Severe viral respiratory infections: are bugs bugging?

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Viral respiratory tract infections (RTI) pose a high burden on the youngest members of our society. Several risk factors are known for severe viral respiratory disease. However, a large proportion of the severe RTI cannot be explained by these risk factors. A growing body of evidence shows that the composition of the microbiota has a major influence on the training of both the mucosal and the systemic immune response and can thus potentially determine susceptibility for severe viral infections. In this review, we discuss the current evidence regarding the influence of bacterial colonization on the severity of viral respiratory infections.

INTRODUCTION

Acute respiratory tract infections (RTI) are the leading cause of mortality and morbidity in infants and young children.[1,2] Both bacteria and viruses can cause serious RTI. Vaccination and the availability of antibiotics substantially reduced the mortality caused by bacterial RTI in developed countries. However, due to the limited availability of antiviral medications and effective vaccines the burden of viral RTI remains high. The leading cause of serious viral RTI in young children is respiratory syncytial virus (RSV),[3] but influenza virus, rhinovirus, parainfluenza virus, adenovirus, and human metapneumovirus can also cause severe respiratory disease.[4,5]

Children are frequently infected with these respiratory viruses, especially during the winter season. In most children, this leads to relatively mild symptoms, presenting as a common cold. However, some children have a more severe course of disease and develop lower respiratory tract symptoms, such as pneumonia and bronchiolitis. These children need to be hospitalized for supportive care and in severe cases mechanical ventilation is needed. This striking diversity in severity of infection is especially evident in RSV infections. Known risk factors to develop severe infection are prematurity, age (<6 months), congenital heart disease and chronic lung disease (bronchopulmonary dysplasia), presence of siblings, and breastfeeding (<1 month).[3,6–8] The contribution of these risk factors to the development of severe disease is not fully understood.[9] In addition, a large proportion of children with severe disease who required hospitalization are previously healthy and have no known risk factors.[10,11]

In recent years, a growing body of evidence has shown that colonization of mucosal tissues can influence the immune system both locally and systemically. We hypothesize that the composition of the microbiome may affect the severity of viral infection. This article aims to discuss the available evidence regarding the microbiome as a determinant for disease severity of viral RTI.

THE MICROBIOME AFFECTS THE IMMUNE SYSTEM BOTH LOCALLY AND SYSTEMICALLY

Mucosal surfaces of the human body provide residence to complex microbial ecosystems, together called the "microbiota". The presence of these bacteria is crucial for our well being. Microbiota have an important role in the digestive process in the intestines, produce vitamins, and provide a barrier to protect against translocation by and infection with pathogens.[12]

Vertebrates have co-evolved with bacteria present in their bodies for nearly half a billion years. Resident bacteria profoundly shape the immune system, while the immune system has to control the microbiota. This has resulted in a mutualistic and symbiotic partnership between the human immune system and these commensal microorganisms.[13]

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Gut microbiota influence the immune system both locally and systemically

The area in the human body with the highest diversity and density of microbes is the gastrointestinal tract.[14] Although there is an enormous variety in taxa and composition between individuals, the gut microbiota is typically dominated by strict anaerobes like *Firmicutes* (e.g., *Lactobacillus*, *Bacillus*, *and Clostridium*) and *Bacteroidetes* (e.g., *Bacteroides*).[15,16] In lower abundances *Proteobacteria* (e.g., *Escherichia*) and *Actinobacteria* (e.g., *Bifidobacterium*) can be found.[15] The composition of the microbiota of the adult gut is mainly influenced by dietary patterns.[17] However, broad-spectrum antibiotics, inflammation, or other stress inducers may influence the composition as well.[17]

The influence of the gut bacteria on the immune system has been extensively studied and reviewed.[17–21] Disruption of the balance in the microbiota (dysbiosis) has been associated with inflammation-linked disorders, e.g., inflammatory bowel disease and airway allergies. A growing body of evidence suggests that the composition of gut commensals has systemic effects and influences the immune response at distant mucosal locations.

Clarke *et al.*[22] have shown that peptidoglycan from the gut translocates to the bloodstream and to the bone marrow. This systemically present peptidoglycan primes the immune system, enhancing neutrophil killing of two important pathogens, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Commensal gut bacteria influence the balance of T-cell subsets, which reaches far beyond the extent of the intestinal lamina propria. In germ-free (GF) mice, colonization with segmented filamentous bacteria skews the immune system towards a pro-inflammatory response, inducing T helper type 17 (Th17) cells and some Th1 cells leading to arthritis and experimental autoimmune encephalomyelitis.[23,24] In contrast, colonization with certain *Clostridial* strains skews towards an anti-inflammatory response, inducing regulatory T cells (T_{regs}), which reduce serum immunoglobulin E (IgE) responses after immunization.[25] Colonization with polysaccharide A–producing *Bacteroides fragilis* results in higher numbers of circulating CD4+ T cells and a higher Th1 response in circulation.[26]

Multiple studies have shown that the gut microbiota also have an influence on the immune response of the airways.[27] The hygiene hypothesis proposes that disruption of the gut microbiota by, e.g., antibiotics, dietary changes, or a reduction in infections due to decreased exposure, induces a disturbance of the immunological tolerance, resulting in enhanced allergic airway diseases. This is supported by experiments showing that antibiotics disturb the microbiota of the gut of mice, which subsequently induces a reduction in Th1 response and a more severe allergic response in the airways.[28]

Collectively, these data suggest that the commensals of the intestines are crucial for training of the immune system both locally and systemically (**Figure 1**).

Microbiota of the respiratory tract

Respiratory viruses enter the human body through the upper respiratory tract. The bacteria present in the airways may therefore be of importance for the host response towards a viral infection. At present, there are a limited number of papers studying respiratory tract microbiota in healthy adults. An overview of these papers can be found in **Table 1**.

It has been shown that *Firmicutes* and *Actinobacteria* dominate the nostrils. These are mainly *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* on genus level, which are typical skin lineages.[32,33] The oropharynx contains, on

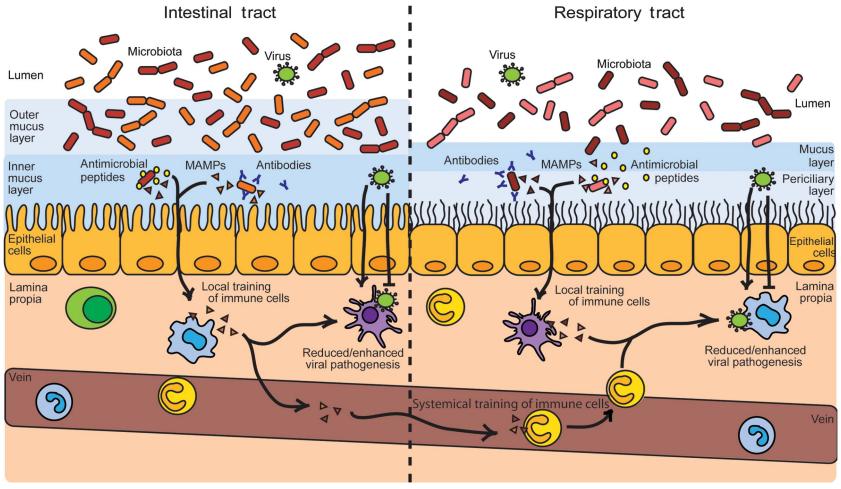

Figure 1 Bacteria and bacterial ligands can train immune cells both locally and systemically. The intestines have a very high diversity and abundance of commensals. Bacteria that invade the inner mucus layer are attacked by antimicrobial peptides and antibodies. Bacterial components are being released and these components can enter the lamina propia where they can locally train the immune cells or enter the bloodstream and train immune cells systemically. These systemically trained immune cells can enter other parts of the human body, e.g., the upper respiratory tract mucosa. The respiratory tract is also colonized by commensals and local training occurs in the lamina propia of the respiratory tract. The local or systemical training of immune cells can enhance or reduce viral pathogenesis, depending on the specific virus and bacteria involved. MAMP, microbe-associated molecular pattern.

Table 1 Overview of current studies on the respiratory tract microbiota

	Study	Location of microbiota	Age of subjects	Number of healthy subjects	Predominant phyla	Predominant genera	Conclusions
Children (<18 years)	Bogaert *et al.*[29]	Nasopharynx	18 months	96 healthy infants	*Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*	*Moraxella*, *Haemophilus*, *Streptococcus*, *Flavobacteria*, *Dolosigranulum*, *Corynebacterium*, *Neisseria*	Children had a high inter-individual variation; therefore no core microbiome could be determined. Winter gave predominantly a *Proteobacteria/Fusobacteria* profile, whereas spring gave a *Bacteroidetes/Firmicutes* profile. The seasonal effect could not be attributed to viral co-infection
	Dominguez-Bello *et al.*[30]	Nasopharynx	5 min	10 healthy newborns	—	Vaginal: *Lactobaccillus*, *Prevotella*, *Sneathia* C-section: *Staphylococcus*, *Corynebacterium*, *Propionibacterium*	Microbiota were undifferentiated over multiple body habitats. Mode of delivery determines the composition of the nasopharynx
	Hilty *et al.*[31]	Nasopharynx	0–2 years	10 healthy infants	—	*Moraxella*, *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Flavobacterium*	Putative commensal families of bacteria were more present in healthy children. Colonization density is lower in bacterial communities with high richness
Adults (>18 years)	Frank *et al.*[32]	Nostril	—	5 healthy adults	*Actinobacteria*, *Firmicutes*	*Propionibacterium*, *Staphylococcus*, *Corynebacterium*	The nostrils harbor a diverse, distinct, and temporally stable microbiota. Cohabitation did not result in convergence of the microbiota
	Lemon *et al.*[33]	Nostril	26–45 years	7 healthy adults	*Firmicutes*, *Actinobacteria*	*Staphylococcus*, *Propionibacterium*, *Corynebacterium*	Nostril microbiota varied between adults. There is an inverse correlation between *Firmicutes* and *Actinobacteria* in the nostrils
	Allen *et al.*[34]	Nasopharynx	18–65 years	10 healthy adults	—	*Streptococcus*, *Staphylococcus*, *Neisseria*	Every adult had a unique bacterial profile in his or her nose, and the bacterial load and profile did not change during experimental rhinovirus infection.
	Chaban *et al.*[35]	Nasopharynx	<1–89 years	65 pandemic H1N1 patients	*Actinobacteria*, *Firmicutes*, *Proteobacteria*	*Corynebacterium*, *Propionibacterium*, *Streptococcus*, *Staphylococcus*, *Variovorax*, *Moraxella*	A limited number of nasopharyngeal microbiome profiles were found. Severity of viral illness could not be assessed. Therefore, the only correlation that could be found was a trend towards higher nasopharyngeal diversity with increasing age
	Ling *et al.*[36]	Nasopharynx	21–24 years	10 healthy adults	*Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*	*Corynebacterium*, *Dolosigranulum*, *Staphyloccocus*, *Lactobacillus*, *Propionibacterium*, *Gardnerella*, *Anaerococcus*, *Prevotella*	A signifiticant inter-individual variation was found. The nasopharynx contains a distinct microbiota
	Charlson *et al.*[37]	Nasopharynx/ lung	—	6 healthy adults	—	*Staphyloccocus*, *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *Veillonella*, *Prevotella*	Composition of the microbiome of the lung is not distinct from the microbiome of the upper respiratory tract but $^{2-4}$log lower in biomass than the upper respiratory microbiome. There is no unique lung microbiome

Table 1 (Continued)

Study	Location of microbiota	Age of subjects	Number of healthy subjects	Predominant phyla	Predominant genera	Conclusions
Garzoni *et al.*[38]	Lung	Mean age 57.6 years	9 healthy adults	—	*Prevotella*, *Streptococcus*, *Neisseria*, *Fusobacterium*, *and Acidaminococcus.*	—
Hilty *et al.*[39]	Lung	Mean age 53 years	8 healthy adults	*Bacteroidetes*, *Firmicutes*	*Prevotella*, *Veillonella*, *Streptococcus*	—
Erb-Downward *et al.*[40]	Lung	40–78 years	3 healthy adults	*Proteobacteria*, *Firmicutes*, *Bacteroidetes*	*Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacterium*	Significant levels of bacteria were found. There is a core pulmonary microbiome that is diverse but has limited richness at the sub-genus level
Morris *et al.*[41]	Lung	18–80 years	45 healthy adults	—	*Streptococcus*, *Prevotella*, *Veillonella*	Most species identified in the lung were also present in the mouth, but there were distinct species that were over-represented in the lung

Search terms: "nasopharynx microbiota", "lung microbiota" and "respiratory tract microbiota". Papers were selected on the following criteria: research papers in English focussing on healthy children/healthy adults, investigating the nostrils/nasopharynx/lungs with minimally three healthy volunteers. In the cases that we did include a study with patients, the results of the control group were separately discussed making it possible for us to interpret these. We only included data on the healthy control group in the table.

phylum level, mainly *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, and on species level, these are mainly *Streptococcus*, *Neisseria*, *Haemophilus*, and *Lachnospira*.[33,37] The nasopharynx contains a combination of the skin lineages found on the nostrils (*Firmicutes* and *Actinobacteria*) combined with lineages typically found in the oral cavity (*Proteobacteria* and *Bacteroidetes*), e.g., *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Neisseria*, and *Prevotella*.[34,36,37]

Determination of the microbiota of the lower respiratory tract is complicated due to technical difficulties. Sampling of healthy donors without contaminating these samples with upper respiratory bacteria is difficult. Moreover, studies performed so far often focussed on individuals with lung disease, e.g., chronic obstructive pulmonary disorder, cystic fibrosis, and asthma, and mostly included small patients groups. Recently, several extensive reviews have been written on the role of the respiratory microbiome in disease.[42,43] Studies have shown that the lungs of diseased and healthy patients show major differences. Healthy lungs contain low amounts of biomass thereby making it hard to detect the microbiota, whereas diseased lungs mostly contain measurable quantities of pathogens typically associated with the disease.[44] A "core airway microbiota" has been described in healthy subjects, containing predominantly *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacterium*, *Haemophilus*, *Veillonella*, *and Porphyromonas*.[40] However, other studies have been more sceptical. One study using healthy donors showed no clear distinction between microbiota of the upper and lower airways. Lung and oropharyngeal microbiota cluster together, separately from the nasopharyngeal microbiota.[37] This may suggest that the bacteria that are found are the result of microaspiration of the microbiome of the upper respiratory tract.[45] Current sequencing techniques do not enable a distinction between live and dead bacteria, which induces a significant bias.[46] The question whether lungs contain a real separate microbiome is yet unanswered.

The influence of airway microbiota on the immune system

The development of GF mice has provided an enormous advantage in the study of the interactions between microbiota and the immune system. However, GF mice lack commensal bacteria in general. Therefore, only the absence of all commensal bacteria can be studied, and no conclusions can be drawn about the lack of microbes at specific locations.

The presence of commensal bacteria is essential for normal cellular maturation, recruitment, and control of airway inflammation.[47] Allergic GF mice have a stronger eosinophil influx, resulting in an increase of Th2 cytokines and thereby show an exaggerated allergic airway response.[48] It has also been shown that GF mice have fewer IgM-producing B cells and CD4+ T cells in the upper airways compared with the conventional mice.[49] Another consequence of the modulation of microbiota can be the lymphocyte response towards bacterial RTI. The presence of commensal bacteria maintained the mucosal immune response towards *Mycoplasma pulmonis* infection.[47]

In contrast to the gut, not much is known about the influence of the nasopharyngeal microbiota on the local immune system. Larsen *et al.*[50] stimulated monocyte-derived dendritic cells (DCs) with selected airway commensal bacteria (e.g., *Veillonella*), pathogenic bacteria (e.g., *Haemophilus* and *Moraxella*), and bacteria present in both healthy and sick lungs (e.g., *Actinomyces*). All bacteria activated DCs to a comparable level based on the surface expression of CD83, CD86, and CD40. However, pathogenic bacteria induced a 3–5-fold greater production of interleukin (IL)-23, IL-10, and IL-12p70. Co-culture experiments showed that *Prevotella* reduced IL-12 production by *Haemophilus* by 50%.

The development of GF mice has proven to be pivotal in studying interactions between commensals and the immune system. So far, to our knowledge, no studies have been performed that recolonized only the respiratory tract. This would enable us to study the effect of specific airway commensals on the development of the respiratory immune system.

THE MICROBIOME HAS AN INFLUENCE ON SEVERITY OF VIRAL INFECTIONS

As stated above, the presence of the microbiota is crucial for the development and maintenance of the human immune system. At the same time, the state of the immune system is important for the susceptibility towards viral infections. Commensal bacteria may either inhibit or enhance viral infections in direct or indirect ways.

Gut commensals directly enhance local enteric virus infections

Recently, two studies have shown that commensal bacteria in the gut can enhance enteric viral infections in a direct manner.[51,52] Kane *et al.*[52] showed that mouse mammary tumor virus, a virus that is transmitted from mother to young through milk and invades through the gut, covers itself with lipopolysaccharide (LPS) from the commensal bacteria present in the gut. This virus–LPS complex is able to stimulate Toll-like receptor 4 (TLR4), which induces IL-6, which subsequently induces IL-10. The LPS-covered virus infects the cells, but due to the IL-10 production the antiviral response is shut off. Antibiotics kills these gut bacteria and thereby prevents viral infection and transmission of the virus.[52] Kuss *et al.*[51] showed that multiple viruses use ligands from the commensal bacteria to enhance their infection. Both poliovirus and reovirus are able to bind LPS and enhance attachment to their target cells and infect them. They showed that this enhanced infection was due to N-acetylglucosamine-containing surface polysaccharides.[51]

Gut commensals protect against systemic and respiratory viral infections

As there have been millions of years of co-evolution between enteric viruses, commensal gut bacteria, and the hosts intestines, it is not surprising to find viruses that use commensal bacteria to aid their pathogenesis. What might be more striking is that the gut microbiota also has a systemic influence on viral infections.

Splenic DCs from GF mice are inhibited in their type I interferon (IFN) production and thus are not able to prime and activate natural killer (NK) cells.[53] As a consequence, their antiviral immunity is severely compromised. Another study also showed that antibiotic treatment of mice led to decreased type I IFN expression and thereby a reduction in the expression of antiviral genes.[54] This resulted in delayed viral clearance after a systemic infection (lymphocytic choriomeningitis virus) and, interestingly, a respiratory infection (influenza). The investigators concluded that host microbiota provide a tonic immune stimulation that establishes the activation threshold of the innate immune system for optimal antiviral immunity.

The influence of the commensal gut microbiota on the occurrence of RTI has been shown before. Clinical studies indicate that probiotics do not influence the incidence of RTI but do reduce the severity of the symptoms and the duration of the illness.[55,56] In mice, the intake of *Lactobacillus plantarum* enhances the type I IFN response after influenza infection and thereby lowers viral titers in the lungs.[57] Other *Lactobacillus* strains can enhance tumor necrosis factor α and IFN-γ production by nasal lymphocytes to influenza infection.[58]

A recently published study indicates that mice given antibiotics have a disturbance in their gut microbiota. Lower influenza-specific antibody titers and lower CD4+ and CD8+ T-cell responses result in higher viral titers in the lungs of these mice.[59] Moreover, it was shown that both intranasal and intrarectal administration of LPS repairs this immune impairment. This mechanism may be a two-hit model in which the intact microbiota first provide signals, e.g., LPS, which lead to pro-IL-1β and pro-IL-18 expression, and secondly, the influenza infection induces the inflammasome, which then converts the pro-forms into IL-1β and IL-18. DCs are then directed towards the lymph nodes and are able to prime the T cells present there. The effect of the commensal gut microbiota towards RTI can also be indirect. Tanaka *et al.*[60] showed that indigenous microbiota of mice maintained the mouse cytomegalovirus (MCMV)-specific CD8+ memory cells in the lungs, probably due to cross-reactivity of the antigenic epitope of MCMV T cells and the enormous variety of peptides in the microbiota. Whether this would be beneficial (faster clearance of MCMV upon reinfection) or possibly detrimental (enhanced immunopathogenesis upon reinfection) is not known.

Respiratory tract microbiota could have a dual role in viral infections

Comparable to the intestinal tract, the commensals, viruses, and host respiratory tract also have a long history of co-evolution, and it may be expected that certain viruses make use of the present commensal bacteria to facilitate infection. Literature on the influence of the specific respiratory tract microbiota on viral RTI is limited. Bacteria or bacterial ligands can either enhance or reduce viral infection rate (top part of **Figure 2**) or they can influence the subsequent immune response of the host towards a viral infection in either an enhancing or reducing way (bottom part of **Figure 2**).

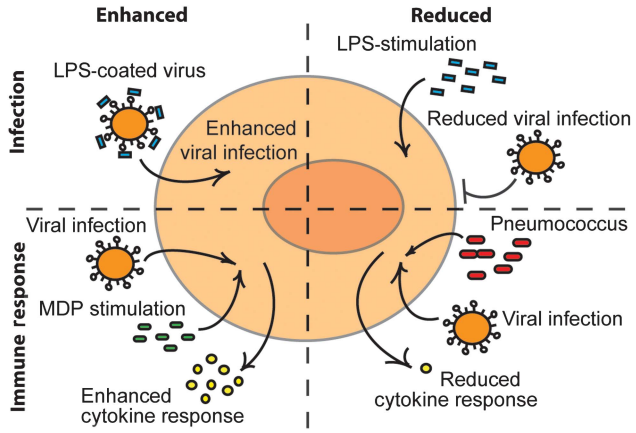

Figure 2 Bacteria or bacterial ligands can influence viral pathogenesis in multiple ways. They can influence viral infection rate itself, as can be seen in the upper part of the figure. Bacteria or bacterial components are able to enhance or inhibit viral infection rate, depending on the specific virus and ligands involved. It can also influence viral pathogenesis in an indirect manner. Most respiratory viruses induce immunopathogenesis. The lower part of the figure shows how bacteria or its components can influence the subsequent immune response of the host towards the viral infection, thereby influencing the pathogenesis. LPS, lipopolysaccharide; MDP, muramyl dipeptide.

As discussed in the section on the influence of intestinal microbiota on enteric viruses, certain viruses are able to coat themselves with LPS thereby enhancing their infection rates.[51,52] However, at this point this has not been described for respiratory viruses. Viral replication in the respiratory tract can be enhanced by *Staphylococcal* enterotoxins or exposure to *S. pneumoniae*.[61,62] One study showed that LPS could also reduce viral infection rates. *In vitro* pre-stimulation of human macrophages with LPS induced an antiviral response, which reduced RSV and influenza infection by 80%.[63] LPS seems to be a ligand that can influence viral pathogenesis in multiple ways. Therefore, the receptor for LPS, TLR4, is of interest. TLR4 has also been described as being a receptor that is able to recognize the F-protein of RSV.[64] Whether or not this dual function of TLR4 is also of importance for the interaction between commensal bacteria and viral infections is not known.

In vitro studies in human primary cells have shown that the pro-inflammatory response to a viral infection can be enhanced by a specific bacterial ligand, muramyl dipeptide (MDP).[65] Multiple respiratory viruses, including RSV, are able to induce type I IFNs, upregulating the receptor for MDP, namely NOD2 (nucleotide-binding oligomerization domain-containing protein 2). Subsequent stimulation by MDP leads to a severely enhanced pro-inflammatory response, which could potentially facilitate the immunopathogenesis of RSV infection.

Respiratory commensal bacteria can also reduce the immune response to a viral infection. The presence of a commensal nasopharyngeal microbiota protected mice against RSV-induced airway hyper-responsiveness. Mice were infected with RSV and subsequently treated with antibiotics, which depleted the nasopharynx from *Streptococcus viridans*. This led to increased numbers of inflammatory lymphocytes, decreased T_{regs} and transforming growth factor-β production and enhanced airway hyper-responsiveness. This effect was limited within local tissues and not systemic.[66] Influenza-infected mice showed an enhanced recruitment of inflammatory monocytes to the lungs when they were stimulated with MDP. This resulted in a reduced pulmonary inflammation, viral load, and mortality.[67]

Pre-stimulation of cultured human airway epithelial cells with *Haemophilus influenzae* induced an upregulation of intercellular adhesion molecule-1 and TLR3. This resulted in an increased binding of rhinovirus and a subsequent stronger IL-8 response.[68] This study shows that certain pathogens can induce a stronger infection as well as an enhanced immune response.

Although indirect, there is also some evidence from clinical studies. Some studies have shown that the effect of the pneumococcal conjugate vaccine is broader than just a reduction in pneumococcal carriage and infection.[69,70] The reduction in pneumococcal carriage and infection also results in a reduction of 31% in viral RTI. Individuals with acute viral infections are more often and more heavily colonized by specific bacteria.[71–73] Therefore, it can be argued that colonization with *S. pneumoniae* increases the risk to get infected with respiratory viruses or that its presence enhances symptoms. However, it cannot be excluded that viral infection also affects colonization rate and spread of pneumococcus.

So far, literature clearly shows that commensal microbiota affect our susceptibility to viral infections. An overview on current studies on the influence of the respiratory microbiota on respiratory viral infections can be found in **Table 2**. This effect can either be indirect, by modulating the immune system, or direct, e.g., viruses that use bacterial ligands to enhance their infection (**Figure 2**). Current evidence suggests that the influence of the gut microbiota on systemic immunity is mostly protective against viral infections, whereas local commensal bacteria and local immune responses, both in the intestines and in the respiratory tract, can either enhance or eliminate the viral infection.

THE FORMATION AND IMPLICATIONS OF THE NEONATAL MICROBIOTA

As shown above, it is well accepted that the presence of commensal microbiota has a major influence on the training of the immune system. Moreover, we provided an overview of the literature demonstrating the influence of this commensal microbiota on viral infections. However, viral RTI pose a big disease burden to very young infants. In this part of the review, we will summarize the literature regarding the initial colonization and how this influences the developing immune system.

The microbiome of infants is subject to enormous changes

During and immediately after birth, the newborn becomes colonized. Newborns receive their first colonizers from their passage through the mother's vaginal tract, contact with their surroundings, nurses, parents, visitors, and by feeding.

Table 2 Overview of the literature showing an influence of bacteria or bacterial ligands on viral respiratory infection outcome

	Study	Type of study	Virus	Bacteria or bacterial ligand	Timing of intervention	Outcome of the study
In vitro studies	Short *et al.*[63]	Monocyte-derived macrophages	Influenza	LPS (Gram-negative bacteria)	Ligand 24 h a.i.	LPS pre-stimulation of monocyte-derived macrophages reduces influenza virus infection and antigen-presentation to CD8+ T cells
	Sajjan *et al.*[68]	Epithelial cells	Rhinovirus	*Haemophilus influenzae*	Bacteria 24 h a.i.	*Haemophilus influenzae* infection increases airway epithelial cell receptor expression, leading to enhanced binding of rhinovirus and a potentiation of rhinovirus-induced chemokine release
	Wang *et al.*[61]	A549 cells	Rhinovirus	*Staphylococcus aureus*	Toxins 8h p.i.	Staphylococcal enterotoxin A/B enhanced rhinoviral replication in airway epithelial cells
	Nguyen *et al.*[49]	Primary epithelial, myeolid, and lymphoid cells	RSV	Pam3Cys (Gram-positive bacteria)	Simultaneously	The synthetic bacterial lipopeptide Pam3CSK4 enhances RSV binding and infection of primary epithelial, myeloid, and lymphoid cells
	Vissers *et al.*[65]	PBMC	RSV	MDP (mostly Gram-positive bacteria)	Simultaneously	Pro-inflammatory cytokine production by PBMC after RSV infection is enhanced when stimulated with MDP
Mice studies	Abt *et al.*[54]	4/6-week-old C57BL/6 mice	Influenza	Not specified	2–4 weeks of antibiotics a.i.	Commensal-derived signals provide tonic immune stimulation that establishes the activation threshold of the innate immune system required for optimal Antiviral immunity
	Coulombe *et al.*[74]	4/6-week-old C57BL/6	Influenza	MDP (mostly Gram-positive bacteria)	Ligand 1–5 days p.i.	MDP treatment of mice infected with influenza significantly reduces mortality, viral load and pulmonary inflammation
	Hori *et al.*[58]	15-month-old BALB/c	Influenza	*Lactobacillus casei*	Bacteria 4 months a.i.	Oral administration of *Lactobacillus casei* activates systemic cellular immunity and local cellular immunity, which ameliorate influenza infection
	Ichinohe *et al.*[59]	C57BL/6 mice	Influenza	Neomycin-sensitive bacteria	4 weeks of antibiotics a.i.	Neomycin-sensitive bacteria regulate the generation of virus-specific CD4 and CD8 T cells and antibody responses following respiratory influenza virus infection
	Maeda *et al.*[57]	7-week-old C57BL/6 mice	Influenza	*Lactobacillus plantarum*	Bacteria 7 days a.i.—6 days p.i.	*Lactovacillus plantarum* enhances protection against influenza virus infection by stimulation of type I interferon production
	Wang *et al.*[75]	C57BL/6	Influenza	*Staphylococcus aureus*	Bacteria on day 3 or day 7 a.i.	Priming with Toll-like receptor 2-ligand$^+$ *Staphylococcus aureus* significantly attenuates influenza-mediated lung immune injury
	Wu *et al.*[76]	BALB/c mice	Influenza	Not specified	Antibiotics 8 days a.i.	The intestinal microbiota critically regulates the TLR7 signalling pathway following respiratory influenza virus infection
	Wyde *et al.*[67]	Mice	Influenza	MDP (mostly Gram-positive bacteria)	Ligand 2 days a.i.	Mice given MDP before influenza challenge had significantly reduced pulmonary virus titers and mortality compared with comparably challenged control mice
	Tanaka *et al.*[60]	5/6-week-old BALB/c mice	Murine cytomegalovirus	Not specified	2 weeks of fecal suspension a.i.	The indigenous microbiota have a crucial role in the expansion and maintenance of viral-specific CD8 memory T cells
	Chiba *et al.*[11]	3-week-old BALB/c mice	RSV	*Lactobacillus rhamnosus*	Bacteria 5 days a.i.	Treatment with *Lactobacillus rhamnosus* modulates the antiviral respiratory immune response and significantly reduce lung viral loads and tissue injuries after the challenge with RSV

Table 2 (Continued)

	Study	Type of study	Virus	Bacteria or bacterial ligand	Timing of intervention	Outcome of the study
	Ni *et al.*[66]	3-week-old BALB/c mice	RSV	*Streptococcus viridans*	Antibiotics from day 0 to day 14 p.i.	Loss of *Streptococcus viridans* induces airway hyperresponsiveness and a decrease in T_{reg} cells in response to RSV infection
Human studies	Dagan *et al.*[70]	Vaccination trial	Not specified	*Streptococcus pneumoniae*	Infants followed 22 months after vaccination	9-Valent pneumococcal conjugate vaccine reduces upper respiratory infections with 15% and lower respiratory problems with 16%
	Madhi *et al.*[69]	Vaccination trial	Influenza, RSV, parainfluenzavirus, adenovirus	*Streptococcus pneumoniae*	Infants followed ~2.5 years after vaccination	9-Valent pneumococcal conjugate vaccine prevents 31% of pneumonias associated with respiratory viruses in children in hospital
	Verkaik *et al.*[62]	Prospective study	Metapneumovirus	*Streptococcus pneumoniae*	Infants followed for first 2 years	Frequent nasopharyngeal carriage of *Streptococcus pneumoniae* was associated with increased seroconversion rates of infants to hMPV at the age of 2 years
	De Vrese *et al.*[55]	Probiotics study	Coxsackievirus, ECHO-virus, rhinovirus, RSV, parainfluenzavirus, metapneumovirus, influenza, adenovirus	*Lactobacillus gasseri*, *Bifidobacterium longum*, *Bifidobacterium bifidum*	Probiotics for 3 or 5 months	Use of probiotics had no effect on the incidence of respiratory infections but significantly shortened duration of episodes, reduced severity of symptoms, and increased cytotoxic T/T suppressor/T helper cell counts

Abbreviations: a.i., before infection; hMPV, human metapneumovirus; LPS, lipopolysaccharide; MDP, muramyl dipeptide; PBMC, peripheral blood mononuclear cells; p.i., post infection; RSV, respiratory syncytial virus; TLR, Toll-like receptor; Treg, regulatory T cells.

Colonization is influenced by multiple factors, such as age,[77] mode of delivery,[30] breastfeeding,[78] antibiotic use,[79] vaccination,[80] season,[29] exposure to smoke,[81] day-care attendance,[82,83] and presence of siblings.[84]

Initial colonizers of the gastrointestinal tract are mainly facultative anaerobes (e.g., *Lactobacilli* and *Enterobacteria*), subsequently obligate anaerobes will follow quickly (e.g., *Bacteroides* and *Bifidobacterium*). Over time, the microbiota increases in diversity and stability and mirrors the adult pattern by the age of 1–3 years.[77,85]

An important determinant of the initial colonizers is the mode of delivery. Newborns who are vaginally delivered have microbiota that resemble the vaginal microbiota (*Lactobacillus*, *Prevotella*) of their mother, while newborns delivered by C-section have microbiota resembling the mother's skin (*Staphylococcus*, *Corynebacterium*).[30] In contrast to adults, the microbiota of newborns are undifferentiated across multiple body habitats, thereby showing that only a subset of these initial colonizers will permanently colonize the infant and will become a part of the distinctive microbiota across multiple body habitats. Several studies have shown that the initial colonizers are important as newborns delivered by C-section are more susceptible to certain pathogens,[86] which may be the result of a delayed colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides*.[87,88]

Other components also influence the colonization in the first months, e.g., breast-feeding and birth weight. Newborns with extremely low birth weights have less *Bacteroidetes*.[89] Infants that are breast-fed show a higher diversity in microbiota and have more *Bacteroidetes*,[78] which are associated with a better health and less allergies later in life.[90]

Hansen *et al.*[91] have shown in mice that there is a time window in which young mice can be efficiently colonized. Inoculation of GF mice at 3 weeks of age changed their microbiota composition to the bacteria that were inoculated. However, GF mice at 1 week of age that were inoculated developed a microbiota, which was comparable to specific pathogen-free mice.

Other investigators studied the time-dependent development of the microbiota in different organ systems.[92] They showed that certain clusters of bacteria were first present in the gut, before colonizing the respiratory tract. This highlights the interaction between these two organ systems and their microbiota. A different diet induced different intestinal microbiota and subsequently different respiratory microbiota.

Not many studies have focused on the upper respiratory tract microbiota in healthy children (**Table 1**). Bogaert *et al.*[29] showed that the most pre-dominant phyla of the nasopharynx of young children are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*. They showed that there were distinct microbiota during fall/winter (mostly *Proteobacteria* and *Fusobacteria*) and spring (mostly *Bacteroidetes* and *Firmicutes*). The majority of the children had a predominant Gram-negative nasopharyngeal community profile, which was even stronger in winter. They hypothesized that the spring-microbiota is more balanced and thereby protective against overgrowth of pathogenic species.

The initial colonizing microbiome is crucial for the development of the immune system

Certain parts of the innate and adaptive immune system of infants are still immature after birth (extensively reviewed by Martin *et al.*[93]). The development of a stable microbiome occurs therefore during a time that corresponds to a critical period of immune development and maturation. While a fetus is in the womb, pro-inflammatory responses are suppressed so as to avoid adverse immunological reactions between mother and child. Children are therefore born with immunological tolerance, making it possible to be colonized by bacteria.

The introduction of GF mice in research clearly shows how important commensal bacteria are for the development of the immune system. GF mice have poorly developed gut-associated lymphoid tissue, less intraepithelial lymphocytes, smaller Peyer's patches, hardly any mature isolated lymphoid follicles and low IgA levels.[93]

Mice studies have shown that a short GF period after birth changes the numbers of T_{regs}, NK, and NKT cells and cytokine levels permanently.[91] In contrast to adult mice, colonization of neonatal GF mice protected them from accumulation of NKT cells in the colonic lamina propia and the lungs. This resulted in a decreased pathology when inflammatory bowel disease or allergic asthma was induced.[94] So a small time window exists in which one can be efficiently colonized, which will modify future immune responses and pathology.

A study in infants showed that breast-fed infants have gut microbiota that are richer in virulence genes, and this is correlated with upregulation of immunity-related genes.[78] Another study showed that infants who were early and intensely colonized by *Bacteroides fragilis* had a downregulated LPS responsiveness.[95]

Strachan[96] was the first to publish that hay fever and eczema (both allergic diseases) were less common in children from larger families. This resulted in the formulation of the "hygiene hypothesis", which poses that a lack of early childhood exposure to infectious agents and symbiotic microorganisms increases susceptibility to allergic diseases, even at distant locations like, e.g., the skin or the upper airways, by suppressing the natural development of the immune system. Epidemiological studies have shown a correlation between the prevalence of asthma and airway allergies and variations in the gastrointestinal tract bacteria, e.g., lower amounts of *Lactobacilli* and *Bifidobacteria*.[97] Several reviews on the use of probiotics in humans to prevent respiratory infections or allergic airway disease show that studies so far have given rise to conflicting evidence.[98,99] The majority of the reviewed studies have shown a beneficial effect on respiratory infections or allergic airway disease.[100–105] However, there are also studies that show no difference.[106,107] One study performed in mice even showed a more severe allergic airway response after neonatal treatment of mice with *Lactobacillus casei*.[108] Most probiotic trials have tried to reduce respiratory burden via oral intake of probiotics.[58,109] However, some studies have shown that nasal administration of probiotics might be more efficient.[110,111]

There is a lack of data on the development of the microbiome and the immune system in young infants. However, it is clear that the initial colonization is crucial for the development of the immune system. In the future, it will be crucial to study the initial colonization and immune development of infants in more detail.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The influence of the intestinal microbiome on the immune system is widely accepted. Evidence suggests that the intestinal microbiome has an influence on systemic immunity and immunity in distant locations, such as the respiratory tract. However, the respiratory tract by itself is also colonized with commensal bacteria, and it is to be expected that these commensals also influence the local respiratory immunity. A growing body of evidence suggests that microbiota influence viral infections in several ways. This influence can be either detrimental or beneficial for viral infections. Again, the basis for this evidence is stronger for intestinal microbiota as compared with that for the respiratory tract. However, by comparison with the intestinal tract one would assume that the respiratory microbiota influences viral RTI. They could enable or prevent direct virus infection, prime the immune system for a viral infection, or perhaps enhance the immune response once a virus has infected epithelial cells. For multiple respiratory viruses, part of the disease pathogenesis is an enhanced immune/inflammatory response. Animal models should be developed to specifically study the influence of the nasopharyngeal microbiota both on local immune development as well as the severity of RTI. Whether the influence comes from functional bacterial groups or perhaps "keystone species"[112] is not known and could be different for each type of viral infection. Research into which specific bacteria are involved will be crucial for future understanding.

Timing and composition of the initial colonization in infants has lifelong consequences for the immune response. The development of the immature immune system happens simultaneously with the quickly changing composition of the microbiota. Therefore, one can imagine that the initial colonization and also the presence of specific bacterial strains during a viral RTI influences the disease severity. Studies in infants have shown that not only pneumococcal vaccination but also the use of probiotics can protect against severe viral RTI. Consequently, host microbiota can no longer be ignored when studying host–viral interactions. A more thorough understanding of the ontogeny of the microbiome in infants is critically required.

Advances in both sequencing technologies and the development of important mice models herald a new era in characterizing the role of the microbiota in the severity of infections. Comprehension of the impact of the microbiota on the susceptibility to severe RTI, together with a better understanding of the dynamics and kinetics of colonization during infancy, will allow new possibilities for the treatment and detection of highly susceptible infants.

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