Immunology
The Journal of cells, molecules, systems and technologies
British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

The impact of lung microbiota dysbiosis on inflammation

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doi:10.1111/imm.13139
Received 4 August 2019; revised 5 October 2019; accepted 15 October 2019.
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Summary

Host–microbiota interaction plays fundamental roles in the homeostasis of mucosal immunity. Dysbiosis of intestinal microbiota has been demonstrated to participate in various immune responses and many multifactorial diseases. Study of intestinal microbiota has moved beyond the consequences of dysbiosis to the causal microbiota associated with diseases. However, studies of pulmonary microbiota and its dysbiosis are still in their infancy. Improvement of culture-dependent and -independent techniques has facilitated our understanding of lung microbiota that not only exists in healthy lung tissue but also exerts great impact on immune responses under both physiological and pathological conditions. In this review, we summarize recent progresses of lung microbiota dysbiosis and its impact on the local immune system that determines the balance of tolerance and inflammation. We discuss the causal roles of pulmonary dysbiosis under disease settings, and propose that the interaction between lung microbiota and host is critical for establishing the immune homeostasis in lung.

Keywords: dysbiosis; inflammation; lung; microbiota; pulmonary disease.

Introduction

Microbiota coexists with host as symbiotic commensals to form a mutualistic relationship. Microbiota has co-evolved with its host and colonizes the host's mucosal tissues, including oral cavity, gastrointestinal tract, skin, vaginal tract and respiratory tract.[1] The National Institutes of Health launched the Human Microbiome Project in 2007 and, since then, microbiome at five major body sites of health subjects and microbiome from three cohorts of microbiota-associated conditions have been characterized.[2,3] However, owing to the traditional notion that lung is sterile and technical challenges of sampling, the lung microbiota was not included into this project.[4] Over the past several years, the field of lung microbiota has been revolutionized by the advance of culture-dependent and -independent techniques.[4]

Several pioneering studies using culture-independent techniques have confirmed that there are diverse communities, although much less than that in the gastrointestinal tract, of microbes even in lung under health conditions,[5–7] and the microbial composition of healthy lung is distinct from that of individuals with pulmonary diseases.[8] With appropriate protocols, bacteria can be isolated and cultured from lung lobes, indicating that there are live microbes in lung.[9] The history and evolution of this field has been well reviewed by Dickson *et al.*[4] Healthy lung usually harbours a small community of bacteria, comprising mainly of four phyla: Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria, which are similar in human and mice. While being a small amount but

Abbreviations: BPT, background predominant taxa; cDCs, conventional dendritic cells; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; DCs, dendritic cells; ICOS-L, inducible co-stimulatory molecule ligand; iNKT, invariant natural killer T-cells; IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharides; OMVs, outer membrane vesicles; pDCs, plasmacytoid dendritic cells; SCFAs, short-chain fatty acids; SPF, specific pathogen-free; SPT, supraglottic predominant taxa; Treg, regulatory T-cells

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important, like the roles that gut microbiota plays in shaping immune system development in the gastrointestinal tract, pulmonary microbiota has been gradually realized to play important roles in maintaining the homeostasis in lung.

As the main function of the respiratory system is gas exchange and the air is not always innocuous, maturation of lung immune homeostasis occurs in a nearly open environment full of microorganisms and inhaled particles. The pulmonary immune system comprises of a network of participants: the lung epithelial cells stand for the first barrier that protects against external harms, while various lung-resident lymphocytes and alveolar macrophages can continually monitor the potential threats. Exposure to external microorganisms after birth helps the host to establish the immune system of the lung. However, the homeostasis state of the pulmonary immune system is not so stable as its interaction with microbiota is dynamic, and it changes with age, genetics and environmental exposure. Dysbiosis of lung microbiota will lead to disturbance of pulmonary homeostasis and predispose the host to the occurrence or progression of lung diseases. Up to now, it is still a challenge to reveal the mechanisms of the generation and alteration of lung microbiota dysbiosis and to figure out its contributions to the development of lung diseases. In this review, we summarize recent studies of both external and internal pressures that lead to lung microbiota dysbiosis. We highlight the effects that dysbiosis of lung microbiota exerts on reshaping the pulmonary immune system and predisposition to lung diseases. Previous studies usually attribute the dysbiosis of lung diseases to the associated consequences; however, there are much more than just associations. We discuss the potential causal microbes out of the chaotic microbiota dysbiosis that contribute to pulmonary immune responses and diseases. Exploration of the causal roles of lung microbiota dysbiosis will help us better understand how the pulmonary immune homeostasis is established and provide potential precise strategies to target dysbiosis for lung disease prevention and therapy.

Generation of lung microbiota

It is generally believed that the fetus *in utero* is sterile,[10] and the lung is filled with bacteria-free amniotic fluid.[11] Immediately after birth, the newborn's mucosal tissues are colonized by bacteria, which are relatively homogeneous across all body sites, but very quickly differentiate into organ-specific communities with body sites serving as the primary determinant of the bacterial composition and its functional capacity.[12] Gollwitzer *et al.*[13] quantified the bacterial load in mouse lungs at multiple time points after birth, and found the bacterial load increases and the composition changes with age. Interestingly, they found the neonatal airways mainly harbour Firmicutes and Gammaproteobacteria, which have been proved to be associated with asthmatic phenotypes in human[6] and mice[14] and, with age, Bacteroidetes gradually expands.

To better understand the microbiology of the lung, one proposed model is the 'adapted island model',[15] where the total microbial burden and relative composition are determined by a balance between immigration of microbes from the 'mainland' and their local distinction.[4] While aspiration of oral cavity microbes from the upper respiratory tract to the lower tract is a major factor that affects the lung microbiota,[16,17] airborne microbes serve as another important contributor. Therefore, the lung microbiota displays great spatial variation between individuals and continual renewal within individuals. Although the compositions of lung microbiota of healthy subjects are relatively stable,[18] various patterns of dysbiosis of lung microbiota are associated with many lung diseases, like idiopathic pulmonary fibrosis (IPF),[19–21] cystic fibrosis (CF),[22,23] chronic obstructive pulmonary disease (COPD),[24] asthma,[25] and cancer.[26,27]

Internal and external factors that lead to lung microbiota dysbiosis

The human lung, mainly comprising of a single epithelial cell layer coated with thin mucus, has approximately 70 m^2 of surface area, which is in nearly direct contact with the exterior environment.[28] It is not exaggerating to say that the lung and its associated microbiota are just one step away from disaster. A set of internal and external factors can perturb the relatively fragile microbial ecosystem of the lung to an extent that exceeds its resistance capability and finally leads to dysbiosis (Fig. 1).

Genetics

Host genetics are critical internal factors that influence the composition of lung microbiota and local immunity. Airways are lined with a thin mucus layer,[29] which inhales external toxins and transports them out of the lung through ciliary beating and cough. MUC5AC and MUC5B are mucin-encoding genes highly expressed in the airways, with the former mainly expressed in proximal airways by surface goblet cells and the latter in surface secretory cells throughout the airways.[29] Roy *et al.*[30] found that Muc5b but not Muc5ac is required for mucociliary clearance, controlling spontaneous infections in the airways and maintaining immune homeostasis in mouse lungs. Muc5b-deficient mice harbour more culturable bacteria in the lungs over time and further more in the spontaneous moribund mice, and 16S rRNA analysis shows significantly increased *Streptococci* and *Staphylococci*, especially one important pneumonia-causing pathogen *Staphylococcus aureus*. Importantly, antibiotics treatment ameliorates the spontaneous infection-

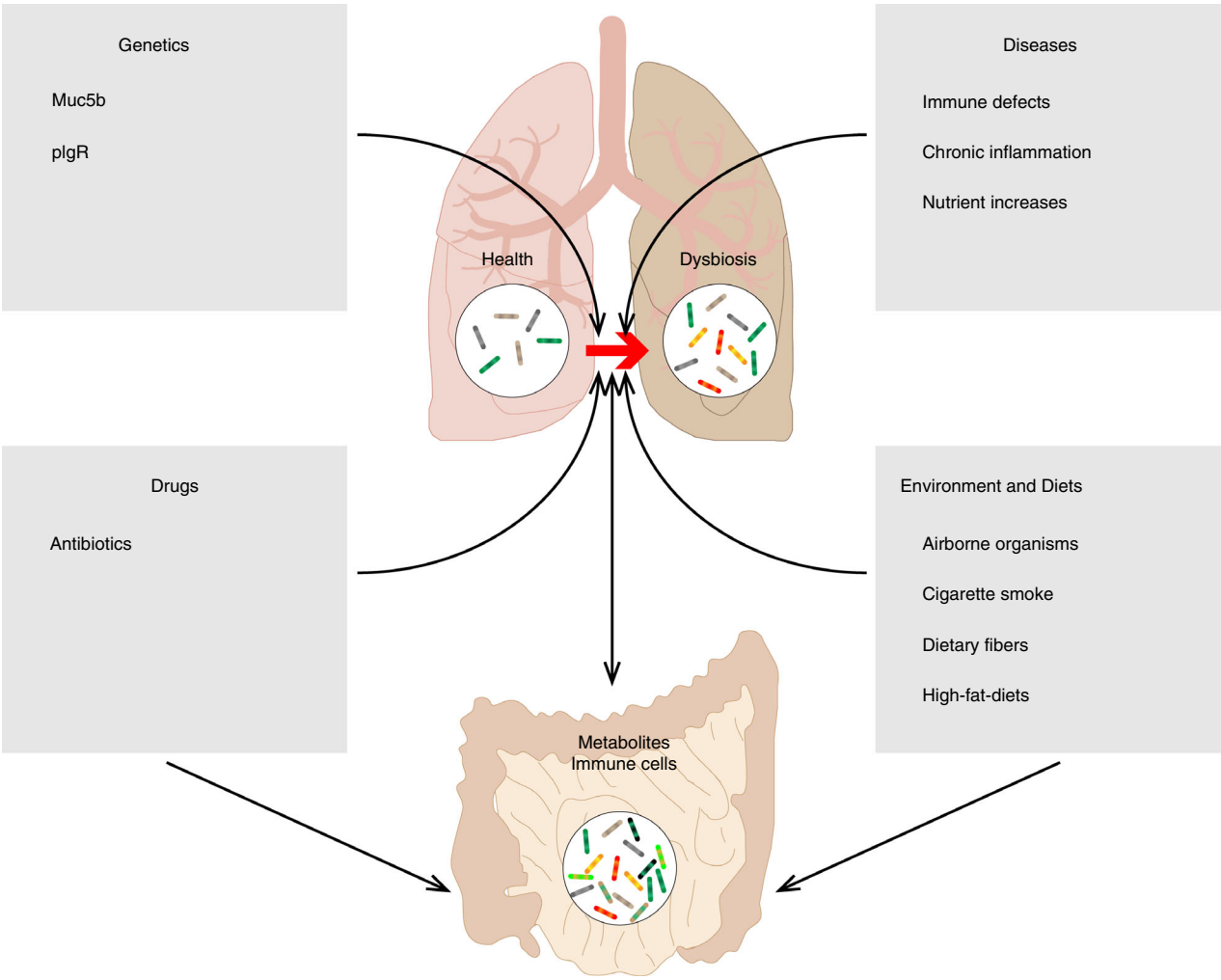

Figure 1. Internal and external factors that lead to lung microbiota dysbiosis. Genetic deficiency of Muc5b or pIgR causes direct barrier disruption in lung; drugs including antibiotics can either target local immune cells to inhibit their activity or target bacteria to prevent their survival; progression of many pulmonary diseases including viral infections and fibrosis is usually accompanied with immune defects, chronic inflammation and nutrient increases; airborne organisms and cigarette smoking may directly or indirectly shift pulmonary bacterial composition while dietary changes are associated with changes of metabolites that have systemic effects on the immune system; the lung may closely interact with the gut to form the lung–gut axis, which is mainly mediated by metabolites changes and status changes of immune cells.

associated mortality of *Muc5b*-deficient mice.[30] Paradoxically, Hancock *et al.* showed in mice that Muc5b overexpression is also related to impaired mucociliary clearance and predisposes the host to Bleomycin-induced fibrosis.[31,32] Mucin overproduction not only disrupts normal ciliary beat frequency and mucociliary transport rate, but may also provide nutrients for the opportunistic pathogens, as mucin is one of the important nutritional sources for pathogens like *Pseudomonas aeruginosa*.[33–35]

In addition to mucin, the airway epithelial cells support an antigen-specific secretory IgA (SIgA) barrier that covers and protects the airway surface.[36] In patients with COPD, widespread structural abnormalities of the airway epithelium are common, and are correlated with decreased expression of polymeric immunoglobulin receptor (pIgR) and disruption of the SIgA barrier in airways.[37] *pIgR*-deficient mice spontaneously develop COPD-like phenotypes as they age, which is correlated with about twofold increase of bacterial taxa in the lung.[38] Further analysis identified 10 taxes like *Prevotella*, *Veillonella* and *Bacillus*, that can discriminate the genotypes of *pIgR*-deficient mice.[38] Importantly, germ-free *pIgR*-deficient mice are completely protected from airway chronic inflammation, remodelling and emphysema compared with mice maintained in standard conditions,[38] and reconstituting the microbiome of germ-free *pIgR*-deficient mice can recover those COPD-like phenotypes. These data suggest that specific immunodeficiency in the lung will cause dysbiosis of lung microbiota with opportunistic

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pathogen infection and lead to disruption of local immune homeostasis.

Environment and diet

People are daily exposed to various sources of airborne microorganisms from both indoor and outdoor environments,[39,40] which may have beneficial or detrimental effects on human health. Previous studies have shown that high microbial diversity in the environment is associated with lower asthma risk, especially in children exposed to a farming environment.[41,42] Birzele *et al.*[43] found that farm exposure is positively correlated with bacterial diversity in mattress dust samples, especially the bacterial genera *Clostridium*, *Facklamia*, and some genus within the family of *Ruminococcaceae*. Interestingly, they found stronger negative association of asthma risk with bacterial diversity in mattress dust compared with that in nasal specimens, indicating that microbial involvement contributes more than just colonization of the upper airway. Another study showed that the protective low asthma risk microbiota has a low abundance of *Streptococcaceae*, which serves as predictor of asthma risk and potential modifiable target for asthma prevention.[44] Despite the fact that airborne microorganisms contribute to the ameliorated asthma, their effects on other pulmonary diseases and the corresponding lung microbiota composition need to be further determined.

As an important trigger of many inflammatory lung diseases, cigarette smoking also exerts a significant effect on the bacterial composition in the lung. Recently, one study showed that exposing mice to smoking for 90 days will cause denser inflammation and congestion in their lungs compared with the non-smoking mice.[45] Importantly, lung microbiota composition is different between the two groups, suggesting that lung microbiota dysbiosis caused by smoking may serve as the aetiology of the associated inflammatory pulmonary diseases. Another study explored the mechanisms underlying the association between cigarette smoking and the increased risk of acute respiratory distress syndrome after severe blunt trauma.[46] They found that smoking is significantly associated with compositional changes of patient lung microbiota both at the time of ICU admission and 48 hr post ICU admission, with some opportunistic pathogens including *Streptococcus*, *Fusobacterium*, *Prevotella*, *Haemophilus* and *Treponema* displaying the most significant enrichment. However, through 16S rDNA sequencing of the microbiome of the upper and lower respiratory tract in healthy nonsmokers and smokers, Morris *et al.*[47] found that the lung microbiome is not significantly altered by smoking.

The intestinal microbiota is remarkably influenced by diet,[48,49] which may also indirectly affect lung microbiota. Dietary fibres have displayed their beneficial roles in ameliorating gastrointestinal disorders, and recently they have also been proved to play a protective role in the lung against allergic airway inflammation.[14] Interestingly, dietary fermentable fibre content can modulate not only intestinal microbiota but also lung microbiota, especially by altering the ratios of Firmicutes and Bacteroidetes.[14] However, whether the changes of the lung microbiota under different dietary styles can ameliorate allergic airway inflammation needs further determination.

Drugs

Inappropriate antibiotics usage can produce long-lasting deleterious effects for human health, which has been well revealed in many gastrointestinal researches, as it may also clear beneficial bacteria and provide niches for outgrowth of opportunistic pathogens.[50–52] Early life or perinatal antibiotics exposure can cause shifts in the intestinal microbiota and predispose the host to Th2- or Th1/Th17-driven allergic airway inflammatory diseases,[53,54] suggesting that antibiotics may also cause lung microbiota dysbiosis. Using different antibiotics to treat mouse lungs has displayed different effects on the progression of Bleomycin-induced fibrosis,[19] and another study also found that vancomycin plus neomycin-treated mice have decreased bacterial load associated with reduced regulatory T-cells and enhanced T-cell and natural killer (NK) cell activation that paralleled a significant reduction of melanoma B16 lung metastasis in their lungs.[55] These data indicate that antibiotics can cause lung microbiota dysbiosis similar to the situations in the gastrointestine[56] and affect pulmonary disease development.

Co-trimoxazole and Azithromycin have been used in clinical trials to treat IPF and achieved beneficial effects,[57,58] whose effects are generally thought owing to their anti-inflammatory activity. However, these drugs are also known as broad-spectrum antibiotics, suggesting that they may exert their beneficial effects by bactericidal functions, as more than one-third of IPF patients are colonized with pathogenic bacteria or *Pneumocystis jirovecii*, most of which are susceptible to co-trimoxazole.[58] These clinic trials provide important insights into future clinical therapy of dysbiosis-associated pulmonary diseases by targeting opportunistic pathogenic bacteria with selective antibiotics.

Diseases

Lung microbiota dysbiosis occurs in nearly all kinds of lung diseases, yet the driving mechanism is largely unknown. Several investigators have now revealed that human immunodeficiency virus (HIV) infection changes the composition of lung microbiota and identified the enrichment of *Prevotella*, *Veilonella* and *Streptococcus* in the lower airways, which positively correlate with advanced HIV infection and HIV-associated pulmonary

diseases.[59] HIV infection is accompanied with immune defects and chronic inflammation, which is likely to cause dysbiosis in the lung. Lozupone *et al*. found that the bronchoalveolar fluid of HIV-positive individuals was frequently enriched with *Tropheryma whipplei*, the aetiological agent of Whipple's disease, which was reduced by effective antiretroviral therapy.[60]

Among many factors affecting microbiota composition, nutrients are most vulnerable to be disrupted. The nutrient supply of the airways, normally scarce in health, but is likely increased by the presence of mucus and vascular permeability during lung injury. Dysbiosis of lung microbiota in both IPF patients and Bleomycin-induced fibrosis models has been widely reported,[19–21] yet the reason remains to be explored. Dysbiosis of lung microbiota after Bleomycin exposure precedes the onset of fibrosis, showing increased bacterial load and alpha diversity.[19] Further analyses show that the Gram-negative Bacteroidetes is the most upregulated bacteria phylum, among which *Bacteroides* and *Prevotella* are the most obviously increased genera. Intestinal *Bacteroides* abundance is strongly correlated with richness of many host amino acids and fatty acid metabolites, whereas *Prevotella* abundance is associated with carbohydrate metabolism in the host. RNA-seq assay shows that the corresponding genes associated with those metabolites are indeed increased in Bleomycin-treated mouse lungs; therefore, like in the gut, those increased metabolites under the situation of lung injury may endow *Bacteroides* and *Prevotella* growth advantage. Another acute lung injury model induced by lipopolysaccharides (LPSs) intratracheal administration also shows increased bacterial loads and remarkably changed composition.[61] Further analyses show that bacteria from families *Xanthomonadaceae* and *Brucellaceae* increase their abundance in the injured mouse lungs, and metabolic profiling of BAL from these mice detects the presence of bacterial substrates suitable for both isolates, suggesting metabolic changes in the pulmonary niches after lung injury may provide these bacteria with a growth advantage.

Lung–gut axis

Except for the above factors, lung microbiota can also be systemically influenced by gut microbiota to form a lung–gut axis that has been discussed in many reviews.[62–64] Reflux and aspiration can promote direct bacterial seeding into the lungs, while metabolism and nutrition are key indirect mediators of the gut–lung axis. Gut microbiota can metabolite dietary fibres into short-chain fatty acids (SCFAs), which enhances seeding of lung with dendritic cells (DCs) that have high phagocytic capability but impaired ability to promote Th2 cell effector function thereby ameliorating the allergic airway inflammation.[14] However, SCFAs only represent a small fraction of metabolites that alter with changes of dietary, antibiotics treatment, or inflammation in the gastrointestine. Thus, the potential roles of other metabolites still remain to be explored.

Interestingly, changes in lung microbiota may also influence the composition of gut microbiota. Influenza virus infection in the respiratory tract is often accompanied by increases of *Enterobacteriaceae* as well as reductions of *Lactobacilli* and *Lactococci* in the intestinal microbiota.[65,66] Another study shows that the dysbiosis in lung microbiota upon administration of LPS in mice is accompanied by disturbances in their gut microbiota due to movement of bacteria from their lung into the bloodstream.[61] As the lung–gut axis has attracted more and more attention, manipulation of gut microbiota or its metabolites may serve as potential clinical strategies to treat lung diseases.

Lung microbiota dysbiosis affects local inflammation

Microbial colonization in the gastrointestinal tract after birth is a critical step for the education of the local and systematic immune compartment,[67] and people gradually find that this dogma also suits the lung mucosal system. Although no viable bacteria have been detected in lung specimens from preterm and term infants, one study proves that even the microbial DNA in the fetus lung shows its effect on the developing pulmonary immune system, and 'dysbiosis' predisposes the fetus to many lung diseases like bronchopulmonary dysplasia,[11] although no obvious differences in pulmonary structure, epithelium thickness or bronchus number are observed between germ-free mice and bacteria-colonized mice, and lungs of germ-free mice have bigger but less alveolar as illustrated by histological examination.[19,68] The lack of microbiota leads to immaturation of the pulmonary immune system, rendering the host susceptible to lung disorders like allergic asthma.[9,69] In addition to the fact that dysbiosis of lung microbiota is associated with progression of nearly all kinds of pulmonary diseases,[4] there are more and more direct evidences to propose that lung microbiota deficiency or dysbiosis, once established, dramatically affects both local pulmonary and systemic landscape of immune cells, thereby creating a feedback loop where local immune cells and microbiota cross-talk with each other (Fig. 2).

Effects on innate immunity

Pulmonary epithelium is the first defense barrier that protects the lung against external toxins, which is coated with thin and mobile mucus.[29] Mucus production in the intestine is a dynamic process that is affected by microbiota,[70] so does it work in the lung. Histological

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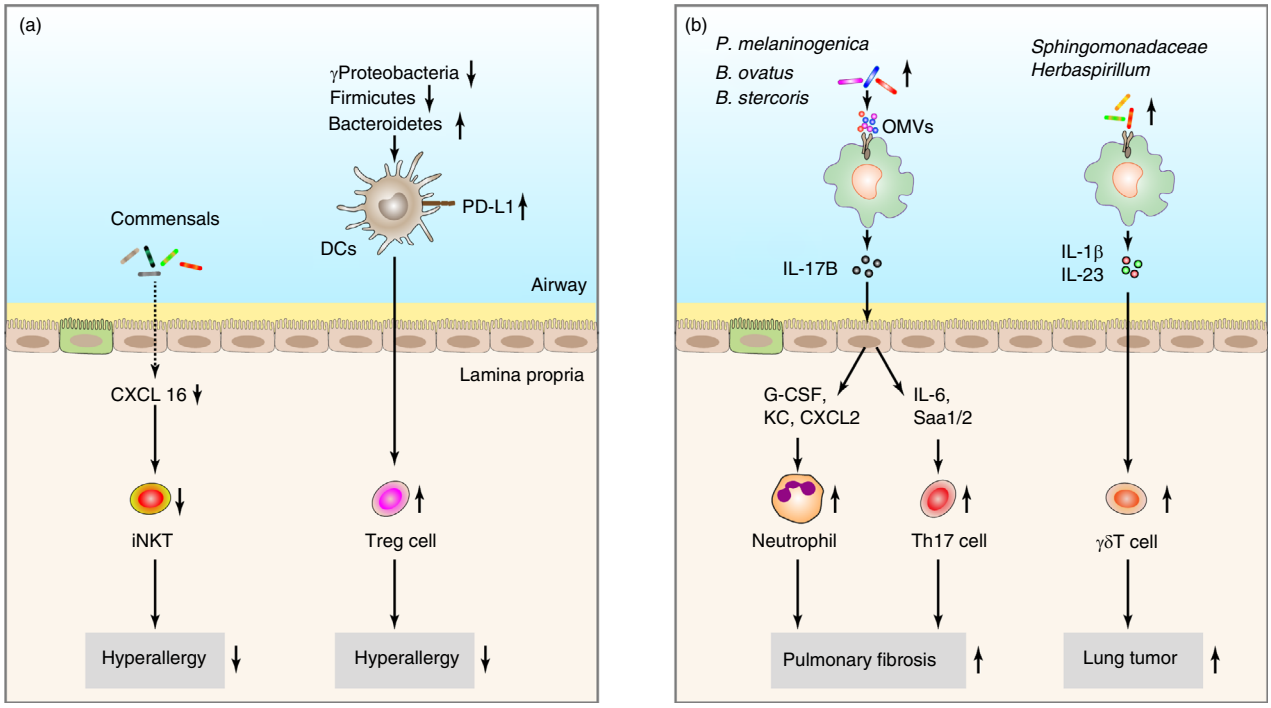

Figure 2. The effects of lung microbiota on the pulmonary immune system. (a) The gradual emergence of pulmonary commensals is associated with decreased allergic responses in the lung. (b) Dysregulated lung microbiota especially the expansion of specific microbes contributes to chronic inflammation that promotes the progression of pulmonary diseases including fibrosis and tumour.

examination reveals that, compared with bacteria-colonized mouse lungs, lungs of germ-free mice have reduced mucus,[68] which is consistent with another study showing that Muc5ac mRNA levels are higher in the lungs of specific pathogen-free (SPF) mice.[9] Interestingly, colonizing germ-free mouse lungs with bacteria *Lactobacillus* spp. restores mucus production to the level of SPF mice.[68] Both intestinal and pulmonary microbiota can promote the expression of several interleukin (IL)-17 family cytokines, like IL-17A and IL-17E,[19,71,72] which have been proved to enhance airway mucus production through increasing the expression of Muc5ac,[73–75] and dysbiosis of lung microbiota that occurred in many chronic lung inflammatory diseases is usually associated with either reduced or increased mucus production.[30,31] Therefore, these data suggest lung microbiota may regulate the local mucosal barrier functions through modulating mucus production.

The healthy lungs are populated with a number of different macrophages, with alveolar macrophages as the dominant subpopulation.[76,77] Alveolar macrophages are vital for maintaining lung health and function by clearing external toxins, debris, surfactant and apoptotic cells, therefore functioning as sentinels under healthy state.[76] During inflammation, alveolar macrophages can mediate bacterial clearance and initiate neutrophil recruitment.[78] Wang *et al.*[79] identified that *S. aureus*, a common lung opportunistic pathogen, recruits peripheral $CCR2^{+}CD11B^{+}$ monocytes into alveoli and polarizes them into M2 alveolar macrophages, which significantly attenuates influenza-induced lung injury. Microaspiration is a common phenomenon mediating seeding of oral bacteria into airways in healthy subjects. Segal *et al.* found that about half of the people they examined are enriched with oral taxa, therefore they classified two distinct lung microbiomes: $\text{pneumotype}_{\text{SPT}}$, characterized by high bacterial load and supraglottic predominant taxa (SPT); and $\text{pneumotype}_{\text{BPT}}$, with low bacterial burden and background predominant taxa (BPT).[17] Of note, alveolar macrophages isolated from $\text{pneumotype}_{\text{SPT}}$ lungs, which are enriched with bacterial taxa like Prevotella, Rothia and Veillonella, show decreased production of IL-6 and MIP-1α under LPS stimulation, indicating an attenuated immune response of alveolar macrophages under the presence of $\text{pneumotype}_{\text{SPT}}$ lung microbiota.[17] Another study shows that lung microbiota dysbiosis in lung transplantation may lead to inflammatory or remodelling profiles in macrophages.[80] Importantly, Firmicutes- and Proteobacteria-driven dysbiosis is associated with low macrophage and high neutrophil percentages and an inflammation gene expression profile while, in contrast, Bacteroidetes-driven dysbiosis is linked to high macrophage and low neutrophil percentages and proremodelling profile.[80]

Dendritic cells bridge innate immunity and adaptive immunity in the lung mucosal system. Under homeostatic conditions, lung mucosa generally contains two functionally different DC subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs).[81] As in the gut, DCs in lungs are constantly exposed to external factors, including commensal bacteria and pathogens, and both cDCs and pDCs express a large number of pattern recognition receptors,[81] and thus it is not surprising that DCs play critical roles in regulating local immune homeostasis through interacting with local microbes. Increased bacterial load in the lungs during the first 2 weeks after birth protects mice from airways hyperresponsive to allergens. Mechanistically, the changed lung microbiota, especially shifts from a predominance of Gamma proteobacteria and Firmicutes to Bacteroidetes, promotes PD-L1 expression on $CD11b^+$ DCs in lung, which then induce Treg cells to ameliorate the airway allergic inflammation.[13] Increased numbers and altered functions of airway mucosal DCs have been found in many chronic inflammatory pulmonary diseases, like asthma[82] and COPD.[83] Lower expression of the inducible co-stimulatory molecule ligand (ICOS-L) on airway DCs is observed in individuals with severe asthma,[84] while cDCs in patients with COPD show prominent expression of Langerhans' cell markers langerin and CD1a and the co-stimulatory molecules CD80 and CD86.[83] Yet, the specific roles of lung microbiota dysbiosis in regulating airway DCs functions need to be further determined. One study shows that lung commensals and pathogenic bacteria exert different effects on human monocytes-derived DCs *in vitro*,[85] and that pathogenic bacteria provoke higher levels of IL-23, IL-12p70 and IL-10 production than commensal like *Prevotella* spp. and *Veilonella* spp. Therefore, dysbiosis of lung microbiota, particularly outgrowth of opportunistic pathogens, may remodel DCs functions to regulate local immunity.

Invariant natural killer T (iNKT) cells are a CD1d-restricted T-cell population, which use an αβ T-cell receptor heterodimer with limited diversity for the recognition of exogeneous (bacterial and nonbacterial) and endogenous lipid antigens.[86] iNKT cells play an important role in the control of commensals including opportunistic pathogenic microbiota.[87] In turn, the microbiota regulates iNKT cells. Olszak *et al.*[88] illustrated that iNKT cells contribute to the enhanced airway allergic inflammation in germ-free mice. The expression and production of the chemokine ligand CXCL16, which is responsible for iNKT recruitment, is increased in germ-free mouse lung and intestine, and colonization of neonatal germ-free mice with a conventional microbiota could decrease CXCL16 production and thus alleviate the hyperallergic responses.[88] Interestingly, regulation of CXCL16 expression and iNKT cells accumulation by the microbiota is independent of the Toll-like receptor adaptor protein Myd88, but depends on epigenetic modulations like CpG methylation.[88] Further study showed that monocolonization with a human bacterium *Bacteroides fragilis* or treatment with a *B. fragilis*-derived glycosphingolipid can block the increased number of iNKT cells in the adult colon but not in the lung,[89] indicating that specific lung microbes are likely responsible for local pulmonary iNKT cells regulation.

Effects on adaptive immunity

Lung-resident γδT cells are known as important effector and regulator cells maintaining pulmonary homeostasis and affecting the progress of many pulmonary diseases including lung cancers and COPD.[90] Recently, Jin *et al.*[27] provided evidence that lung microbiota is disordered in lung tumour-bearing mice, displaying increased bacterial load and reduced bacterial diversity in the airways. Several bacterial taxa, including *Herbaspirillum* and *Sphingomonadaceae*, *Aggregatibacter* and *Lactobacillus*, are dramatically expanded in tumour-bearing lungs. Importantly, the tumour development is significantly correlated with the dysbiosis of lung microbiota but not intestinal microbiota.[27] Mechanistically, dysregulated lung microbiota provokes activation of resident γδT cells to promote lung adenocarcinoma development. Lung commensal microbes could stimulate the production of IL-1β and IL-23 from myeloid cells in a Myd88-dependent manner, and then induce the proliferation and activation of specific γδT cells that produce IL-17 to promote tumour-associated inflammation.[27] Interestingly, the differentiation and activation of γδT cells are discounted in germ-free mice and antibiotics-treated mice, which are significantly protected from lung cancer development induced by *Kras* mutation and *p53* loss.[27] Lung microbiota dysbiosis is associated with progressive respiratory diseases like COPD. Yadava *et al.*[91] explored whether there is a causal relationship of the dysbiosis and COPD progression by using a LPS/elastase intranasally treated mouse model. They found both microbial abundance and diversity were decreased in the COPD-like mouse lungs with an outgrowth of the genera *Pseudomonas*, *Lactobacillus* and a reduction of *Prevotella*. Intranasal transfer of the enriched microbiota from LPS/elastase-treated mice into antibiotics-treated mice could induce IL-17-producing γδT cells development, and recapitulate the IL-17 inflammatory responses.[91] Together, these data suggest an important role of γδT cells in mediating the effect of lung microbiota dysbiosis on the progression of chronic inflammatory lung diseases.

Th17 cells, the major cell sources of IL-17, play critical roles in regulating pulmonary homeostasis and diseases.[92] Our recent work shows that the dysregulated lung microbiota in mice with Bleomycin-induced fibrosis can remarkably induce Th17 cells and the following IL-17 production, and then promote fibrosis progression.[19]

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Th17 cells are diminished in the lungs of either antibiotics-treated mice or germ-free mice, suggesting a critical role of the disordered microbiota in regulating Th17 cells development. Importantly, several bacterial species out of the genera *Bacteroides* are identified to dramatically induce Th17 cells when intranasally transferred into antibiotics-treated or germ-free mice. Mechanistically, these bacteria can act on alveolar macrophages in a Myd88-TLR2/4-dependent manner to secret IL-17B, another IL-17 family member, which in turn stimulate lung epithelial cells to produce Th17-promoting cytokines like IL-6 and Saa1/2. Other investigations have also found correlations of the lung microbiota dysbiosis with Th17 cells in severe asthma. Huang *et al.* found in severe asthma patients that Th17 cells are positively correlated with a number of taxa, mainly Proteobacteria like *Pasteurellaceae*, *Enterobacteriaceae* and *Bacillaceae*.[93] Interestingly, a proportion of taxa showing correlation with expression of FKBP5, an indicator of steroid responsiveness, also belong to Proteobacteria but not the same families as that correlated with Th17 cells,[93] suggesting a multifactorial effect of dysbiosis on allergic inflammatory responses. Moreover, although lung microbiota from pneumotype$_{SPT}$ individuals blunts alveolar macrophages TLR response as mentioned above, it is positively correlated with a proinflammatory phenotype characterized by multiple Th17-related cytokines like IL-1β, IL-6 and Th17 lymphocytes.[17]

Regulatory T (Treg) cells are critical for maintenance of pulmonary immunological tolerance to airborne allergens and for restrain of deleterious immune responses to self and non-self antigens.[13,94] A limited number of lung microbiota in neonatal mice render the host susceptible to airway allergic inflammation, and the increased bacterial load after birth could promote development of Helios$^-$ Treg cells, which decrease the hyperallergic responsiveness.[13] The positively correlated increase of lung microbiota and Treg cells is mainly mediated by the induced expression of PD-L1 on DCs,[13] indicating a mechanism through which lung commensal microbes modulate Treg cells development and allergic responses. At the same time, Treg cells are downregulated in many chronic inflammatory pulmonary diseases, including CF [95] and COPD,[96] and their decrease correlates with the disease progression. Maybe it is the lung microbiota dysbiosis that leads to the impaired Treg cells, resulting in deficient capability to restrain the local inflammatory responses. One recent study shows that aerosol administration of vancomycin and neomycin for 5 days significantly affects lung microbiota diversity, displaying reduced population of the genus *Streptococcus*, which are common Gram-positive Firmicutes commensal of the lung, and increased bacterial genera belonging to less represented members of Proteobacteria and Actinobacteria.[55] The compositional changes are accompanied with reduced Treg cells, which contribute to a significant reduction of melanoma B16 lung metastasis.[55]

The causal microbes that contribute to pulmonary diseases

Dysregulated lung microbiota is associated with the pathogenesis of nearly all kinds of chronic inflammatory pulmonary diseases, which has been discussed in many reviews. However, whether the dysbiosis is a consequence or cause of these diseases remains largely unknown. Here we briefly categorize recent progresses exploring the causal microbes of these diseases.

Our recent work shows that lung microbiota is dysregulated in Bleomycin-induced fibrosis mice, and the fibrotic phenotypes are reduced in either antibiotics-pretreated or germ-free mice.[19] In order to identify whether there's specific commensal microbes contributing to fibrosis, individually intranasal inoculation of several bacterial species, including the most significantly expanded bacteria *Bacteroides ovatus*, *Bacteroides stercoris*, *Prevotella melaninogenica* and *Ruminococcus gnavus* in Bleomycin-treated lungs, and the most abundant bacteria *Streptococcus* sp. and *Lactobacillus murinus* in healthy lungs, into antibiotic-pretreated mice and germ-free mice shows that *B. ovatus*, *B. stercoris* and *P. melaninogenica* remarkably enhanced the fibrotic responses.[19] Mechanistically, these profibrotic bacteria can secret outer membrane vesicles (OMVs), containing ligands for TLR2/4, to stimulate alveolar macrophages, which then produce IL-17B to promote fibrosis through inducing Th17 cells and neutrophils.[19] Therefore, specific microbes could modulate disease progression by producing OMVs.

Another recent study shows that pulmonary local microbiota is disordered along with the development of lung adenocarcinoma, and most importantly the dysbiosis can provoke local inflammation by inducing the proliferation and activation of γδT cells.[27] *Kras* mutation and *p53* loss-induced lung cancer development is dramatically ameliorated in germ-free mice or antibiotics-pretreated mice.[27] To further confirm the important role of lung microbiota in promoting tumour development, they isolated and cultured several bacterial species including *Lactobacillus*, *Streptococcus* and *Staphylococcus* from the late-stage lung tumours and then intratracheally inoculated into another cohort of mice shortly after tumour initiation. The inoculation of these bacteria cocktails dramatically enhanced the expansion of IL-17-producing γδT cells in a TLR-Myd88-dependent manner and accelerated tumour development.[27] Consistently, local administration of TLR ligands like LPS and peptidoglycan also triggered the expansion of IL-17-producing γδT cells in the lung.[27] However, the specific commensal microbes contributing to tumour development remain to be defined.

Moreover, intranasal inoculation of microbiota isolated from LPS-treated mice intensifies IL-6-induced lung inflammation in naive mice,[61] while intranasal transfer of fluid enriched with the pulmonary microbiota isolated from LPS/Elastase-treated mice, a mouse model to mimic human COPD diseases, enhances IL-17A production in the lungs of antibiotics-treated or germ-free mice.[91] Despite that the specific composition of lung microbiota needs further exploration, these studies elucidate that lung microbiota, especially the set of inborn opportunistic pathogens outgrew in the environment of inflamed lungs but not the external infectious pathogens, play causal roles in many pulmonary diseases.

Conclusions and future perspectives

Significant progresses have been made in the past several years, which elucidate the critical roles of the lung microbiota in establishing local immune homeostasis and protection against external threats. The bidirectional and geographically isolated lung structure endows lung microbiota with fragility and dynamic, therefore dysbiosis occurs under various deleterious factors, which once established will lead to acceleration of pulmonary diseases. On the other hand, the relatively low abundance of lung microbiota makes it a challenge to isolate, culture and identify the functional microbes. However, the low biomass does not undermine its potential critical roles in shaping local lung immunity.

A deep understanding of the interactions between lung microbiota dysbiosis and the local immune system makes modulation of pulmonary microbiota an insightful therapeutic strategy to treat lung diseases just like already suggested in the intestine like *Bifidobacterium bifidum*, which has been demonstrated to activate TLR2 and endow DC cells with the capability to induce Treg cells thereby protecting against experimental colitis,[97] and *Lactobacillus plantarum*, which combined with fructo-oligosaccharide enables long-term colonization and protection against sepsis in neonates.[98] Interestingly, aerosolization with *Lactobacillus rhamnosus* or *B. bifidum* was recently proved to be able to decrease the immunosuppressive cells and increase the activity of effector cells, thereby preventing tumour implantation in the lungs.[55] Moreover, intranasal administrations of several different bacterial species of the genus *Lactobacillus* have also been reported to protect the host against the influenza infections.[99–101]

Compared with the research of microbiota in other mucosal tissues, especially the intestine, our understanding of lung microbiota is still limited and a set of conceptual questions still need to be answered. One question is what are the nonbacterial components of lung microbiota like fungi and virus? Fungal[102,103] and viral [65,79] infections are common in many lung diseases, deciphering their composition and interaction with the local immune system will help us understand the primary cause of particular diseases and develop more efficient therapies. The second question is how to deeply identify the causal commensals of a lung disease. Chronic lung diseases are associated with lung microbiota dysbiosis, allowing for the complexity of the composition, searching for the real 'assailant' remains a challenge. Triangulation of microbe–phenotype relationships has been shown to be an effective method for reducing the noise inherent in microbiota studies and enabling identification of causal microbes in colitis.[104] However, there is still no such efficient method as to lung research except for testing the candidate bacteria by inoculation of them individually into bacteria-depleted mice. Another question is how these causal bacteria exert their detrimental effects. To answer this question, we need to combine the genomic, transcriptomic and metabolomic analysis to identify the mechanisms by which the dysregulated microbiota promotes the pathogenesis of lung diseases. The last question is how to restore the normal lung microbiota after dysbiosis. The prerequisite of restoring the dysregulated microbiota is finding out the main triggers among the various deleterious factors and developing specific targeting therapies. Extensive fecal microbiota transplantation trials aimed at restoring intestinal microbiota diversity have been conducted in an attempt to combat the associated diseases, showing success rates of over 80% in treating infection with *Clostridium difficile*, a nosocomial pathogen often recalcitrant to treatment with antibiotics.[105] Likewise, intranasal or aerosol administration of normal microbiota into patient lungs may be a potential choice to restore lung microbiota to treat the associated pulmonary disorders.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (91842306, 81830018 and 81430036), National Key R&D Program of China (2018YFA0507402), and the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB19000000).

Disclosure

The authors declare no competing financial interests.

Author contributions

D.Y. wrote the manuscript; Y.Q., X.S. and Y.X. edited the manuscript.

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