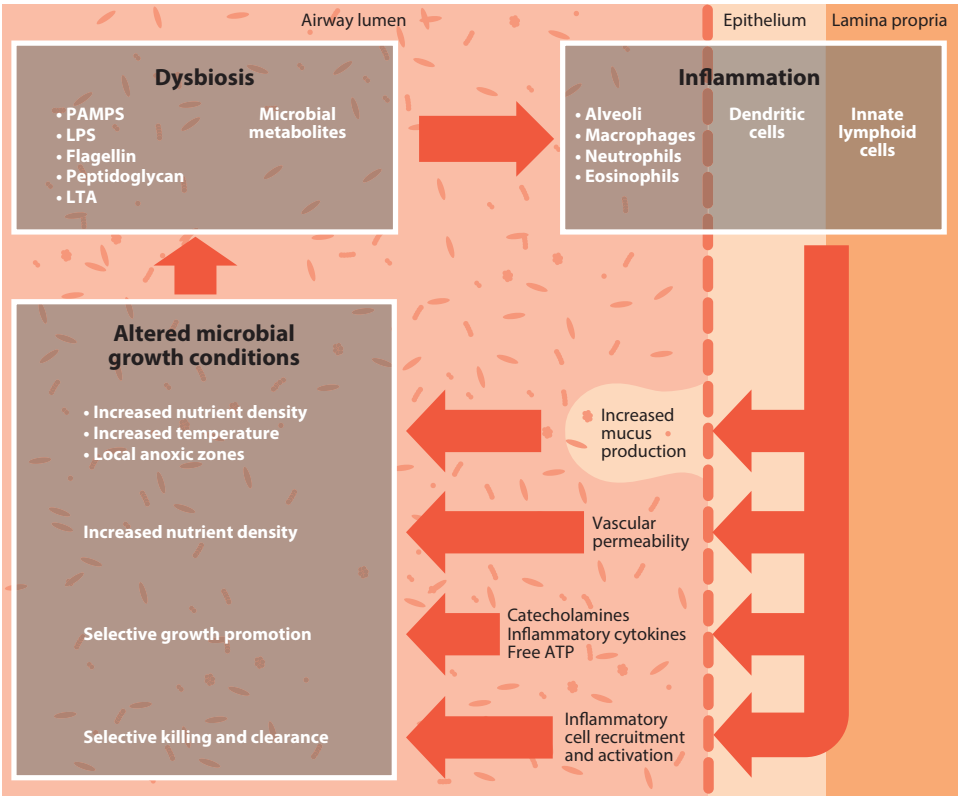

Figure 4

The dysbiosis-inflammation cycle. Inflammation of the airways alters environmental growth conditions of airway microbiota via positive and negative selective pressures. Disordered growth conditions result in a disordered microbiome, which provokes further airway inflammation via pathogen-associated molecular pattern (PAMP)–pattern recognition receptor (PRR) interactions, via microbial metabolite signaling to leukocytes and epithelial cells, and via other pathways. This cascade results in a self-amplifying cycle of airway inflammation and respiratory dysbiosis. Adapted from Reference 28.

alteration in microbial behavior, and the indirect effects of the dysregulated inflammatory response (128).

Respiratory Exacerbations Are Not Acute Infections of the Airways

As discussed above, acute infections of the respiratory tract have clear and intuitive features in culture-independent analysis: elevated bacterial burden coupled with decreased community diversity. A revelation of the field is how thoroughly and consistently exacerbations of chronic lung disease lack these defining features. Eight culture-independent studies of three different diseases (COPD, CF, and bronchiectasis) have compared patients' respiratory specimens taken at times of clinical stability and during exacerbation (35, 104, 107, 129–133). All have reported the same finding: There is no change in bacterial burden or community diversity during exacerbation. This revelation is consistent with numerous clear clinical differences between exacerbations and respiratory infections (e.g., lack of correlation between in vitro susceptibility and response to therapy, lack of association with sepsis, inconsistent or absent responsiveness to antibiotics) (28).

These findings do not imply that respiratory microbiota are uninvolved in the pathogenesis of exacerbations. We propose instead that, in contrast to infections of the airways, exacerbations are acute occasions of respiratory dysbiosis: disorder and dysregulation of the respiratory ecosystem, coupled with a dysregulated host immune response, resulting in negative effects on host biology. The paired-specimen studies mentioned above show that at the time of exacerbation, the bacterial communities in patients' airways shift away from Bacteroidetes (the most abundant phylum in the lung communities of healthy subjects) and toward Proteobacteria and other disease-associated taxonomic groups (28). Exacerbations are triggered by an inflammatory insult (e.g., viral infection, allergic exposure), which initiates a cascade of host inflammatory responses that accelerates the dysbiosis-inflammation cycle depicted in **Figure 4**. Homeostasis is restored only after the positive feedback loop is severed. This ecological model of exacerbation pathogenesis provides a plausible explanation for why the airway inflammation of exacerbations persists long after direct exposure to the inflammatory precipitant has ended.

Pneumonia Is an Emergent Phenomenon Arising from the Complex Adaptive System of the Respiratory Ecosystem

In our conventional understanding of pneumonia, changes in any of a few key factors (size of bacterial inoculum, virulence of pathogen, strength of host defenses) should yield proportionate, predictable changes in the frequency and severity of the infection. Yet this model presupposes a linear system of pathogenesis, in which small changes in initial conditions yield proportionately small changes in outcomes. Viewed through the modern lens of ecology, however, pneumonia instead emerges abruptly (and unpredictably) from the complex adaptive system of the respiratory ecosystem (20). Complex adaptive systems are defined by the presence of diverse entities that interact with each other within a common space, that exhibit interdependent actions, and that possess the capacity to adapt to changes in conditions (134, 135). Complex adaptive systems, unlike linear systems, defy reductionist modeling and are better modeled via computational techniques (e.g., agent-based modeling). A modern approach to pneumonia will attempt to explain how the abrupt ecological order of acute infection (high bacterial biomass, low community diversity) emerges from the dynamic homeostasis of a preexisting ecosystem. Identification of the self-accelerating positive feedback loops of host response and microbial growth discussed above will be central to advancing this systems-based understanding of pneumonia pathogenesis (20).

FUTURE ISSUES

1. Does the lung microbiome define clinically important phenotypes of chronic lung disease?
2. Can the respiratory microbiome be manipulated for therapeutic purposes?
3. Do current and potential therapies for lung disease achieve their benefit by way of microbiome alteration (e.g., macrolides)?
4. Can the complex dynamics of the respiratory ecosystem and the host response be modeled using advanced, nonlinear techniques?
5. Can the human lung microbiome be studied using animal modeling?
6. Can the microbiome be integrated with other -omes (e.g., the genome, the transcriptome, the metabolome) to identify novel mechanisms of the pathogenesis of lung disease?

7. How do nonbacterial microbiota (fungi, viruses, archaea) contribute to the biology of respiratory health and disease?

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