The dynamic lung microbiome in health and disease

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Abstract

New methods and technologies within the field of lung biology are beginning to shed new light into the microbial world of the respiratory tract. Long considered to be a sterile environment, it is now clear that the human lungs are frequently exposed to live microbes and their by-products. The nature of the lung microbiome is quite distinct from other microbial communities inhabiting our bodies such as those in the gut. Notably, the microbiome of the lung exhibits a low biomass and is dominated by dynamic fluxes of microbial immigration and clearance, resulting in a bacterial burden and microbiome composition that is fluid in nature rather than fixed. As our understanding of the microbial ecology of the lung improves, it is becoming increasingly apparent that certain disease states can disrupt the microbial–host interface and ultimately affect disease pathogenesis. In this Review, we provide an overview of lower airway microbial dynamics in health and disease and discuss future work that is required to uncover novel therapeutic targets to improve lung health.

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Introduction
Humans and microbes have symbiotically coevolved over hundreds of thousands of years, resulting in a human microbiome that is home to a wide range of microorganisms, including bacteria, yeasts, archaea, fungi, protozoa and viruses. These microbes, as well as their local environment, have a crucial role in immune system regulation and maturation, digestion, the production of certain vitamins, and the prevention of disease. We now know that microbes occupy virtually all surfaces of the human body, and the respiratory tract is no exception.

The upper and lower respiratory tracts differ in their microbial composition and biomass (Fig. 1). The upper respiratory tract, which includes the nasal cavity, paranasal sinuses, pharynx and supraglottic portion of the larynx, is largely colonized with bacteria. Moreover, within the upper respiratory tract, there are topographical differences in microbial composition. For example, the dominant taxa in the nasal cavity and nasopharynx include Moraxella, Staphylococcus, Corynebacterium, Haemophilus and Streptococcus species, whereas the oropharynx exhibits high abundance of Prevotella, Veillonella, Streptococcus, Leptotrichia, Rothia, Neisseria and Haemophilus species (Fig. 1). By contrast, the lower respiratory tract, comprised of the trachea and lungs, exhibits a relatively low biomass, with a major role in lower airway mucosal immunology (discussed below). Such low biomass is maintained by rapid microbial clearance via a number of physiological mechanisms (Fig. 1), enabling the lower respiratory tract to perform its most crucial function: the exchange of oxygen and carbon dioxide.

Dysbiosis, defined as deviation from a normal microbial composition, is associated with a number of adverse biological phenomena, sometimes with clinical consequences. In the lung, dysbiosis can have a significant impact on the development and progression of respiratory diseases, emphasizing the clinical need to understand the biology of the lung microbiome. Over the past decade, there has been a rapid expansion of studies applying culture-independent genomic methods to profile the lung microbiome (Box 1). As we discuss below, several studies have now shown that certain microbial signals are associated with distinct lower airway inflammatory endotypes and disease processes.

The lung microbiome in health and disease is often conceptualized as a pendulum that swings between two static states — from a paucity of microbes in the lung airways to patterns of resilient microbial colonization. However, it is possible that these extremes are not mutually exclusive but rather snapshots of a dynamic microbial system. In this Review, we describe the evolutionary nature of the lung microbiome and the underlying mechanisms by which sustained dysbiosis might affect respiratory disease progression. We hope to challenge the conceptualization that the lung microbial environment is a static entity and emphasize the dynamic balance between microbial immigration and clearance. As discussed below, the dynamic nature of the lung microbiome has been documented by experimental mouse models of microaspiration and might be an important distinguishing feature from the microbial behaviour seen in other mucosae with high biomass, such as the oral cavity and gut, where microbial communities are highly resilient. We argue that focusing on elucidating the dynamic nature of the lung microbiome will be critical to understanding mechanisms of lung disease and for the development of novel therapeutic targets involving the microbial–host interface.

The origins of the lung microbiome
Once thought to be a sterile environment, it is now understood that the lungs are frequently exposed to a variety of microorganisms. Human data support that microbial exposures start early in life, within days to weeks after birth, and have important implications for immune system maturation. In a study of lower airway samples from infants, differences in the composition of the microbial environment were associated with distinct changes in the host immune tone. Within the first few weeks of life, domination of the lower airway microbial community by either Staphylococcus or Ureaplasma species represents two distinct signatures of the lung microbiome in association with the mode of neonatal delivery. Lower airway enrichment with Ureaplasma, a common genital tract commensal, is associated with vaginal delivery, whereas enrichment with Staphylococcus, a common skin commensal, is associated with caesarean delivery, providing an early example of the role of the environment on the lung microbiome. As neonates mature, their lung microbiome shifts towards enrichment with a more diverse mixture of oral commensals such as Streptococcus, Prevotella, Porphyromonas and Veillonella.

These longitudinal changes in the lung microbiome affect the regulation of immunoglobulins and innate immune responses, and are thus postulated to be one of the mechanisms by which lower airway immune maturation occurs. Microbial changes in other parts of the respiratory tract may also affect the immune tone in early life. For example, microbial diversity and enrichment of the neonatal hypopharynx with microbes such as Prevotella and Veillonella species have been linked to the subsequent development of reactive airway diseases such as asthma, suggesting that early changes in the microbial environment, even if occurring among commensal taxa rather than among obvious respiratory pathogens, may predispose infants to certain respiratory conditions later in life.

A dynamic microbial–host interface
As with any mucosal surface in the human body, microbial exposures are not invisible to the host immune system. Indeed, the microbial products found in the lower airways derive from a balancing act between microbial immigration and microbial clearance. Unlike other mucosae, however, the lungs must maintain a lower bacterial burden in order to facilitate gas exchange, their main physiological purpose. Functionally, the conditions of the lower airways are distinct from those of other mucosae with regard to oxygen tension, protein content, presence of surfactants and other environmental factors. Furthermore, in order to maintain a low bacterial burden, there is a significant reliance on mechanisms that facilitate the clearance of microbes that reach the lower airways, including mechanical mechanisms, such as mucociliary clearance and cough, as well as innate and adaptive host immune responses (Fig. 1).

Unlike the oral cavity and gut lumen, both of which have a very high bacterial burden containing millions of viable and replicating microbes, the lower airways must maintain a thin bronchial epithelial fluid lining to facilitate effective gas transfer. Such unique conditions favour a more dynamic homeostatic state of the lung microbiome. Specifically, the microbiomes of the oral cavity and gastrointestinal tract tend to be much more static, dominated by reproducing site-specific microbial communities, changes in which are interpreted as a dysbiotic signature. However, the lower airway microbiome is frequently changing, with episodic exposure to microbes and their rapid clearance, resulting in a dynamic physiological process that defines eubiosis (Fig. 2). This is supported by several human cross-sectional investigations showing that traces of microbial DNA from oral commensals, despite a failure to isolate these bacteria by culture, are common in the healthy lung. Genetic evaluation of the lower airway microbiota (by 16S rRNA gene sequencing, shotgun metagenomic sequencing...
Lower airway immune tone: the lung–lung axis

Multiple investigations have shown the importance of gut microbial communities in setting the host immune tone, which affects lung immune responses as part of the gut–lung axis. Much less studied, lung microbial communities also affect lung immune responses. Indeed, existing cross-sectional human data, combined with data from murine models, suggest that there is frequent, episodic lung exposure to oral commensals that are subsequently rapidly cleared (Fig. 2). Models of microaspiration support that microbial clearance occurs at variable rates and is dependent on the species of the microbes aspirated. Importantly, even with rapid lower airway clearance of aspirated oral commensals, such events lead to a durable and dynamic change in the lower airway immune tone. For instance, mouse aspiration with a mixture of oral commensals (Prevotella melaninogenica, Veillonella parvula and Streptococcus mitis) leads to activation of CD4+ and CD8+ T cells, recruitment of IL-17-producing T cells (T helper 17 (T_h17) and γδ T cells), and other counter-regulatory immunological responses such as an increase in regulatory T cells and immune-checkpoint inhibitor markers on T cells. Importantly, this microbiome-dependent immunological priming is a durable phenomenon that persists beyond the transient presence of aspirated microbes and goes on to significantly impact innate immune defences against respiratory pathogens. If such responses are representative of normal lung microbial dynamics, these dynamic changes may reflect true lung ‘eubiosis’.

The above experimental preclinical data is in line with multiple observations from cross-sectional studies, illustrating again that distinct lung microbial signatures are associated with lower airway immune tone. For example, one study of mice with similar genetic backgrounds but heterogeneous microbial compositions found that the composition of the lower airway microbiome was associated with dynamic changes which may reflect true lung ‘eubiosis’.
Proteobacteria species were associated with activation of T\textsubscript{H}17-cell subtypes in the lung\textsuperscript{10}. In another study of patients with severe asthma, as well as with the recruitment of both T\textsubscript{H}17 cells and neutrophils,\textsuperscript{11} Proteobacteria species were associated with activation of T\textsubscript{H}17-associated pathways\textsuperscript{11}. Collectively, these data suggest that the lung microbiome, despite constantly being in flux with rapid clearance of microbes acquired via microaspiration or environmental exposures, leaves important signatures and imprints on the lower airway immune tone.

The studies described above give some insight into how the human lung might respond to sporadic exposure to certain microbes. However, numerous unanswered questions remain. It is unclear what happens when the frequency of microbial exposures increases. For example, it is unknown whether the balance between pro-inflammatory effects and counter-regulatory mechanisms becomes altered as aspiration events become more frequent. Whether recurrent exposure leads to chronic inflammation is also unclear. Despite a paucity of answers, data suggest that persistent exposure to certain microbes can trigger mechanisms leading not only to increased inflammation but also to immune exhaustion (Fig. 2). For example, in individuals where the lower airway microbiome is dominated by oral commensals, there

Box 1

Use of culture-independent methods in clinical practice

The use of high-throughput sequencing methods is still in its infancy but not far from reaching clinical use. For example, culture-independent techniques are evolving as a promising approach to detect respiratory pathogens among patients suspected of having pneumonia. This approach promises not only a more rapid diagnosis than with traditional culture-based methods but also the identification of respiratory pathogens among patients with culture-negative pneumonia\textsuperscript{95-97}. For example, a recently developed culture panel using a multiplex PCR assay on sputum and bronchoalveolar lavage samples can provide diagnostic data within hours\textsuperscript{96} and has been shown to substantially increase the rate of microbiological diagnosis\textsuperscript{96-99}. Targeted methods can also detect atypical bacterial and viral pathogens as well as certain antimicrobial resistance genes. Further, combining microbial and host genomic signals has the added potential benefit of accurately differentiating lower airway pulmonary infection from other pathological conditions\textsuperscript{100-102}.

Targeted approaches are limited by the finite number of targets evaluated. With the development of nanopore sequencing technologies, such as the MinION, real-time evaluation of RNA or DNA can be performed on a clinical sample, thus providing immediate genomic detection of a pathogen\textsuperscript{103}. Next-generation highly multiplexed methods may lead to an expanded ability to identify patients with bacterial, viral or fungal infections. Further, their use in combination with host transcriptome sequencing methods may provide important prognostic information among patients with pulmonary infections. For example, among critically ill patients with COVID-19, a combination of microbial and host genomic signals from the lower airways provides significant insights about the pathophysiological derangements occurring in these patients that may explain individual risk for poor clinical outcomes\textsuperscript{104}. As the application of multi-omics becomes more widespread, it is possible that we will be better able to understand the phenotypic and endotypic characteristics that carry clinical significance across several lung conditions. For now, more studies employing longitudinal investigative approaches are needed before the adoption of culture-independent methods into clinical practice becomes a reality.

Culture-independent methods could also change our view of the effects of therapies commonly used in clinical practice. Characterization of the lower airway microbiome is challenging our current understanding of broad-spectrum and narrow antibiotic use, uncovering supposedly ‘off-target’ microbial effects. In cystic fibrosis, inhaled tobramycin, commonly given to target colonization with Pseudomonas, has broad effects on non-pathogenic microbes, which is an important consideration when interpreting results of antibiotic use\textsuperscript{102-105}. For example, in the early stages of chronic obstructive pulmonary disease, chronic use of azithromycin leads to significant changes in the composition of the lower airway microbiome, which can be associated with the release of microbial metabolites with anti-inflammatory properties\textsuperscript{106}. Thus, some of these off-target effects may lead to important mechanisms affecting disease progression. Further, azithromycin therapy in lung transplant recipients with chronic rejection has been shown to improve lung function, suggesting that host–microbial interaction may play a role in its pathogenesis\textsuperscript{107}.

There are of course limitations to culture-independent methods that can severely limit their applicability in clinical practice. One example is that multiplex PCR assays cannot accurately distinguish DNA from viable versus non-viable organisms. Thus, initial detection does not necessarily indicate a need to treat nor does ongoing detection always necessitate prolonging antimicrobial therapy in a patient already being treated. Another limitation is that culture-independent methods applied on samples with a low microbial biomass are far more prone to sequencing noise and DNA contamination. In this regard, microbial RNA metatranscriptome sequencing methods may be particularly useful as detection of microbial RNA generally suggests the presence of viable microbes and given that RNA is rapidly degraded after cell lysis.

Despite evolving data that supports use of mixed bacterial transfers, such as faecal microbiota transplantation, in treating gastrointestinal conditions like Clostridium difficile infection\textsuperscript{108}, irritable bowel syndrome\textsuperscript{109} and ulcerative colitis\textsuperscript{110}, much less is known about the impact of intentionally altering the lung microbiome in respiratory diseases. Interestingly, there is evolving literature to suggest that restoring the normal gastrointestinal microbiome with the use of probiotics and prebiotics has therapeutic benefit in a number of respiratory diseases such as lung cancer, asthma, tuberculosis and cystic fibrosis (the so-called ‘gut–lung axis’\textsuperscript{111}); however, therapeutic interventions directly targeting the lung microbiome, through prebiotics or probiotics, are lacking and will need to be explored further in future investigations.

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is a blunting of the Toll-like receptor 4 (TLR4) response of alveolar macrophages\(^\text{10}\). In another human study, microbial products, such as short-chain fatty acids (SCFAs), had significant immunomodulatory properties and blunted IFNγ and IL-17 responses to pathogen-associated molecular patterns\(^\text{13}\). Finally, in mice, experimental exposure of the lower airways to oral commensals not only triggers an increase in inflammatory cytokines but also an increase in immune-checkpoint inhibitor markers, such as PD1, among T cells and recruitment of regulatory T cells\(^\text{4}\). These findings collectively suggest that the dynamic nature of microbial–host interactions in the lung likely changes as the frequency of exposure to microbes, rate of elimination and type of immune response change over time.

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**Fig. 2 | Lung microbial dynamics in eubiosis and dysbiosis.**

**a** Eubiosis

A series of dynamic events promote eubiosis in the lower airways. Aspiration of a microorganism triggers the activation of leukocytes that orchestrate bacterial elimination by macrophage and neutrophil activity. After a transient upregulation of adaptive immunity, the immune tone returns to baseline with a low level of detectable residual microbial DNA.

**b** Early dysbiosis with immune imbalance

Recurrent aspirations lead to changes in the lower airway microbial environment that can slowly become irreversible.

**c** Established dysbiosis with immune exhaustion

Increased mucus production facilitates persistent colonization and increased bacterial burden and is associated with acute and chronic lower airway disease. Microbial immunomodulatory products, such as short-chain fatty acids (SCFAs), and host immune responses, such as a T helper 1 (TH1)/TH2 imbalance, an aggravated TH17 response and pro-inflammatory cytokines, can perpetuate neutrophilic inflammation, increased regulatory T (Treg) cell response and impaired immune surveillance. These changes can ultimately promote increased bacterial burden.
Dysbiosis in respiratory inflammatory diseases

Microbial dysbiosis is present in various lung diseases, such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), in which a reduction in the diversity of bacterial composition has been linked to disease progression\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\). However, whether microbial dysbiosis itself is the cause of disease or a result of the disease process is not clear. For example, in lung disease, pathophysiological changes in the lung architecture and impaired mucus clearance mechanisms might result in microbial dysbiosis rather than being caused by it. Alternatively, dysbiosis may play a causative role in disease through upregulation of inflammatory signals (such as NF-κB, Ras, IL-17 and PI3K) or blunting of TNF and IFNγ production in response to pathogens in the lower airways\(^9\)\(^10\)\(^11\). In this section, we review some examples of dysbiotic signatures identified among pulmonary diseases commonly described as inflammatory and discuss the potential role of lower airway microbes in their pathogenesis.

Chronic obstructive pulmonary disease

COPD serves as a useful example of the evolutionary nature of the lung microbiome and its impact on respiratory disease progression. Initial microbiome studies in patients with stable COPD demonstrated a clear association between the presence of potentially pathogenic microbes (PPMs), such as *Haemophilus* species, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and gram-negative enteric bacteria, and the presence of neutrophilic inflammation and increased expression of cytokines, such as IL-8 and TNF, in the lower airways\(^12\)\(^13\)\(^14\). Lower airway colonization with PPMs has been correlated with increased severity of daily symptoms in COPD and progressive radiographic changes\(^15\)\(^16\). Nonetheless, precisely how these microbial events contribute to early stages of disease onset is poorly understood. Smoking alone does not seem to be associated with a significant change in the lower airway microbiome\(^17\)\(^18\); however, this situation changes as smokers start to develop early pathophysiological evidence of COPD. In a recent study of the lower airway microbiome in patients with early COPD, enrichment with *Streptococcus*, *Staphylococcus*, *Prevotella* and *Gemella* (strains that are part of the normal oral microbiota) was associated with lung function abnormalities, including an impaired response to bronchodilator therapy\(^19\). In support of a causative role of the microbiome in COPD, longitudinal enrichment with oral commensals in repeated lower airway samples results in the development of COPD-like changes in a macaque preclinical model\(^20\).

As COPD progresses, chronic inflammation results in impairment of the lung innate immune defences, which in turn pave the way for increased bacterial burden of both new and existing pathogens. Multiple investigations have shown that, in moderate-to-severe COPD, there is enrichment with Gamma-proteobacteria (which includes many PPMs such as *Haemophilus* and *Moraxella* species)\(^21\)\(^22\)\(^23\)\(^24\)\(^25\)\(^26\). In another study of patients with COPD, the presence of Proteobacteria-dominated microbes in sputum was associated with poorer lung function, worse scores on the Medical Research Council dyspnoea scale, and more frequent exacerbations\(^27\). In a multicentre trial involving longitudinal sampling from three large cohorts of patients with moderate to severe COPD, the airway microbiome (as assessed in sputum samples) showed clear associations between its composition and the inflammatory endotype\(^28\). Specifically, the *Haemophilus*-dominated sputum microbiota behaved rather stably over time and was associated with elevated IL-1β and TNF levels. These findings are supported by another study of patients with stable COPD, which similarly showed that colonization with *Haemophilus influenzae* was associated with increased airway inflammation\(^29\). It is therefore possible that these patients might benefit from targeted antimicrobial therapies to mitigate chronic inflammation. In contrast, patients with a ‘balanced’ microbiome profile, characterized by enrichment with several oral commensals such as *Veillonella* and *Prevotella*, exhibited a more dynamic microbiome over time and had elevations in serum and sputum IL-17A\(^30\). This subgroup was more susceptible to greater microbiome shifts during exacerbations. Thus, it is possible that these patients may benefit from a different, perhaps distinctly targeted, anti-inflammatory therapeutic approach or closer monitoring of airway ecology and pathogen acquisition, particularly at times of acute exacerbations. Importantly, increases in the relative abundance of some oral commensals, such as *Campylobacter* and *Granulicatella*, were associated with intra-patient switches from neutrophilic to eosinophilic inflammation\(^31\). Taken together, these findings are consistent with a model in which recurrent microaspiration of oral secretions starts occurring at a higher rate in early stages of disease, which contributes to lower airway inflammation; subsequently, further impairment of microbial clearance results in immune exhaustion and in eventual lower airway colonization with respiratory pathogens, a phenomenon that has been well documented in later stages of disease through both genomic-based and culture-based methods\(^32\)\(^33\)\(^34\)\(^35\)\(^36\). Thus, multiple forms of dysbiosis can be seen in patients with inflammatory airway diseases such as COPD (Fig. 3a).

Asthma

Several investigations have focused on the airway microbiome in asthma. Although studies in early childhood with lower airway samples are lacking, multiple studies have shown associations between changes in the microbiome and development of wheezing and asthma diagnosis\(^37\)\(^38\)\(^39\)\(^40\). Even among children with an established asthma diagnosis, compositional changes in the upper airway microbiome are associated with disease control\(^41\). Evaluation of the lower airway microbiome of patients with asthma has been performed more extensively in adults. Several studies have now shown that the composition of the lower airway microbiome is associated with distinct clinical features among patients with asthma, including airway bronchial responsiveness and severity\(^42\)\(^43\)\(^44\)\(^45\). It is now understood that asthma comprises a group of very heterogeneous endotypes with a large proportion of patients with eosinophilic or type 2 (T2)-high inflammation that is more susceptible to steroids and targeted therapies. In contrast, treatment is less effective in patients with a non-T2-high inflammatory endotype (that is, patients with mostly neutrophilic airway inflammation) for reasons that remain unknown\(^46\). One intriguing hypothesis is that the lung microbiome may have a major role specifically among patients with a non-T2-high inflammatory neutrophilic endotype. For example, evaluation of the lower airway microbiota using airway brushings obtained from asthmatics showed that bacterial composition was not associated eosinophilic inflammation. In contrast, significant associations were found between the enrichment with Proteobacteria and other taxa and T\(_{H}17\) inflammatory markers, likely resulting in neutrophilic recruitment\(^47\). Thus, better understanding of the molecular mechanism affecting these associations may allow for the discovery of novel treatable traits among patients with asthma presenting with more refractory forms of the disease.

Idiopathic pulmonary fibrosis

The lung microbiome has important implications for disease progression and survival in various other pulmonary diseases. There are now several
studies showing an association between lower airway bacterial burden and disease progression-free survival among patients with IPF (Fig. 3b). In particular, one study reported a correlation between alveolar inflammatory and fibrotic cytokines and lung microbiota diversity and composition, and lower Shannon diversity indices (within-sample diversity) were associated with higher concentrations of IL-1Ra, IL-1β, CXCL8, MIP1α, G-CSF and EGF. In addition, alveolar concentrations of IL-6 were positively correlated with relative abundance of the lung Firmicutes phylum, whereas alveolar IL-12p70 was negatively correlated with relative abundance of the lung Proteobacteria phylum. Other IPF studies have also shown clear associations between lung dysbiosis and both peripheral blood mononuclear cell transcriptomic expression of immune pathways and progression-free survival, suggesting that microbial–host interactions have an important role in the pathogenesis of IPF.
of pulmonary fibrosis. However, a major limitation of these human studies is the inability to test the directionality of observed associations. In a bleomycin murine model of pulmonary fibrosis, microbial dysbiosis preceded peak lung injury and persisted until the development of fibrosis. In contrast, germ-free mice exposed to bleomycin-induced pulmonary fibrosis had a mortality benefit compared to conventional mice (Fig. 3b). In another preclinical murine model, *Bacteroides* and *Prevotella* species were associated with increased fibrotic pathogenesis through IL-17R signalling. Taken together, these data suggest that certain microbial exposures may act as persistent stimuli for repetitive alveolar injury, ultimately contributing to pulmonary fibrosis and continual inflammatory injury in this disease.

**Acute respiratory distress syndrome**

Both in preclinical models of sepsis and in humans with established acute respiratory distress syndrome, the lung microbiome likely plays a major role in the pathogenesis of the disease (Fig. 3c). Using culture-independent methods, a study from 2016 demonstrated that enrichment of the lower airways with gut-associated bacteria correlated with concentrations of alveolar TNF, a key mediator of alveolar inflammation in acute respiratory distress syndrome. In addition, a recent study of patients with COVID-19 on mechanical ventilation demonstrated worse clinical outcomes among patients whose lower airway microbiome was enriched with *Mycoplasma salivarium*, another oral commensal. Additionally, a recent large study of over 300 patients on mechanical ventilation demonstrated that enrichment of the lower airways with *Staphylococcus* or *Pseudomonadaceae* was associated with increased lower airway inflammation and worse clinical outcomes, including poorer 30-day survival and longer time to liberation from mechanical ventilation. Taken together, these data suggest that microbial dysbiosis may have an important role in the dysregulation of local and systemic inflammation that consequently impacts clinical outcomes in acute respiratory failure.

**The lung microbiome and immune surveillance**

Although much research has focused on how the lung microbiome promotes inflammatory states, its effects on immune surveillance are much less understood but are likely very important. Among patients with cystic fibrosis, a reduction in alpha diversity, which quantifies microbial diversity within a sample, is directly correlated with disease severity and colonization with bacterial pathogens. In many cases, this is likely due to the emergence of a dominant pathogen that increases in relative abundance. In addition, decreased microbial diversity sometimes precedes the development of cystic fibrosis exacerbations. These data suggest that a reduction in biodiversity over time can render the lower airways more susceptible to colonization with a dominant pathogen such as *Pseudomonas* or *Burkholderia* species.

HIV

As another example of dysbiosis that might result in decreased host immune surveillance, people living with HIV continue to experience higher rates of respiratory infections and lung cancer despite widespread use of antiretroviral therapy (ART). It is hypothesized that residual levels of HIV in deep tissues leads to chronic immune activation and lung inflammation. This longstanding pro-inflammatory state can eventually contribute to immune cell exhaustion and pulmonary...
Pathogen Dysbiosis Pathogen

Figure 4 | Dysbiotic events rendering increased host susceptibility to pathogens. a. In cases of acquisition of a dominant pathogen, dysbiotic changes occurring prior to pathogen seeding may promote host susceptibility. b. In cases of non-dominant respiratory pathogens, changes in the lower airway microbiome might continue as a dysbiotic signature once pathogens are established in the lower airways causing lung injury. c. Alternatively, non-dominant pathogens could lead to a new dysbiotic signature that favours pathogen survival and contributes to lung injury.

immune dysregulation, thereby making people living with HIV be at higher risk for pulmonary infections and malignancies. Several studies have tried to identify dysbiotic signals present in HIV, including a multicentre trial that described the presence of high levels of *Tropheryma whippelii* in the lungs of some individuals with HIV; of note, however, this has also been observed in smokers and other cohorts, and thus may not be unique to HIV. Further, the lung microbiome in people living with HIV, especially among those with advanced HIV, shows an increased burden of *Prevotella* and *Veillonella* species. Even with the immune reconstitution that occurs after initiation of ART, there is persistent enrichment of the lower airway microbiota with these oral commensals. This pattern of microbial dysbiosis is associated with subclinical inflammation, characterized by an increase in neutrophils and lymphocytes. Further, patients with HIV who are treatment naive have been shown to have decreased biodiversity and greater intersample diversity compared to healthy controls, suggesting that this dysbiotic signature may be prominent in patients with HIV and may confer increased susceptibility to pneumonia. In patients with HIV treated with ART, enrichment of the lower airways with oral taxa was associated with increased production of microbial SCFAs. As shown in one longitudinal cohort study of South African patients with HIV treated with ART, SCFAs have direct inhibitory effects on T cell function via the suppression IFNγ and IL-17 production and are associated with an increased risk of tuberculosis. Given the well-known immunoregulatory properties of SCFAs and the evidence that these molecules could be produced through fermentation of aspirated oral anaerobes, it is plausible that this may be one potential mechanism by which the lower airway microbiome impairs host immune surveillance to pathogens.

**Cancer**

Beyond associations with susceptibility to pathogens, several investigations have suggested a link between lower airway dysbiosis and lung cancer. Epidemiological studies and culture-dependent techniques have shown that chronic or recurrent bacterial infections are common in patients with lung cancer over the course of their disease, and post-obstructive pneumonia can negatively impact their response to therapy and overall survival. Studies have reported a correlation between certain respiratory pathogens, such as *Mycobacterium tuberculosis*, with lung cancer, and a systematic review found a significantly increased lung cancer risk associated with pre-existing tuberculosis, independent of tobacco exposure. This might be due to tuberculosis being associated with inflammatory mediators, such as TNF, and excessive extracellular matrix turnover. Mechanistically, the increase in pathogenic bacteria can lead to chronic inflammation through the persistent generation of inflammatory mediators, thereby affecting cellular apoptosis and increasing risk for DNA mutations. Bacterial metabolites, such as reactive oxygen and nitrogen species, can cause direct DNA damage and disrupt a variety of signalling pathways, thereby generating a pro-carcinogenic environment (Fig. 3d). A number of bacterial metabolites, including SCFAs, have also been implicated in both the regulation of host metabolism in the lungs and in signalling pathways. Thus, it is possible that alterations in bacteria-derived molecules can affect the tumour microenvironment and oncogenic signals. This delicate balance between tolerance and immune activation in the lung microenvironment can be disrupted by the overuse of antibiotics for the treatment of infections. For example, preclinical models using broad-spectrum antibiotics demonstrated the importance of commensal bacteria in supporting the host immune response against cancer, defined an important role for γδ T 17 cell responses in the mechanism and showed deleterious effects of antibiotic treatment on cancer progression.

With the growing use of culture-independent methods, we can now appreciate microbiome differences between patients with and without lung cancer diagnoses. The microbiota of lung tumours is characterized by a lower alpha diversity compared to non-malignant lung tissue samples, an association that seems to be independent of tobacco use, environmental exposures and clinical staging. Among taxonomic signatures, associations have been described between lung malignancy and increased relative abundance of oral commensals such as *Streptococcus*, *Prevotella*, *Veillonella*, *Rothia*, *Megasphaera* and *Acidovorax*. Other less common respiratory pathogens, such as *Staphylococcus* and bacteria from the Firmicutes and Saccharibacteria (formerly known as TM7) phyla, have also been found in lower airway samples from patients with lung cancer. Microbial differences have also been noted among various types of cancer. One study found that *Brevundimonas*, *Acinetobacter* and *Propionibacterium* were more enriched in lung adenocarcinoma whereas *Enterobacter* was more enriched in squamous cell carcinoma. Another study demonstrated...
an association between the *Acidovorax* genus and squamous cell carcinoma among smokers, particularly in tumours harbouring TP53 mutations; it is also likely that some of these microbial exposures contribute to a host immune response that may affect carcinogenesis. Indeed, human data show that enrichment with supraglottic-predominant taxa, such as *Streptococcus, Prevotella* and *Veillonella*, is associated with upregulation of transcriptomic pathways that are known to have a major pathogenic role in carcinogenesis such as ERK and PI3K. Upregulation of the PI3K pathway occurs early in lung carcinogenesis and may affect proliferation, apoptosis and cell survival as well as the induction of PDL1 expression. Thus, these data demonstrate how a dysregulated microbiota might increase the risk of lung cancer development. Furthermore, exposure of KRAS-mutated bronchial epithelial cells to either the supernatant of these taxa or their heat-killed products leads to similar upregulation of transcriptomic signatures to those seen in human data. Overall, although the specific bacterial taxa identified varied somewhat from one study to another depending on the sample type and patient cohort, the consistent observation is that lung cancer is associated with a dysregulated local microbiome characterized by reduced alpha diversity and altered bacterial composition.

The lung microbiome may have a role in the progression of lung cancer or its risk for recurrence. Among patients with surgically resected early-stage non-small-cell lung cancer (NSCLC), recurrence was associated with a higher diversity and enrichment of *Bacteroidaceae, Lachnospiraceae* and *Ruminococcaceae* in the lung tissue microbiome. In another study of patients who underwent surgical resection of NSCLC, bronchoalveolar lavage enrichment with *Sphingomonas, Psychromonas* and *Serratia* and a reduced abundance of *Cloacibacterium, Geobacillus* and *Brevibacterium* were observed in patients who had early lung cancer recurrence compared to those without recurrence in the subsequent 3 years. Importantly, pre-surgery bronchoalveolar lavage microbiome was associated with the tumour expression of genes involved in cell proliferation, immunity and signal transduction. Distinct microbial differences can also be noted among patients with different stage of disease. For example, the genus *Thermus* was reported to be more abundant in tumour tissues from patients with advanced stage disease (TNM stages III–IV), and *Legionella* abundance was higher in patients who developed metastases. Further, in a recent study, a lower airway dysbiotic signature characterized by enrichment with oral commensals was more prevalent in individuals with TNM stage III–IV NSCLC compared to those with the disease at earlier stages, and a similar microbial signal was associated with poor prognosis and increased risk for mortality. Mechanistically, multi-omics integration of the microbiome and host transcriptomic data from the lower airways of patients with lung cancer showed that several of these oral commensals, including *Veillonella* and *Streptococcus* species, were associated with upregulation of the IL-17, PI3K, MAPK and ERK pathways in the airway transcriptome.

Preclinical mouse models of lung adenocarcinoma show that lower airway dysbiosis, induced by intratracheal aspiration of some of these oral commensals, can lead to T<sub>IL-17</sub> inflammation, increased expression of immune-checkpoint inhibitor molecules (PD1) by T cells, accelerated tumour progression and mortality. Using germ-free preclinical lung cancer models, others have shown that commensal taxa stimulate Myd88-dependent IL-1β and IL-23 production from myeloid cells, activate IL-17 production by γδ T cells and lead to a pro-inflammatory state that induces tumour proliferation. Furthermore, preclinical evidence suggests that direct blocking of the IL-17 pathway through anti-IL-17 monoclonal antibodies can slow tumour growth in the setting of lower airway dysbiosis induced by aspiration of oral commensals. These findings clearly link local microbiota-immune cross-talk to lung tumour development and thereby identify candidate cellular and molecular mediators that can be targeted for anticancer therapy.

The evidence described above shows some lack of consistency in the specific microbial taxa associated with pulmonary malignancy. Inconsistencies between studies may be due to socio-demographic differences inpatient cohorts or differences in research techniques employed, a phenomenon similarly noted in studies of gut microbiome signatures associated with malignancies. For example, while gut microbiomic signatures have been found to be associated with inflammatory tone and susceptibility to immunotherapy in melanoma and NSCLC, the specific taxa identified have been inconsistent among different studies. It is also possible that taxonomic signals are markers of functional microbial differences but evaluation of this hypothesis is prevented by a lack of studies focusing on microbial metabolism in the lung (for example, using approaches such as metagenome, metatranscriptome, metabolome or proteome). However, despite these different taxonomic signals, the host signals associated with lower airway dysbiosis seem to similarly point towards a dysregulated immune response with a pro-inflammatory T<sub>IL-17</sub> state and increased expression of immune-checkpoint inhibition, which might impair effective immune surveillance of nascent malignant cells.

Importantly, tumour microbiome studies are often limited by a very low microbial biomass and are thus prone to sequencing noise and DNA contamination from kits and reagents, although various methods have been proposed on how to effectively deal with both of these issues. Thus, although recent studies indicate that the lung microbiome may soon become a critical diagnostic and preventive biomarker for lung cancer stage, genotype and risk factor stratification, further mechanistic and large-scale clinical studies are required to dissect its precise role in tumorigenesis.

**Conclusions and future directions**

Studying the lung microbiome in varying disease states has identified microbial signatures associated with diagnosis, illness severity and prognosis. However, a lack of consistent findings across studies makes it difficult to clearly define lung dysbiosis. It is quite possible that even within a single disease process there are many forms of dysbiosis, a concept that has previously been referred to as the ‘Anna Karenina principle’ in reference to Tolstoy’s writings that “happy families are all alike; every unhappy family is unhappy in its own way.” Even less established is how we define lung microbial eubiosis. Part of the difficulty may reside in the nature of the lung microbiome, with its low diversity, composition and resilience in the face of environmental challenges. As the use of culture-independent and multi-omics methods becomes more widespread, understanding these principles is essential for the successful development of novel diagnostic and therapeutic modalities targeting microbial–host interactions.
In Fig. 5, we illustrate an overarching speculative view of how the delicate balance between microbial immigration (driven by inhalation, microaspiration and mucosal dispersion) and microbial elimination (driven by cough, mucociliary transport and immune mechanisms) establishes a complex microbial–host interface. This interface is likely affected by many factors, including those related to both the host and the environment. The exact effects that each of these factors has on the microbiome are relatively unknown, although we can speculate about the existence of some beneficial and other detrimental factors that might change throughout the course of our lives (Fig. 5).

We postulate that factors such as vaginal delivery, exercise and a healthy diet contribute towards lower airway eubiosis, either directly or via the gut–lung axis. Conversely, obesity, an unhealthy diet and frequent antibiotic use could disrupt the lower airway microbiome in an unfavourable manner. The effect of breastfeeding versus formula feeds is also unknown, with only a single randomized controlled trial showing no difference in the incidence of gastrointestinal and respiratory infections in breastfed versus formula-fed infants, although this study did not specifically investigate the microbiome.

Finally, we surmise that lower airway immune resilience and the diversity of the lung microbiome likely change as we age, and the effect of external factors on the lung microbiome may therefore change across the lifespan. Well-designed controlled studies are needed to evaluate the potential effects of these environmental factors on the lung microbiome and host immune response. Careful consideration of age, sex and other important clinical conditions are key to a better understanding of how the lower airway microbiota influences lower airway immune tone. Thus, future investigations will need to move away from cross-sectional designs and correlative studies towards longitudinal sampling approaches that account for topographical characteristics and experimental conditions (Box 2). Further, although understanding the human lung microbiome requires human studies, preclinical models allow for better control of microbial and host conditions as well as the implementation of experimental approaches under controlled circumstances. These mechanistic approaches are needed to understand how the lung microbiome affects host susceptibility to inflammatory processes, infections and malignancy.

Next, we must consider the potential therapeutic impact that modulating the lung microbiome might have on respiratory diseases. Currently, many existing and widely used interventions beyond antibiotics, such as inhaled corticosteroids and cystic fibrosis transmembrane conductance regulator modulators, already alter the respiratory microbiota. In fact, there are now several potential mechanisms that have been proposed to explain how corticosteroid therapy might impact epithelial barrier function and consequently alter airway ecology, including the increased expression of epithelial genes involved in tight junction promotion and the upregulation of IL-17 and TNF inflammatory pathways. Beyond corticosteroids and cystic fibrosis transmembrane conductance regulator modulators, several other interventions have been trialled to modulate the lung microbiota with varied success. For example, oral probiotics have been studied in the context of both cystic fibrosis and ventilator-associated pneumonia, although these likely target shifts in the gut microbiome and their effects on the respiratory microbiome remain unknown.

Fig. 5 | Potential modifiers of lower airway microbiome course. The dynamic nature of the lower airway microbiome is ever evolving. The overarching factors that control the balance of the microbiota are microbial immigration via inhalation and microaspiration and microbial elimination via cough, mucociliary transport and immune responses. We postulate that factors such as vaginal delivery, exercise and a healthy diet may contribute towards eubiosis in the lower airway, whereas obesity, an unhealthy diet and frequent antibiotic use could disrupt the lower airway microbiome in an unfavourable manner. Finally, we surmise that lower airway immune resilience and the diversity of the lung microbiome (a symbol of health in its own right) follows a quasi-sinusoidal path, peaking in adulthood.
Bacteriophage therapy has similarly been trialled, although its use is still in its infancy\(^\text{116}\). Other studies have employed both inhaled and systemic antibiotics to modulate the respiratory microbiota but results are mixed across various pulmonary diseases\(^\text{25}\).

Unlike the gut microbiome, in which probiotics can be used to restore normal gastrointestinal flora, no such respiratory probiotic currently exists. However, in a preclinical model, episodic instillation of oral commensals into the lower respiratory tract modified host susceptibility to respiratory pathogens\(^\text{1}\). While these types of investigations may provide the biological plausibility that microbiome modifications in the lung could provide health benefits, mechanistic studies that include both preclinical work and individuals with respiratory diseases are needed. These type of investigations in turn might allow us to identify microbial signatures associated with less inflammation or decreased pathogen susceptibility. Until we understand what a beneficial ecological target might look like — a so-called ‘ideal’ respiratory microbiome — we are limited in our ability to implement safe and meaningful clinical trials.

In conclusion, there is a need to embrace the dynamic nature of microbes visiting the lower airways, particularly as it relates to host immune tone and the pathogenesis of multiple different disease processes. Novel clinical applications need to account for the evolutionary nature of the lung microbiome where eubiosis and dysbiosis truly exist on a spectrum.

**Box 2**

**Lung transplantation as an opportunity to examine longitudinal changes in the microbiome**

Despite the dramatic increase in studies utilizing culture-independent methods to profile the lung microbiome, most research to date provides only a static view of the lung microbiome, usually at a given stage of disease. Prior studies have shown differences after lung transplantation in both bacterial load and composition in comparison to healthy controls and non-lung transplant recipients with chronic lung disease\(^\text{109–112}\). However, even in lung transplantation, where patients routinely undergo surveillance bronchoscopies to monitor for signs of infection and rejection, most studies have only explored whether the composition and function of the lung microbiome at a pre-specified time point is associated with subsequent disease development and progression. For example, a recent prospective study demonstrated increased risk for chronic lung allograft dysfunction (CLAD) and death among asymptomatic lung transplant recipients that develop increased lung bacterial burden\(^\text{111}\). In addition, prior observational studies have suggested that Actinobacteria dominance (Propionibacterium and Corynebacterium species) early after lung transplantation is associated with lower rates of chronic and acute rejection and less inflammation\(^\text{110}\), whereas infection with Chlamydia pneumoniae is associated with higher rates of acute and chronic rejection\(^\text{115}\).

Furthermore, one study found that the presence of certain pathogens from the Firmicutes and Proteobacteria phyla was associated with increased inflammatory gene expression (such as TNF and COX2) whereas enrichment with Bacteroidetes was associated with a tissue remodelling profile (such as MMP12, TIMP1 and PDGFD)\(^\text{116}\). Collectively, these data suggest that microbial dysbiosis likely has implications on allograft function and survival after lung transplantation. How the dynamic nature of the microbial–host interface changes over time remains unclear.

Longitudinal evaluation of changes occurring in the lung microbiome may provide further insight into the evolving microbial–host interaction and how it affects disease progression. A number of studies have demonstrated that dynamic changes to the lung microbiome occur after transplantation, in which a reduction of alpha diversity correlates with a decline in lung function, increase in infection rates and allograft failure\(^\text{116}\). Different taxa tend to dominate the lung microbiome in the first year after transplantation, with Bacteroidetes being the most abundant phylum, followed by Firmicutes, Proteobacteria and Actinobacteria\(^\text{116}\). Over time, there is often enrichment with Proteobacteria, particularly Pseudomonas, which has been linked to development of CLAD in multiple studies\(^\text{109–112}\). One study in particular revealed two species within the genus Pseudomonas — P. aeruginosa and P. fluorescens — and observed lower biodiversity, more bacterial DNA, greater neutrophilia and increased clinical symptoms among patients with a higher relative abundance of these taxa\(^\text{117}\). Interestingly, another study found that recolonization of the allograft with Pseudomonas in individuals with cystic fibrosis was not associated with CLAD\(^\text{118}\). Thus, further longitudinal investigations are needed to better understand the factors that determine risks for complications among patients undergoing lung transplantation.

Given the frequency with which lung transplant recipients undergo surveillance bronchoscopies, there is an untapped opportunity to longitudinally examine the evolutionary nature of the lung microbiome after transplantation and study its relationship to lower airway immune tone and clinical outcomes. Further, there are several unique factors occurring after lung transplantation that make it a highly useful disease model to study microbial–host interactions. First, sample acquisition can be made prior to development of disease, including both bacterial, viral, and fungal infections and inflammatory complications (that is, acute and chronic rejection). Second, the samples are often rich in microbes due to their impaired mechanical clearance, such due to a defective afferent cough reflex, anastomotic mechanical barriers and mucociliary dysfunction. Third, frequent microaspiration results from a high incidence of oropharyngeal dysphagia, gastroesophageal reflux disease and gastroparesis after lung transplantation. Finally, significant heterogeneity in prescribed immunosuppressive regimens exists, thus altering the host response to varying degrees.
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